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Ancestral Dependence on Breast Stemness and Nucleotide Excision Repair in Breast Cancer Stem Cells in Novel Human Model Systems

Omar Ibrahim
Nova Southeastern University

ANCESTRAL DEPENDENCE ON BREAST STEMNESS AND NUCLEOTIDE EXCISION REPAIR IN BREAST CANCER STEM CELLS IN NOVEL HUMAN MODEL SYSTEMS

By

OMAR M. IBRAHIM

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Dissertation Advisor: Jean J. Latimer, Ph.D.

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CERTIFICATE OF APPROVAL

Ph.D. Dissertation

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OMAR MOHAMMED IBRAHIM

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Examining Committee:

Dr. Jean J. Latimer, Ph.D. — Date: 1/22/18
Chairperson of Dissertation Committee

Dr. Stephen G. Grant, Ph.D. — Date: 1-24-18
Dissertation Committee Member

Dr. Ana Maria Castejon, Ph.D. — Date: 1/22/18
Dissertation Committee Member

Dr. Peter M. Gannett, Ph.D. — Date: 1.24.18
Dissertation Committee Member

Dr. Jose Lopez, Ph.D. — Date: 1-18-18
Dissertation Committee Member

Nova Southeastern University
Health Professions Division
College of Pharmacy
Pharmaceutical Sciences
Fort Lauderdale, FL

CERTIFICATION

We hereby certify that this dissertation, submitted by Omar Mohammed Ibrahim, conforms to acceptable standards and is fully adequate in scope and quality to fulfill the dissertation requirement for the Doctor of Philosophy degree.

Approved:

Michelle A. Clark, Ph.D. — Date: 1/24/18
Department Chairperson

Dr. Peter M. Gannett, Ph.D. — Date: 01.25.18
Associate Dean, Research and Graduate education

Lisa Deziel, Pharm.D., Ph.D. — Date: 01/26/2018
Dean

An Abstract of a Dissertation Submitted to Nova Southeastern University
in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

ANCESTRAL DEPENDENCE ON BREAST STEMNESS AND NUCLEOTIDE EXCISION REPAIR IN BREAST CANCER STEM CELLS IN NOVEL HUMAN MODEL SYSTEMS

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Precocious puberty, measured by thelarche or menarche, is a well-established breast cancer risk factor. Using a unique and patented tissue engineering system we established cell lines from 48 primary cultures of normal breast tissue from reduction mammoplasties and used them as an *in vitro* model of thelarche, quantifying episphere formation and ductal differentiation. Differential ductal formation was observed based on ancestry. In light of African American (AA) precocious thelarche both in vivo and as we observed *in vitro*, we hypothesized that AA women would exhibit an increased proportion of breast stem cells, with greater potency for differentiation. Five AA cell lines and five European-derived white (EW) cell lines were analyzed. AA cell lines had significantly higher percentages of breast stem cells and these stem cells were more potent than those from EW cell lines, indicating innate differences in stem cells might be responsible for ancestral differences in thelarche. We adapted our tissue engineering system for xenoestrogenic assay development. The formation of epispheres was dose-responsive with estradiol and bisphenol A. Development of a new assay is crucial for banning consumer chemicals that cause cancer related changes in breast development. We then investigated breast cancer cell lines for the role of Nucleotide Excision Repair (NER) in disease progression. Late stage breast cancer cell lines (pre- or post-treatment) manifested significantly higher NER capacity than stage I breast cancer or reduction cell lines. Increased NER capacity may be responsible for chemotherapeutic resistance, consequential of tumor progression. We hypothesized that the driver of higher repair capacity was due to the cancer stem cell populations within these cell lines. Cancer stem cells were flow sorted from breast cancer-derived cell lines and subjected to the functional Unscheduled DNA Synthesis (UDS) assay to assess NER capacity. Cancer stem cells had increased NER capacity compared with non-stem cells from the same cell lines. These data are consistent with breast cancer stem cells driving the increased NER capacity in late stage breast tumors. Developing therapies specific to the breast cancer stem cell compartment, albeit an elusive target, may provide a new treatment modality for resistant, late stage breast tumors.

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List of abbreviations and symbols

AA	African American
AC	Adriamycin and cyclophosphamide
Ago2	Argonaut-2
ALL	Acute lymphoblastic leukemia
API	Asian/pacific islander
ATM	Ataxia Telangiectasia Mutated
BCDIN3D	BCDIN3 Domain Containing RNA Methyltransferase
BMI	Body mass index
C/EBP-β	CCAAT enhancer binding protein-β
CAF	Cyclophosphamide, doxorubicin (adriamycin), and fluorouracil
CAK	CDK-activating kinase
Cat#	Catalog number
CCNH	Cyclin H
CD24	cluster of differentiation 24
CD44	cluster of differentiation 44
CD49f	Cluster ofdifferentiation / integrin alpha-6 / ITGA6
CDK7	Cyclin-dependent kinase-7
CEF	Cyclophosphamide, epirubicin, and 5-fluorouracil
CENT2	Centrin-2
CHK	Checkpoint kinase
CI	Confidence interval
CK17	Cytokeratin 17
CK5	Cytokeratin 5
CLL	Chronic lymphocytic leukemia
CMF	Cyclophosphamide, methotrexate, and 5-fluorouracil
CNS	Central nervous system
CSA	Cockayne syndrome complementation group-A
CSB	Cockayne syndrome complementation group-B
CSC	Cancer stem cell
CUL4A	Cullin-4A
DDB1	DNA binding protein-1
DDB2	DNA binding protein-2
DGCR8	DiGeorge syndrome critical region gene 8
DMEM	Dulbecco’s Modified Eagle Medium

DNA	Deoxyribonucleic acid
E2F1	E2F transcription factor-1
EGFR	Epidermal growth factor receptor
ERBB2	erb-B2 receptor tyrosine kinase 2
ERCC1	Excision repair cross-complementation group 1
EW	European-derived white
FBS	Fetal bovine serum
FFs	Foreskin fibroblasts
FOXO1	Forkhead box O1
FOXO3	Forkhead Box O3
FSC	Forward scatter
GCN5	General control of amino acid synthesis 5
GTFIIH	General transcription factor IIH
HBSS	Hanks buffered saline
HER2/neu	Human epidermal growth factor receptor 2
HIF-1α	Hypoxia inducible factor-1α
HR	Hazard ratio
ID#	Identification number
IL33	Interluekin 33
KM	Kaplan Meier
MGMT	O-6-Methylguanine-DNA Methyltransferase
MLH1	MutL homology 1
MNAT1	Ménage a trois homolog-1
MRD	Minimal residual disease
mRNA	Messenger RNA
MRP1	Multidrug resistance-associated protein-1
MSH2	MutS homology 2
MSH6	MutS homology 6
MWRI	Magee-Womens Research Institute
MYC	Myelocytomatosis virus
NER	Nucleotide excision repair
OCT3/4	Octamer-binding transcription factor
ORF	Open reading frame
PARP	poly adenosine diphosphate-ribose polymerase
PBS	Phosphate buffer saline
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PDCD4	Programmed cell death protein-4
PI	Propidium iodide

PTEN	Phosphatase and tension homolog
RAD23B	RAD23 homolog B
RAS	Retrovirus-associated DNA sequences
RASA1	Protein activator-1
RB1	Retinoblastoma
RBX1	Ring-box-1
RFC	Replication factor C
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
ROCK1	Rho associated coiled-coil containing protein kinase-1
RPA1	Replication protein A1
RPA2	Replication protein A2
RPA3	Replication protein A3
RREB1	RAS responsive element binding protein-1
RT-PCR	Reverse transcriptase polymerase chain reaction
RTKN	Rhotekin
siRNA	Small interfering RNA
SPRED1	Sprouty related EVH1 Domain Containing-1
SSC	Side scatter
TBS	Tris-buffered saline
TBST	Tris-buffered saline, 0.1% Tween 20
TFIIH	transcription factor II H
TNBC	Triple negative breast cancer
TNM	Tumor-node metastasis
TRBP	TAR RNA-binding protein
TTD-A	Trichothiodystrophy complementation group-A
UTR	Untranslated region
UV	Ultraviolet
WBC	White blood cell counts
XAB2	XPA binding protein-2
XP	Xeroderma pigmentosum
XRCC1	X-ray repair cross complementing-1

Chapter 1

Introduction

1.1 Problem statement

1.1.1 Premature thelarche

The age of menarche (time of first period) has been decreasing for decades in the United States and Europe. Some of this effect can be attributed to better nutrition, less exercise and higher body mass index; however, there are indications that modulable factors such as environmental exposures contribute as well. Menarche and thelarche (the onset of breast development) are linked. For every year of premature thelarche, the risk of breast cancer increases by 5-20%. African American girls in the United States undergo menarche and thelarche an average of 2 years earlier than girls of European-derived White ancestry.

1.1.2 Risk factors in African American populations

Research, overall, in minority groups have lagged behind that of European-derived White women. The majority of large cohort studies in breast cancer have been primarily done and are still being done on European-derived White women (Long et al., 2013; Michailidou et al., 2015). There are known differences in breast cancer risk factors and how they affect women of different ancestries differently. Models used to predict and assess breast cancer risk are much less accurate and tend to underestimate the risk of breast cancer in minority populations such as African American women (Adams-

Campbell et al., 2009). There is a lack of scientific literature on the etiology and treatment of African American breast cancer.

African American women have a higher incidence of breast cancer than European-derived White women before age 40, while the opposite is true after age 40. Although socio-economic factors have, in the past, been attributed to these differences, data exists to support that there may be intrinsic biological differences in African American breast tissue compared with white breast tissue. Evidence for this hypothesis includes the fact that a higher percentage of estrogen receptor negative breast tumors are present in African American women (Pacheco, Gao, Bumb, Ellis, & Ma, 2013).

1.1.3 Breast cancer

Breast cancer is currently the second most prevalent cancer in women and is second in cancer-related deaths (Howlader et al., 2017). The majority of treatment options for breast cancer are invasive requiring biopsy, surgery, radiation, and/or chemotherapy. While recent success in targeted and hormonal therapies have helped to improve breast cancer mortality rates, there are still tumors that are not cured. Breast cancer recurrence after decades, and treatment of resistant tumors remain a large problem. Certain molecular subtypes of breast cancer lack any targeted treatment and do not respond to any hormonal therapy (Dawood, 2010). Treatment in these types of breast cancer has seen little advancement. In fact, breast cancers without hormone and growth factor receptors, such as triple negative breast cancer suffer from worse overall survival rates. Studies have shown that triple negative breast cancer has a 5-year overall survival

as low as 75%, 15% less than the current breast cancer 5-year overall survival rate of 90% regardless of type (Howlader et al., 2017; Ovcaricek, Frkovic, Matos, Mozina, & Borstnar, 2011).

1.1.4 Treatment resistance

Recurrence in breast cancer often results in very poor outcomes and is much more difficult to treat (Witteveen, Kwast, Sonke, IJzerman, & Siesling, 2015). Mechanisms of resistance in cancer treatment include increased DNA repair capacity. In cells with increased DNA repair, DNA damage caused by chemotherapy agents will be repaired with greater efficiency and therefore cancerous cells are more likely to survive chemotherapy treatment and continue to grow and proliferate (Helleday et al., 2008). Nucleotide excision repair is primarily responsible for repair of DNA damage induced by the cancer chemotherapeutic agents cyclophosphamide and doxorubicin and therefore increased resistance (Reed, 1998; Wood, 2011). Mutations in nucleotide excision repair genes have prognostic value, as they have been used successfully to predict the of outcome cyclophosphamide and doxorubicin response (Andersson et al., 1996; Saffi et al., 2010). Nucleotide excision repair deficiency is intrinsic in stage I breast cancer and plays a role in genomic instability of early stage breast tumors (Latimer et al., 2010). However, we know very little, if anything, about the role of nucleotide excision repair in the progression of breast cancer.

1.1.5 Model systems and cancer stem cells

There are over 60 commercially available cell lines that are used as surrogates to study breast cancer *in vitro*. However, the use of these cell lines to represent breast cancer overall has been called into question. The majority of these cell lines were originally derived from late stage breast cancer metastases, whereas the current majority of incident breast cancer is presently diagnosed as stage 1 breast cancer. The mechanisms of resistance have been studied using these cell lines. However, the need to explore and understand resistance mechanisms to better treat breast cancers of varying stages still exist.

Within a tumor, there are specific cells termed cancer stem cells that are theorized to have increased DNA repair mechanisms and exhibit increased resistance to genotoxic insult (Pavlopoulou et al., 2016). Contradictory studies have also been published on their supposed resistance to ionizing radiation (S.-Y. Kim et al., 2012; Yang, Sun, He, Cao, & Jiang, 2015). Very little has been done to study the function of nucleotide excision repair in cancer stem cells. In fact, the current literature is void of studies determining nucleotide excision repair functional capacity in breast cancer stem cells. This is unfortunate because, nucleotide excision repair is implicated in treatment resistance and better characterization of breast cancer stem cells may resolve conflicting results seen in the literature (Bowden, 2014; Holohan, Van Schaeybroeck, Longley, & Johnston, 2013).

1.2 Purpose statement

The purpose of this dissertation research was to determine if there are differences in stem cell behavior or number between European-derived White and African American women. This may be pertinent to the differences in the timing of breast differentiation witnessed in these ancestral groups. Then to develop a medium throughput assay for human non-diseased breast differentiation for the testing of putative hormone disruptors in culture. Using our culture model system, which produces *in vitro* like constructs, we aimed to measure disruptions in secondary breast structures due to exposure to putative estrogenic compounds. We ascertained the DNA Nucleotide Excision Repair capacity in a survey of breast tumor cell lines commonly used as well as several developed in our explant system (Appendix A). To further this work, our final aim was to determine DNA repair in breast cancer stem cells within these cell lines.

1.3 Study objective and hypotheses

Hypothesis 1: There is greater percentage and/or potency of breast stem cells in African American breast reduction explants than in European White breast reduction explants that is biologically intrinsic. These intrinsic biological differences will drive breast differentiation in our *in vitro* model of thelarche.

Specific aim 1: Determine the proportion of breast stem cells in African American and European-derived White explants. Using flow cytometry, we will compare the percentage of breast stem cells (CD24-, 44+, 49f+) of African American women to European White women in these cultures to identify differences based on ancestry.

Specific aim 2: Determine the potency of breast stem cells in these explants by sterile flow sorting a single breast stem cell (CD24-, 44+, 49f+) into each well of a 96 well plate for short time culture. We will also compare the ability of a single breast stem cell to differentiate in culture to determine whether African American women have more potent stem cells than European-derived White women.

Specific aim 3: Optimize our 3D multicellular *in vitro* breast differentiation system for chemical testing that can be used to assess putative xenoestrogens. We will identify the smallest chamber and fewest cells necessary for testing on multi-well plates and determine the shortest possible time to determine hormone disruption. The goal will be achieved by determining the speed and accuracy in scoring epispheres, as a measure of changes in episphere formation.

Rationale for Aims: Minority groups are severely underrepresented in breast cancer research. This study will help to gain insight into the etiology of breast cancer using a model of thelarche. Thelarche is a well-established risk factor for breast cancer.

Hypothesis 2: Nucleotide excision repair functional capacity will be increased in breast cancer stem cells compared to breast cancer non-stem cells.

Specific aim 1: Ascertain the capacity of breast cancer stem cells from early and late stage tumor cell lines to perform nucleotide excision repair using the unscheduled DNA synthesis assay. Then compare breast cancer stem cells to breast cancer non-stem cells to ascertain whether breast cancer stem cells have increased nucleotide excision repair capacity.

Rationale for this aim: Breast cancer stem cells are more resistant to genotoxic insults than other cells in the tumor. However, the nucleotide excision repair capacity in breast cancer stem cells is unknown. The nucleotide excision repair pathway is a mechanism of cancer resistance and needs to be further elucidated in breast cancer. Because of its role in resistance, it is possible that nucleotide excision repair could be made into a drug target for future treatment options.

1.4 Barriers, issues, limitations:

1.4.1 Thelarche, ancestry and breast stem cells

Our model system of thelarche is not completely established and we had to limit the work to formation of specific structures seen in culture, termed epispheres. The number of reduction mammoplasty cell lines was limited for the African American population and we had to limit the study to 5 African American and 5 European White. Furthermore, the number of post-menopausal cell lines was limited to 3 post-menopausal samples for stem cell analysis. Despite a very complicated and difficult flow cytometry process to isolate normal stem cells and culture them, we were successful in studying both percentage and potency.

1.4.2 Cancer progression and cancer stem cells

Cancer stem cells are extremely difficult to maintain as stem cells. Most groups are not able to do this, and they have created molecular methods of arresting differentiation that have raised many questions about the validity of these cells representing cancer stem cells (Sajithlal et al., 2010). In spite of this we have been

successful at plating them directly after flow cytometry on 100% basement membrane extract in conditioned medium and perform the biochemical Unscheduled DNA synthesis assay 24 hours later.

1.4.3 Assumptions

In this study we have assumed that one or more of the non-diseased breast reduction derived cell lines would be capable of reproducible episphere formation for the development of an endocrine disruptor test system. We also assumed that with experimental repetition that cancer stem cells would maintain their "stemness" long enough to be assessed for nucleotide excision repair functional capacity and that the presence of a bimodal peak of repair capacity would be evidence of differentiation.

Summary

Our study sheds light on African American women, an under-represented group, to better understand breast differentiation and how it relates to breast cancer. We have also developed a testing system for endocrine disruption. This testing system will fill a gap in future breast cancer prevention strategies. We have previously determined the nucleotide excision repair capacities in respect to stage of breast cancer cell lines/explants. We have furthered this by determining the nucleotide excision repair capacity in cancer stem cells. This fills a large void in the scientific literature and brings new findings into a controversial area of cancer stem cells. Nucleotide excision repair is a mechanism of treatment resistance that can be used by carcinogenic cells. Better

understanding of this mechanism can lead to improved treatments for breast cancer, especially in late stage resistant tumors.

Chapter 2

Literature review

2.1 Breast anatomy

The breast is comprised of the mammary gland, which is a modified sweat gland that produces milk. Lactiferous ducts and lobes make up the mammary gland (Figure 2.1) (Hoda, Bogi, Koerner, & Rosen, 2014). Lobes are made up of smaller lobules which are the milk producing structures in the breast. Lobules and lobes are connected by a series of branching ducts the lead milk from the back of the breast where the lobules are located, through the lactiferous ducts to the nipple (Hoda et al., 2014). Lobules are made up of clusters of hollow spheres called alveoli (Blackburn, 2014).

2.1.1 Microanatomy of the breast

The breast is composed of epithelial and stromal components and together, the ducts and lobules constitute the epithelial portion. The epithelium is composed of polarized luminal epithelial cells (Hoda et al., 2014). These cells which surround the lumen of the ducts and lobules are the cells that secrete milk into the lumen of the alveoli (Figure 2.1). At the base of the luminal cells that form these alveoli are basal myoepithelial cells (Figure 2.1C) that contract and aid in the release of milk, in response to oxytocin binding to the oxytocin receptor (Sternlicht, 2005). Lobules are immediately surrounded by delimiting fibroblasts. Fibroblasts produce paracrine factors that support the epithelium (Hoda et al., 2014).

Lobules also called terminal ducal lobular unit (TDLU), are spherical in shape due to the compact arrangement of multiple short branching's of blind-ended ducts, enclosed in a specialized stroma (Hoda et al., 2014). TDLUs are made up of an inner luminal epithelial layer and an outer layer of myoepthelial cells, all enclosed by a basement membrane. TDLUs, 20-40, are grouped together to form a single lobe (Pandya & Moore, 2011). Each breast consists of 15-20 lobes and each lobe has its own duct (Blackburn, 2014; Pandya & Moore, 2011). On the stromal side of the basement membrane lie fibroblasts and loosely textured collagen, which is traversed by small blood vessels, peripheral blood lymphocytes, macrophages, and plasma cells (Hoda et al., 2014). The majority of the breast is fat tissu e made up of adipocytes that fills the area around the epithelium, fat cells are necessary for the formation of epithelial structures in explanted breast tissue into host animals (Hoda et al., 2014; Pandya & Moore, 2011).

The breast is a highly vascularized and lymphatic organ, and it contains hundreds of lymph nodes around the lobules and ducts. The purpose of these lymph nodes is to provide the suckling infant with antibodies and live lymphocytes before it has a working immune system. The lymph nodes of the breast drain into over 20 axillary lymph nodes, the lymph nodes located under the arm (Figure 2.1) (Vidi, Bissell, & Lelièvre, 2013). There are 6 axillary lymph node groups that are recognized by surgeons (Pandya & Moore, 2011).

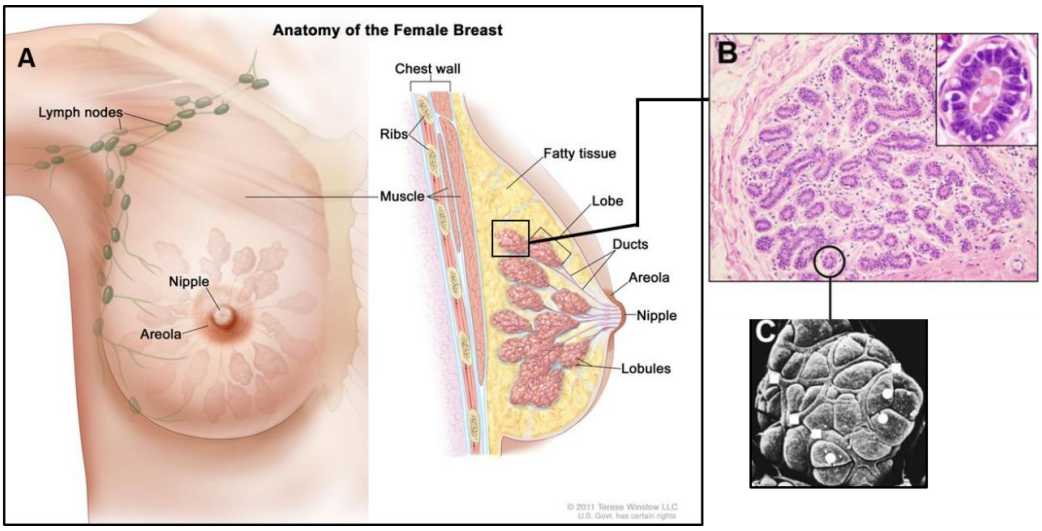


Figure 2.1 **Breast anatomy.** (A) The basic anatomy of the breast (B) Hematoxylin and eosin stained cross section of a breast TDLU. A single alveolus is shown in the inset in the upper right corner, polarized luminal epithelial cells surround an inner lumen. (C) A micrograph of a human alveolus that shows luminal epithelial cells (white circles) surrounded by myoepithelial cells (white squares). Adapted with permission from Vidi et al. 2013 and cancergov.org.

2.2 Breast development

2.2.1 Prenatal and perinatal development

The development of the female breast begins during embryogenesis and undergoes massive changes throughout life. Proliferation of a pair of mammary ridges from ectoderm in the thoracic region are the first signs of fetal breast development. The mammary crest (also called the milk line) is formed from the thickening of epidermis on the ventral surface in the embryo after about 5 weeks (Sternlicht, 2005). The mammary crest atrophies and leaves behind two buds. Breast development begins from these primary buds (Blackburn, 2014). While the development of the fetal mammary gland is independent of hormone stimulation, B-cell lymphoma 2 protein (BCL2) plays a major role in the inhibition of apoptosis and is expressed through prenatal development in the breast (Nathan, Anbazhagan, Clarkson, Bartkova, & Gusterson, 1994).

Around 15 weeks the primary buds will have penetrated into the upper dermis and mesenchymal cells penetrate into the supportive cells of the breast (Javed & Lteif, 2013). Secondary buds will form from the primary buds and will become the lactiferous ducts. Both luminal and basal epithelial cells can be distinguished at this point. Growth of solid epithelium columns will give rise to lobes in the mammary grand.

By 24 weeks, fetal breasts become apparent with tubular structure surrounded by dense connective tissue. Around this period, the breast will become more collagenized and less cellular and the ligaments of Cooper will form. After 28 weeks, a major lactiferous duct will form a mammary pit, which will eventually become the nipple and the development of branching lobule and alveolar glandular structures will take place (Javed & Lteif, 2013). By birth, the infant will have anywhere from 15-20 lobes each with a lactiferous duct leading to the mammary pit (Blackburn, 2014; Javed & Lteif, 2013).

Postnatally the breasts may secret colostrum and enlarge under the influence of placental maternal hormones. This enlargement will subside in the first and second week as hormones disappear. After this disappearance of maternal hormones, the glands will shrink and return to an inactive state (Sternlicht, 2005). At this point, the expression of BCL2 is also lost in the breast (Nathan et al., 1994). Lactiferous ductal structures will persist and branch, however, no alveolar differentiation will occur. The breast remains rudimentary until the production of estrogen and progesterone from the ovary begins at the onset of puberty (Javed & Lteif, 2013; Sternlicht, 2005).

2.2.2 Breast development at puberty

Thelarche or postnatal breast development occurs just before or during puberty (Cabrera, Bright, Frane, Blethen, & Lee, 2014). Thelarche involves elongation of ducts and thickening of the epithelium. Lobule, alveolar, and hormonally responsive periductal stroma differentiation also occurs (Javed & Lteif, 2013). While development of the breast at this stage is highly dependent on hormones, paracrine and autocrine signaling are used to communicate within cells of the lobules. The start of menstruation is called menarche and is highly associated with thelarche, which usually occurs soon after (Hoda et al., 2014; Javed & Lteif, 2013).

A report released by Sandra Steingraber and published by the breast cancer foundation in 2007, is a comprehensive review that highlights the drastic change seen over the past 40 years in the age of puberty. Figure 2.2 shows that the average age of menarche fell from 1790 to 1980 (Euling, Selevan, Pescovitz, & Skakkebaek, 2008; Wyshak & Frisch, 1982). Currently the average age for menarche is 12.6 (range 12.4–12.8) for white non-Hispanic girls and 12.2 (range 12.0–12.4) for African American girls. Furthermore, the average age of thelarche in young females now rests at 10.3 for European-derived white girls and 9.5 for African American, with many in both ancestries beginning before the age of 8 (Cabrera et al., 2014; Euling et al., 2008; Steingraber, 2007). Decreased age of puberty has both emotional and biological implications, including breast cancer risk.

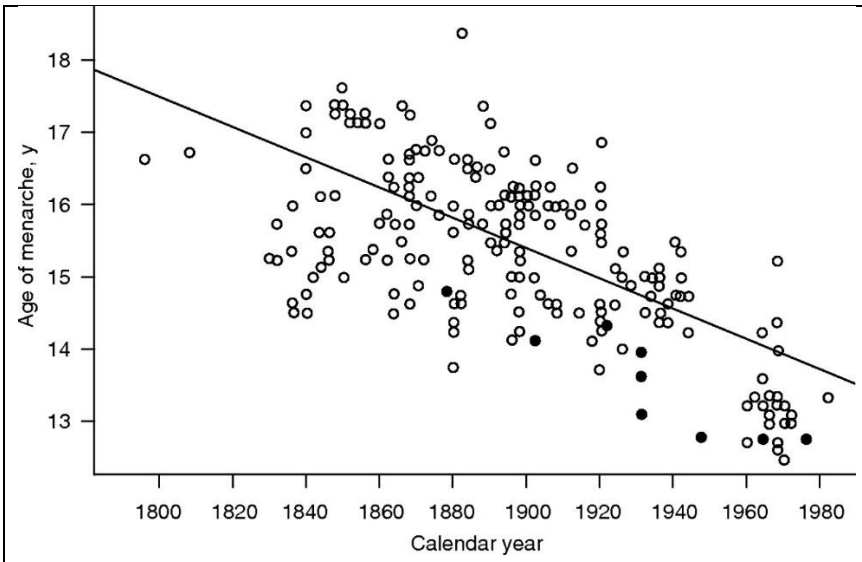


Figure 2.2 **The falling age of puberty.** Data from Europe (open white circles) and USA (closed circles) shows the age of puberty has been in steady decline in over 200,000 patients from 1790 to 1980. Reproduced with permission from Wyshak et al., 1982, Copyright Massachusetts Medical Society

The menstrual cycle is generally divided into 3 phases the follicular phase, ovulatory phase, and the luteal phase (Barrett & Raybould, 2010). The follicular phase lasts about 9 to 23 days; during this stage, estrogen increases and in the breast this signals parenchymal proliferation of the ductal system (Ramakrishnan, Khan, & Badve, 2002). The development of ovarian follicles occurs in this stage. The ovulatory phase lasts 1 to 3 days, and is marked with sharp increases in estrogen from the follicle. The luteal phase lasts 13 to 14 days and during this phase progesterone levels rise. During the luteal phase under the influence of progesterone, the breast ductal system is dilated and differentiation of alveolar cells into secretory cells occurs (Ramakrishnan et al., 2002). If pregnancy does not occur the hormone levels fall, and menses occurs.

2.2.3 Pregnancy and menopause

In preparation for lactation the breast transforms during pregnancy. During the first trimester, the alveoli and ducts increase in lobules. Due to the increase in epithelium the breast becomes denser. Stromal vascularity increases, and the breast will become infiltrated with mononuclear cells. In the second and third trimester, lobular growth will continue through cellular enlargement and proliferation of cells and lobules well become distended with secretion. After birth and breast feeding, lactation declines and the breast lobules will undergo involution and decrease in size and density (Tiede & Kang, 2011).

There are 4 well-defined types of lobules based on how well they are developed (Tiede & Kang, 2011). Type 1 lobules only have a single short terminal duct ending in a cluster of alveoli. Types 2, 3, and 4 all have terminal ducts that branch into multiple clusters of alveoli, each with progressively increasing amounts of branching and increasing numbers of alveoli. Type 4 will eventually develop secretory acinar structures and is only reached in women who have gone through pregnancy and breastfed (Tiede & Kang, 2011). However, Type 4 lobules will regress to type 3 lobules following the end of lactation (Baer et al., 2009; Tiede & Kang, 2011). In virgin and nulliparous women type 1 lobules are the most common type (Tiede & Kang, 2011). then, during menopause the glandular nature of the breast will become subdued with cell death in ducts and alveoli. Finally, the breast will then become more acellular with an increase in stromal components.

2.3 Stem cell

2.3.1 Normal breast stem cells

Stem cells are cells that are undifferentiated and have the ability to self-renew or differentiate into different cell types. Embryonic stem cells are omnipotent or have the ability to differentiate into all cell types as well as self-renew. Adult stem cells are cells that maintain some pluripotency and can differentiate into only few cell types as well as self-renew. Adults stem cells are found throughout the body and aid in many normal functions. In the breast, breast stem cells are thought to be located in the myoepithelial/basal layer of the ductal architecture and give rise to both luminal epithelial cells and myoepithelial cells. The mammary gland is unique in that it does not fully mature, and will only undergo some ductal growth differentiation until puberty. After puberty, it will undergo cyclical changes during menstruation and pregnancy. During the luteal phase of menstruation, alveoli epithelial cells proliferate and are poised to begin a pregnancy. If a pregnancy is not established, these cells die, the alveolus involutes and calcification occurs in the place where the cells have died. During pregnancy, cells in the breast grow and proliferate then involute when lactation is complete. This constant remodeling of the tissue requires a source of cells that can give rise to both cell types, luminal epithelial and myoepithelial cells, that comprise the functional unit of the gland as well as being able to self-renew (Tiede & Kang, 2011). These special cells have been termed breast stem cells and are necessary for the healthy development and maintenance of the mammary gland. The existence of these cells in human breasts have been inferred

from rodent models and *in vitro* studies dating back to the 1950's (Daniel, Young, Medina, & DeOme, 1971; Deome, Faulkin, Bern, & Blair, 1959). In syngeneic C3H mice the mammary fat pad was cleared, and serially transplanted with normal mammary gland. The fat pad of the host would support the normal function of the grafted mammary cells (Deome et al., 1959). What was eventually discovered is that all portions of the mammary gland could repopulate and become a normal functioning mammary gland (Daniel et al., 1971). Serial transplantation involved taking the cells from one rodent, C3H mice, and moving it to another to repopulate the second rodent. However, this was found to be a finite process, and with normal mammary cells, 5 to 8 transplant generations were possible. In contrast, cells transplanted from precancerous/cancerous donors could be transplanted indefinitely (Callahan & Smith, 2000; C. W. Daniel, Aidells, Medina, & Faulkin, 1975; Gilbert H. Smith, Pauley, Socher, & Medina, 1978). Furthermore, Young et al. showed that age, parity, and hormonal status of the donor did not alter the mammary regenerative potential (Young, Medina, DeOme, & Daniel, 1971).

The fact that cells from any portion of the mammary gland and from donors of any age, all pointed to the existence of cells with a stem like capabilities. This was eventually proven and it was shown that a single rare cell in the mouse mammary gland can be transplanted to repopulate an entire functioning mammary gland (Shackleton et al., 2006). Breast stem cells have been defined as a distinct cell type that is able to re-establish the mammary epithelium by asymmetric division into more differentiated cell types, at least luminal epithelial or myoepithelial cells (Van Keymeulen et al., 2011).

Furthermore, mammary stem cells have the ability to undergo symmetric division and only a single daughter cell will differentiate (Chen, Liu, & Song, 2017; Knoblich, 2008). Cells with similar capabilities have been verified in humans as well (Gudjonsson et al., 2002; J Stingl, Eaves, Zandieh, & Emerman, 2001; John Stingl et al., 2006).

While the gold standard of identifying breast stem cells remains the ability to reconstitute the mammary gland in xenograft mice models, the emergence of fluorescence activated sorting (FACS) has been crucial to our understanding and identifying breast stem cells especially in humans (Chen et al., 2017). Multiple surface markers have been employed in the use of purification of stem cells and are summarized in Table 2.1.

Table 2.1 **Breast stem cell markers used in flow cytometry.**

Marker	Source	Associated with	Reference
Stem cell antigen-1 (SCA-1)	Hematopoietic stem	Mammary progenitor cell	Welm et al., 2002. Gudjonsson et al., 2002
CD24*	Sialoglycoprotein; Cell adhesion molecule	Cancer differentiated cell	Hajj et al., 2002
CD44	Glycoprotein; Cell adhesion molecule	Cancer stem/progenitor cell	Hajj et al., 2002
Integrin beta-1 (CD29)	Cell adhesion molecule	Mouse cancer stem/progenitor cell	Shackleton et al., 2007
Integrin alpha-6 (CD49f)	Cell adhesion molecule	Bipotent luminal/myoepithelial progenitor	Stingl et al., 2001
Epithelial cell adhesion molecule (EPCAM/ESA)	Cell adhesion molecule	Bipotent luminal/myoepithelial progenitor	Stingl et al., 2001; Gudjonsson et al., 2002
RH123 efflux	Increased efflux of chemical dye	Bipotent luminal/myoepithelial progenitor	Stingl et al., 1998
Hoechst-33342 dye efflux	Increased efflux of chemical dye	Multipotent	Zhou et al., 2001; Smalley et al. 2003
Aldehyde dehydrogenase (ALDH1)	Increased enzymatic activity	Cancer stem/progenitor cell	Ginestier et al., 2007

***in mouse models CD24, in combination with other markers, is a positive marker for stem/progenitor cells able to generate a functional mammary gland from a single cell (Shackleton 2007).**

****Most markers are used in combinations with multiple positive and negative markers for stem/progenitor cells, ALDH1 is often used alone or in combination with other markers.**

Markers of stem and cell type are often used in conjunction with each other.

Early work in rodents by Welm et al. (2002) identified stem cell antigen-1 stained cells as a mammary stem cell rich population with increased Hoechst efflux, a stem cell characteristic (Welm et al., 2002; Zhou et al., 2001). Sting et al. (2001), discovered 3 types of human mammary progenitor cells *in vitro*, these progenitor cells could produce luminal epithelial, myoepithelial, or both. Cells positive for CD49f and Epcam showed bipotent progenitor differentiation while cells that did not express these markers showed a decrease in potency (Stingl et al., 2001). The development of the mammosphere culture system involved the use of ultralow attachment plates to grow and maintain human mammary epithelial cells. These cells would grow in suspension as spherical structures termed mammospheres (Dontu, Abdallah, et al., 2003). Mammospheres were found to be enriched in stem cells that were maintained in their undifferentiated state. Transcriptional profiling of these mammospheres confirmed the presence of breast stem cells with similar gene profiles to those of earlier markers (Dontu, Al-Hajj, Abdallah, Clarke, & Wicha, 2003; Gudjonsson, Rønnov-Jessen, Villadsen, Bissell, & Petersen, 2003).

2.3.2 Breast cancer stem cells

Eventually it was hypothesized that breast stem cells could be the origin of many tumors and produce more heterogeneous tumors than more differentiated cells (Tu, Lin, & Logothetis, 2002). Stem cells are also more embryonic and share marked similarities with cancer stem cells, or tumor initiating cells such as increased differentiation potential (Kim & Ryu, 2017). First discovered in acute myeloid leukemia (AML) it was found that

cancer cells are heterogeneous (Bonnet & Dick, 1997). There was a subpopulation of cells that were more primitive or embryonic in behavior and more strongly resemble stem cells than other cells in the tumor (Costa, Le Blanc, & Brodin, 2006). Bonnet et al. showed that a specific subset of cells from AML with CD34 positive and CD38 negative markers, when transplanted into non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice would reform the original cancer population. In contrast cells that were CD34+ 38+ could not do this. Thus, the first evidence of more potent cells with in the tumor was found, these cells were coined cancer stem cells.

cancer stem cells are thought to be more treatment resistant and metastatic than other cells within the tumor. After finding cancer stem cells in AML, it was later confirmed that breast cancer stem cells also exist in breast tumors. Human breast cancer stem cells could be grown *in vivo*, and when CD24-/44+/Lineage- (Lineage markers include: CD2, CD3, CD10, CD16, CD18, CD31, CD64, and CD140b) were transplanted into NOD/SCID mice, they could repopulate the original tumor in these mice. Cells that did not display these protein markers, breast cancer non-stem cells, were unable to repopulate the tumor when placed into NOD/SCID mouse (Dontu, Al-Hajj, et al., 2003).

There is evidence that stem cells can be the cell of origin in cancer as they are possible targets for insult (Lim et al., 2009; Miller et al., 1989; Proia et al., 2011). They have intrinsic longevity and activation of epithelial to mesenchymal transition can lead to the formation of cancer stem cells from normal cells (Smith, 2002; Tiede & Kang, 2011). Epithlelial to mesenchymal transition is a process where epithelial cells lose their contact

and adhesion with other cells as well as their polarity which makes them become more migratory, and is highly associated with cancer stem cells and invasiveness (Lee, Hwang, & Choi, 2016). Activation of the Ras-mitogen activated protein kinase pathway via increased oncogenes Ras or transdermal growth factor β1 or by increased expression of transcription factors Snail and/or twist have changed normal human mammary epithelial cells into breast cancer stem cells via an epithlelial to mesenchymal transition mediated process.

Functionally, to be considered a cancer stem cell the putative stem cell must be able to form a tumor in NOD/SCID mouse and then be passagable in additional mice. Under certain stresses it is possible for differentiated tumor cells to revert into more primitive cancer stem cells via epithelial to mesenchymal transition (Owens & Naylor, 2013). A simplified schematic of the advanced cancer stem cell model is outlined in Figure 2.3.

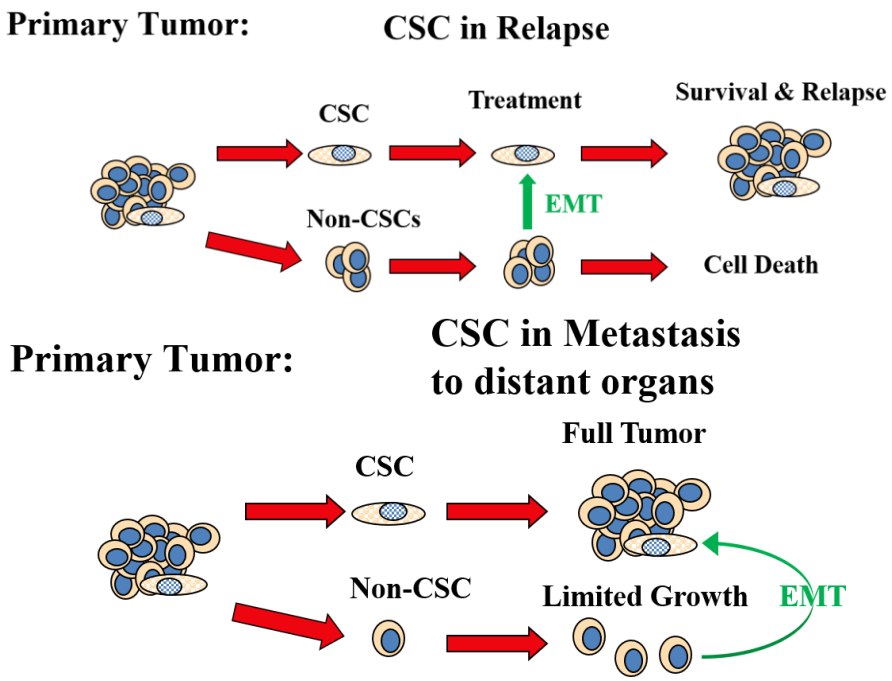


Figure 2.3 **The cancer stem cell theory.** In metastasis: cancer stem cells are the cells that are believed to be responsible or have the capability to move to distant organs and create full tumors. In Relapse: cancer stem cells are believed to be responsible or have the capability to evade cell death and resist treatment then repopulate the tumor. In special circumstances selective pressures can trigger (hypoxia, chemotherapy, etc.) differentiated non-cancer stem cells can undergo EMT and become cancer stem cell (green arrows). Epithelial to mesenchymal transition (EMT), cancer stem cell (CSC).

2.4 Breast cancer

2.4.1 Breast cancer incidence and mortality

Breast cancer is a disease that occurs in one mutated cell that proliferated uncontrollably. It is estimated that there will be about 255,180 newly diagnosed cases of breast cancer in 2017, and 63,410 of these cases will be non-invasive carcinoma in-situ. The Surveillance, Epidemiology, and End Results (SEER) program has been instituted by the National Cancer Institute and has collected clinical, pathological and demographic

data on cancer patients since 1973. Based on the most recent SEER statistics, the lifetime risk of developing breast cancer in women is 12.32%, and while men can also develop breast cancer, the risk is much lower at 0.13% (Desantis et al., 2016). Breast cancer is the second leading cause of cancer-related deaths in women with an expected 40,610 mortalities in 2017 (N Howlader et al., 2017). While the mortality rate has fallen 39% since 1989, they have remained steady in recent years for women under the age of 50. The current breast cancer 5-year survival rate is 90% (N Howlader et al., 2017). Both the increases in breast cancer incidence and the decline in mortality can be attributed to introduction of mammographic screening and better treatment options (Glass, Lacey, Carreon, & Hoover, 2007). Clinical outcome is highly dependent on early detection as as well as other key factors including molecular type, stage, grade, and age.

2.4.2 Breast cancer risk factors

There are many factors that are either positively or negatively associated with the risk of developing breast cancers. These factors fall into two categories, those that are modifiable and those that are non-modifiable. Non-modifiable risk factors are those you cannot change, such as gender, age, race, and family history. Modifiable risk factors are those that can be adjusted such as weight, dietary intake, breastfeeding, alcohol consumption, smoking, and pregnancy (Table 2.2). Non-modifiable breast cancer risk factors are gender, age, race, breast density, age of menarche, genetic susceptibility,

Table 2.2 **Breast cancer risk factors**

Relative risk	Non-modifiable factor	Modifiable factor
>4.0		
	• Age (65+ vs. <65 years, although risk increases across all ages until age 80) • Biopsy-confirmed atypical hyperplasia • Certain inherited genetic mutations for breast cancer (BRCA1 and/or BRCA2) • Ductal carcinoma in situ • Lobular carcinoma in situ • Personal history of early-onset (<40 years) breast cancer • Two or more first-degree relatives with breast cancer diagnosed at an early age • Breast density >75% by area	• None
2.1-4.0		
	• High endogenous estrogen or testosterone levels (post-menopausal) • Mammographically extremely dense (>50%) breasts compared to less dense (11%-25%) • One first-degree relative with breast cancer	• High-dose radiation to chest
1.1-2.0		
	• Ashkenazi Jewish heritage • Early menarche (<12 years) • Height (>5 feet 3 inches) • Late menopause (>55 years) • Mammographically dense (26%-50%) breasts compared to less dense (11%-25%) • Non-atypical ductal hyperplasia or fibro adenoma • Personal history of breast cancer (40+ years) • Personal history of endometrium, ovary, or colon cancer • Any benign breast disease	• Alcohol consumption • Diethylstilbestrol exposure • High socioeconomic status • Late age at first full-term pregnancy (>30 years) • Never breastfed a child • No full-term pregnancies • Obesity (post-menopausal)/adult weight gain • Recent oral contraceptive use • Recent and long-term use of menopausal hormone therapy containing estrogen and progestin • Active and passive smoking

(Boyd et al., 2007; Gierch & Vogel, 2004; N Howlader et al., 2017; Yaghjyan et al., 2011)

and family history. Men can in fact develop breast cancer, however, it is very rare and associated with a breast cancer susceptibility antigen 2 (BRCA2) mutation. The lifetime

risk of a man having breast cancer is 1 in 1000, while the risk in females compared to males is 100 times greater, about 1 in every 8 women will be diagnosed with breast cancer (Howlader N, Noone AM, Krapcho M, Miller D, Bishop K, Altekruse SF, Kosary CL, Yu M, Ruhl J, Tatalovich Z, Mariotto A, Lewis DR, Chen HS, Feuer EJ, 2013). The most prevalent risk factor for female breast cancer is age, and the probability of developing invasive breast cancer in the next 10 years increases with age and is greatest in women that are 70 years old (N Howlader et al., 2017). Caucasian and African American women have the highest incidence of breast cancer while Asian, Native American, and Hispanic women have a lower overall risk of breast cancer (Copeland et al., 2014; Desantis et al., 2016; Gierch & Vogel, 2004).

Breast density is the proportion of fat to fibrous and glandular tissue (dense tissue that appears light on a mammogram) that can be seen and quantified on the mammogram. Breasts that are more than 75% dense by area are at a 4 to 6 fold greater risk of developing breast cancer (Boyd et al., 2007; Yaghjyan et al., 2011).

Medical history is also a risk factor with breast cancer rates being higher in women who have been diagnosed with ductal carcinoma in situ (DCIS), lobular carcinoma in situ (LCIS), or atypical hyperplasia (Donaldson et al., 2017). Patients who have had previous biopsies, including number and outcome are also at increased risk for developing breast cancer. This increase is most prevalent in women who are over the age of 50 (Gail et al., 1989; Nichols, Lee, & Roh, 2017).

Only about 10% of all breast cancers diagnosed are due to known inherited genetic mutations (Figure 2.5), another 15% to 20% have family history, but no known inherited mutation (Schrader, Sharaf, Alanee, & Offit, 2015). The most common mutation in inherited cases is in 2-breast cancer susceptibility 1 and 2 (BRCA1, BRCA2) genes. Both are tumor suppressor genes whose protein is involved in the double strand break repair as well as mismatch repair (Chapman, Taylor, & Boulton, 2012; Wu, Lu, & Yu, 2010). The risk of developing breast cancer in these patients is 72% for BRCA1 mutation carriers and 69% for BRCA2 mutation carriers BRCA2 (Kuchenbaecker et al., 2017; Lippi, Mattiuzzi, & Montagnana, 2017).

Furthermore, there have been three founder mutations discovered in Ashkenazi Jewish women in BRCA1 and BRCA2 genes. As many as 11% of all breast cancers in the Ashkenazi Jewish population are due to these founder mutations, this is substantially higher than other populations (Gabai-Kapara et al., 2014). Overall, inherited genetic mutations make up a small number of all diagnosed breast cancers, these include the high penetrance mutations such as BRCA1 and BRCA2. Currently breast cancer is largely a random and exposure driven disease. Family history is another important factor and the risk of breast cancer increases with each first-degree relative diagnosed with breast cancer. While family history and genetic susceptibility can be very closely related due to the inheritance of genetic mutations, they can also be due to shared environmental affects as well.

Modifiable factors that affect your risk for breast cancer are parity, age at first birth, lifetime duration of breast feeding, age at menopause, body mass index, use of oral contraceptives, use of hormone replacement therapy, smoking, and use of alcohol (Howell et al., 2014; Morimoto et al., 2002). These factors are summarized in Table 2.2. While the association of these factors is established with breast cancer, they do not always hold true for different stages and types of cancers and between different populations (Anderson, Schwab, & Martinez, 2015; Barnard, Boeke, & Tamimi, 2015; Turkoz et al., 2013). For example, parity seems to be protective for breast cancer but when comparisons are corrected for types of breast cancer, there may actually be an increased risk for triple negative breast cancers and decreased risk of luminal A breast cancers (Barnard et al., 2015; Phipps, Malone, Porter, Daling, & Li, 2008; Tamimi et al., 2012).

Most recently, a large-scale project was put together by four consortia, the Collaborative Oncological Gene-environment Study (COGS), to identify susceptibility loci in breast cancer, as well as other hormone-related cancers. COGS used the Genome Wide Association Studies (GWAS) to find areas in the genome with the greatest potential to be associated with risk then genotyped over 200,000 single nucleotide polymorphisms (SNP) in these areas in women of European descent. SNPs are changes in a single nucleotide at a specific position in the DNA, or locus. By doing this, 41 novel loci were found to be associated with breast cancer risk (Michailidou et al., 2014). Studies such as this have contributed to the discovery of over 80 loci that are associated with breast

cancer risk (Michailidou et al., 2015). However, these studies have been lacking populations other than women of European descent, a major limitation. Furthermore, precisely how these SNPs are being used to help calculate risk is still being studied (Gail, 2015; Howell et al., 2014).

These risk factors have been put together to create assessment tools that attempt to predict breast cancer risk and give counseling to individual women as part of a prevention strategy. The most well-known of these models is the breast cancer risk assessment tool, or the Gail model, created by the national cancer institute (NCI) available at https://www.cancer.gov/bcrisktool/. The tool asks eight questions based on key breast cancer risk factors to calculate lifetime and five-year breast cancer risks. Possible interventions can be implemented based on this model for risk reduction including modifying lifestyle factors or even drug interventions (Howell et al., 2014; Kushi et al., 2012; Moyer & U.S. Preventive Services Task Force, 2013). While the Gail model is one model of risk assessment there are other models have been developed to include more in-depth familial traits or modifiable risks, such as the Claus model, Pfeiffer cohort study, Tyrer-Cuzik model (IBIS), and the Breast and Ovarian Analysis of Disease Incidence and Carrier Estimation Algorithm (BOADICEA) (Antoniou, Pharoah, Smith, & Easton, 2004; Claus, Risch, & Thompson, 1994; Pfeiffer et al., 2013; Tyrer, Duffy, & Cuzick, 2004).

2.4.3 Menarche, thelarche and breast cancer risk.

Menarche and thelarche are often used as surrogates of puberty to determine breast cancer risks. Precocious menarche, generally defined as younger than eleven years old, is associated with increased risk of developing breast cancer (Collaborative Group on Hormonal Factors in Breast Cancer, 2012; Kelsey, Gammon, & John, 1993; Latronico, Brito, & Carel, 2016; G. D. Mishra et al., 2017; Ritte et al., 2013). In fact, studies have shown that each one year delay in menarche is associated with 5-10% reduction in breast cancer risk (Collaborative Group on Hormonal Factors in Breast cancer, 2012; Hsieh, Trichopoulos, Katsouyanni, & Yuasa, 1990). Furthermore, breast duct development is also associated with breast cancer risk, rendering thelarche an important risk factor as well. This is a particular concern because the age of menarche and thelarche is declining overall in the past one hundred years (section 1.1.2). African American girls on average begin thelarche almost a full year earlier than European white girls (Euling et al., 2008; Steingraber, 2007).

2.4.4. Breast cancer subtypes and staging

The severity of breast cancer is defined by the pathological stage, which is based on tumor size, lymph node status, and metastasis. Breast cancer is staged from 0-IV, as described in Table 2.3 based on the American Joint Committee for Cancer. The implementation of mammographic screening in 1989 has led to a decrease in late stage breast cancer at diagnosis and incidence of invasive breast cancer, in fact, stage I breast cancer is now the most often diagnosed stage (Siegel, Miller, & Jemal, 2016).

Table 2.3. **Breast cancer staging system** (AJCC)				
Stage		TNM system		
		T	N	M
0		Tis	N	M
I	IA	T1	N0	M0
	IB	T0	N1mi	M0
		T1	N1mi	M0
II	IIA	T0	N1	M0
		T1	N1	M0
		T2	N0	M0
	IIB	T2	N1	M0
		T3	N0	M0
III	IIIA	T0	N2	M0
		T1	N2	M0
		T2	N2	M0
		T3	N1	M0
		T3	N2	M0
	IIIB	T4	N0	M0
		T4	N1	M0
		T4	N2	M0
	IIIC	Any T	N3	M0
IV		Any T	Any N	M1

TX	Primary tumor cannot be assessed
T0	No evidence of primary tumor
Tis	Carcinoma in situ
Tis (DCIS)	Ductal carcinoma in situ Tis
(LCIS)	Lobular carcinoma in situ
Tis (Paget's)	Paget's disease of the nipple NOT associated with invasive carcinoma and/or carcinoma in situ (DCIS and/or LCIS) in the underlying breast parenchyma.
T1	Tumor ≤ 20 mm in greatest dimension
T1mi	Tumor ≤ 1 mm in greatest dimension
T1a	Tumor > 1 mm but ≤ 5 mm in greatest dimension
T1b	Tumor > 5 mm but ≤ 10 mm in greatest dimension
T1c	Tumor > 10 mm but ≤ 20 mm in greatest dimension
T2	Tumor > 20 mm but ≤ 50 mm in greatest dimension

Table 2.3 continued.	
T3	Tumor > 50 mm in greatest dimension
T4	Tumor of any size with direct extension to the chest wall and/or to the skin (ulceration or skin nodules)
T4a	Extension to the chest wall, not including only pectoralis muscle adherence/invasion
T4b	Ulceration and/or ipsilateral satellite nodules and/or edema (including peau d'orange) of the skin, which do not meet the criteria for inflammatory carcinoma
T4c	Both T4a and T4b
T4d	Inflammatory carcinoma
Distant Metastases (M)	
M0	No clinical or radiographic evidence of distant metastases
cM0(i+)	No clinical or radiographic evidence of distant metastases, but deposits of molecularly or microscopically detected tumor cells in circulating blood, bone marrow, or other nonregional nodal tissue that are no larger than 0.2 mm in a patient without symptoms or signs of metastases
M1	Distant detectable metastases as determined by classic clinical and radiographic means and/or histologically proven larger than 0.2 mm
Regional Lymph Nodes (N)	
NX	Regional lymph nodes cannot be assessed (for example, previously removed)
N0	No regional lymph node metastases
N1	Metastases to movable ipsilateral level I, II axillary lymph node(s)
N2	Metastases in ipsilateral level I, II axillary lymph nodes that are clinically fixed or matted; or in clinically detected ipsilateral internal mammary nodes in the absence of clinically evident axillary lymph node metastases
N2a	Metastases in ipsilateral level I, II axillary lymph nodes fixed to one another (matted) or to other structures
N2b	Metastases only in clinically detected ipsilateral internal mammary nodes and in the absence of clinically evident level I, II axillary lymph node metastases
N3	Metastases in ipsilateral infraclavicular (level III axillary) lymph node(s) with or without level I, II axillary lymph node involvement; or in clinically detected ipsilateral internal mammary lymph node(s) with clinically evident level I, II axillary lymph node metastases; or metastases in ipsilateral supraclavicular lymph node(s) with or without axillary or internal mammary lymph node involvement
N3a	Metastases in ipsilateral infraclavicular lymph node(s)
N3b	Metastases in ipsilateral internal mammary lymph node(s) and axillary lymph node(s)
N3c	Metastases in ipsilateral supraclavicular lymph node(s)

Historically, breast cancer sub-types have been pathologically determined based on the expression of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (Her2Neu) (Verhaak et al., 2010). Further, molecular subtypes of breast cancer, based on gene expression patterns of the tumor, highly overlap with these receptor biomarkers. Breast cancer is composed of many different subtypes. In fact, there are at least 21 histologically different types of breast cancer, with at least 5 molecular subtypes based on receptor status (Bernard et al., 2009; Blows et al., 2010; O'Brien et al., 2010; Verhaak et al., 2010). Patient stratification into these sub-types has led to better treatment and survival (Anderson et al., 2015).

Classifying breast cancer based on gene expression profiles has helped to further define breast cancer in clinically relevant ways. Expression microarray has been employed to profile breast cancers based on their intrinsic gene expression profiles. Of these studies, the first was done by Perou et al., (2000), they defined 5 molecular subgroups of breast cancer based on gene expression: Luminal A, Luminal B, Her2-enriched, Basal-like (ER-, PR-, and HER2-), and Normal-like subtype (Perou et al., 2000). Further gene profiling has led to the addition of "Claudin low" as a further molecular subtype of Basal like (Bernard et al., 2009; Hu et al., 2006). The clinical utility of these molecular subtypes has been shown, in that they respond differently to chemotherapy and have different risks of local and regional relapses (Rouzier et al., 2005; Voduc et al., 2010). Clinically, the expression based on immunohistochemical staining of ER, PR, and Her2Neu have been used as surrogates of these intrinsic

molecular subtypes and are most commonly grouped into four categories: Luminal A, Luminal B, Her2-enriched, and Triple Negative (Howlader et al., 2014; Prat et al., 2013). These subtypes are further described in (Table 2.4).

Table 2.4 Breast cancer subtypes.

	ER	PR	Her2 Neu	Prevalence	Secondary markers	Targeted treatment	Characteristics
Luminal A	+	+	-	73%	Ki67(-) CK8(+) CK18(+)	Targeted endocrine treatment	Favorable prognosis
Luminal B	+	+	+/-	10%	Ki67(+) CK8(+) CK18(+/) CCNB1(+)	Targeted endocrine treatment	Highly proliferative, intermediate prognosis
Triple negative	-	-	-	12%	CK5(+) CK17(+) CD44(+) EGFR(+/-)	No targeted therapy	Risk at younger age, aggressive, further divided into Basal like, claudin-low, and normal like
Her2Neu enriched	-	-	+	5%	Ki67(+)	Targeted monoclonal antibodies	Risk at younger age, highly aggressive

(Eroles, Bosch, Pérez-Fidalgo, & Lluch, 2012; Nadia Howlader et al., 2014; Lehmann et al., 2011; Prat et al., 2013)

Luminal cancers are thought to originate in the luminal epithelial cells. Luminal A breast cancers are positive for both ER and PR and they are negative for Her2. Luminal A cancers are the most often diagnosed and have the most favorable prognosis. This is due to tumor response to hormonal treatment, and the less aggressive nature of the

tumor. Luminal B tumors differ in that they may also be HER2-positive and are highly proliferative, as indicated by high amounts of ki67 staining and other proliferation-related markers (Cheang et al., 2009; Tran & Bedard, 2011). Her2-enriched tumors are negative for both ER and PR, but highly express Her2. While these tumors are highly proliferative and aggressive, targeted treatment against Her2-positive tumors have drastically improved patient outcomes (Blows et al., 2010). Triple negative breast cancers are negative for ER, PR, and Her2, and the majority are of the Basal-like subtype based on their gene expression profile (Cheang et al., 2008). Triple negative breast cancer generally has poorer outcomes, lacks targeted treatment, and is highly associated with pre-menopausal breast cancer. Furthermore, African American women are more likely to be diagnosed with this type of breast cancer, as well as Ashkenazi Jewish women and those that carry the BRCA1 mutation (Dawood, 2010; Desantis et al., 2016; Lehmann et al., 2011; Rosenthal, Moyes, Arnell, Evans, & Wenstrup, 2015).

2.4.5 Breast cancer and ancestry

The five racial ethnic groups reported in the Surveillance, Epidemiology, and End Results Program database include European-derived White, African American, Asian/Pacific Islander (API), Hispanic, and American Indian/Alaskan Natives (AI/AN). There are distinctive differences in both incidence rates and mortality in breast cancer based on ancestry (Figure 2.4).

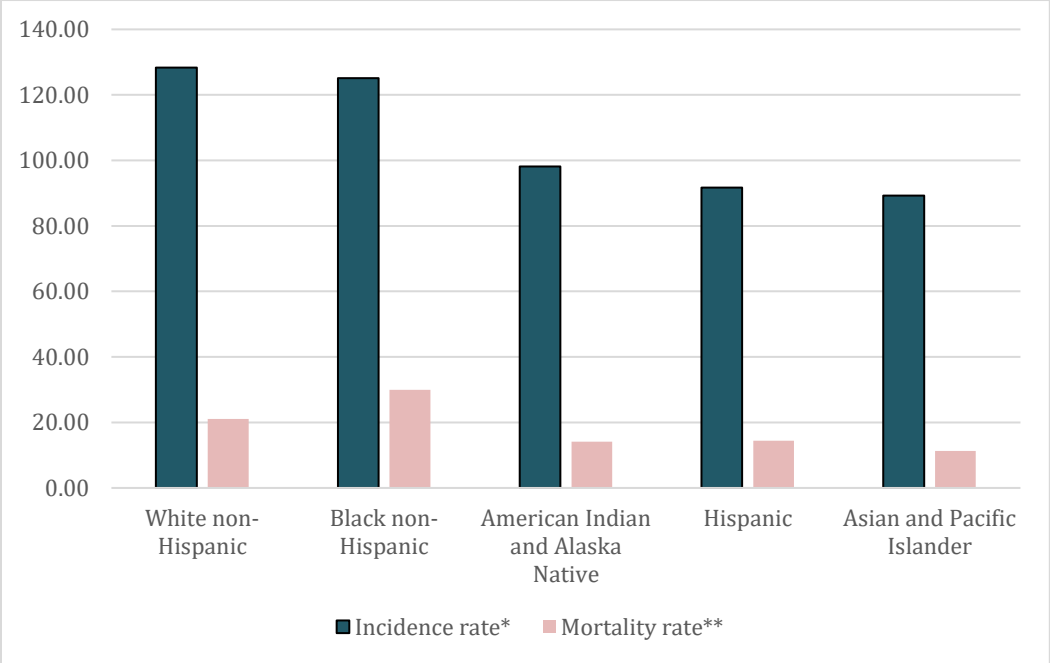


Figure 2.4 **Breast cancer incidence and mortality rates among 5 ancestral categories.** Average annual rate per 100,000, age adjusted to the 2000 US standard population. *Incidence rates, 2009-2013: North American Association of Central Cancer Registries (NAACCR), 2016. **Mortality rates, 2010-2014: National Center for Health Statistics (NCHS), Centers for Disease Control and Prevention, 2016

From 2008 to 2012, the incidence rates of breast cancer have increased for both African American women and Asian/Pacific Islanders, while there was no significant change in the other ancestral categories (Desantis et al., 2016). Furthermore, African American and European-derived White women incidence rates have converged in 2012. Overall mortality rates have declined in all but AI/AN women, where it remains unaffected, however, rates in AI/AN are not as consistent due to the lack of high quality data such as is available for the other ethnic groups. While African American and European White women now share similar incidence rates, the long-term mortality rate in African American women is 42% greater than that of European White (Desantis et al.,

2016). The median age of both diagnosis (58) and mortality (62) of breast cancer is lower in African American women when compared to European White (62 and 69, respectively).

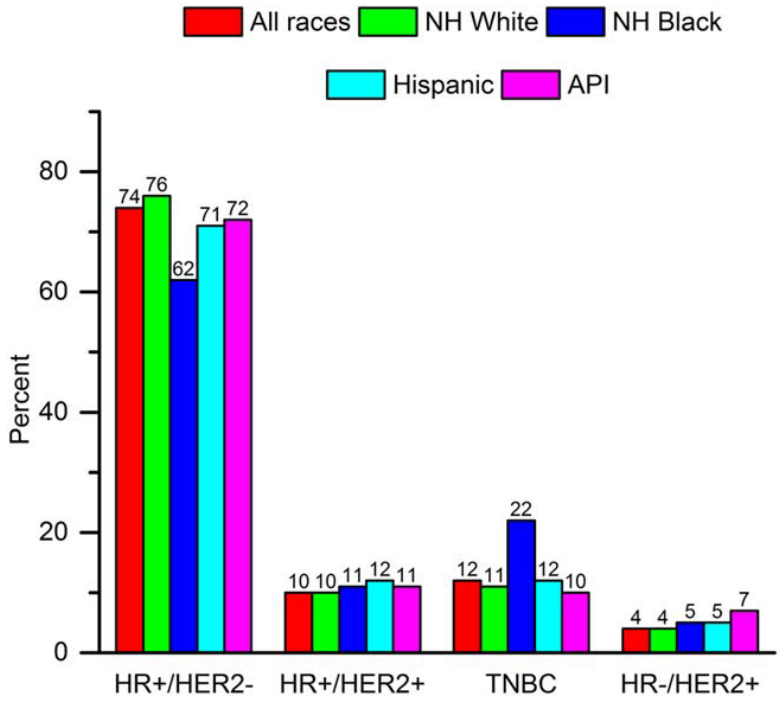


Figure 2.5 **Frequency of breast cancer subtypes based on 5 ancestral categories.** NH indicates non-Hispanic; API, Asian/Pacific Islander; HR, hormone receptor; HER2, human epidermal growth factor receptor 2; TNBC, triple-negative breast cancer. Source: North American Association of Central Cancer Registries CiNA Analytic File, 1995 to 2012. From Desantis et al., (2016), reprinted with permission.

The lower age of mortality in African American women is due to many contributing factors including access to health care, socio-economic status (Bradley, Given, & Roberts, 2002), increased diagnosis of advanced stage tumors (Desantis et al.,

2016), and higher incidence of triple negative breast cancers (Bradley et al., 2002; Carey et al., 2006; Lund et al., 2010; Morris et al., 2007; Stark et al., 2010; Stead et al., 2009). The Carolina breast cancer study is a population study of women diagnosed with invasive breast cancer in North Carolina. The study began phase one in 1993 and was the first study to find increased incidence of triple negative breast cancer in African American women (Carey et al., 2006; Newman et al., 1995). Most recent SEER data shows that the frequency of triple negative breast cancer is 22% in African American and 12% in European White women from 1995 to 2012 (Figure 2.5) (Desantis et al., 2016).

It is important to note that while differences in the frequency of triple negative breast cancer are significant and have been well verified in several studies, the overall survival of patients with regard to ancestry are inconsistent. While studies do show mortality rates are increased in African American women, they do not all control for prognostic markers such as triple negative breast cancer (Woodward et al., 2006). There are studies that show when comparing triple negative breast cancer of African American women to European White women there is no significant difference in mortality (Dawood, 2010; O'Brien et al., 2010; Pacheco et al., 2013; Sturtz, Melley, Mamula, Shriver, & Ellsworth, 2014). In contrast, increased hazard ratios in matched ER status breast cancers of different ancestries showed African American women had increased mortality when controlled for subtypes (Albain, Unger, Crowley, Coltman, & Hershman, 2009). The interaction was strongest in patients with ER-positive breast cancers: African American ER-positive breast cancer showed higher mortality than ER-positive European-

derived White breast cancer. However, no interaction was significant in this study. Another study by Lund et al also showed increased mortality when triple negative breast cancers of African American women were compared to triple negative breast cancers of European White women, with a hazard ratio of 2.0 (CI 95% 1.0-3.7) after controlling for other variables (Lund et al., 2009). They also showed that triple negative breast cancer tumors in African American women had increased expression of p16, p53, Cyclin E, and low Bcl-2 and Cyclin D (Lund et al., 2009). In 2007 a study based on the California cancer registry from 1999 to 2003 showed that at each stage triple negative breast cancer had the worst survival. Overall, African American women had the poorest 5-year relative survival for triple negative breast cancer in stages II-IV when compared to European-derived White women triple negative breast cancer with the same stages (Bauer, Brown, Cress, Parise, & Caggiano, 2007). In summary, triple negative breast cancers consistently have the worst survival outcomes, and African American women with triple negative breast cancer suffer from worse survival than European-derived White women with triple negative breast cancer.

Contributing factors for breast cancer that have been shown to be significantly different between African American and European White women include age of first parity, multiparity, breast feeding, contraceptive use, education, smoking, caffeine intake, alcohol consumption, and obesity (Table 2.5) (Carey et al., 2006; Hall, Moorman, Millikan, & Newman, 2005). Multiple childbirths and earlier age of first child are frequently seen in African American women and may increase their risk of breast cancer

(Danforth Jr, 2013; Hall et al., 2005). Breast feeding is a risk factor that is inversely related to triple negative breast cancer risk, and multiple studies show African American women breast feed significantly less than European White women (Hall et al., 2005; Millikan et al., 2008; Sturtz et al., 2014). Not only do African American women differ in which risk factors affect them the most, but how the risk factors affect them also differs (Table 2.5).

Table 2.5 **Risk factors prevalence and odds based on ancestry**

	AA compared to EW	**AA compared to AA controls**	**EW compared to EW Controls**	**Pattern of risk**
Age at first full-term pregnancy	Younger	No association with risk	Later pregnancy increases BC Risk	Different
Parity	Less likely to be nulliparous	In pre-menopausal nulliparous slightly decreases BC risk	Nulliparous increases BC risk	Different
Breast feeding	More likely to never have breast fed	Reduced BC risk	Not significantly associated with reduced BC risk	Different
Oral contraceptive	Longer use of contraceptive	Slight increase in BC risk	Slight increase in BC risk	Same
Body size	Larger body mass index	No association with risk	No association with risk	Same
Waist to hip ratio	Large waist/hip ratio	Larger ratio increases BC risk	Larger ratio increases BC risk	Same
Education level	Lower levels of education	No association with risk	No association with risk	Same
Alcohol consumption	Less alcohol consumption	No association with risk	No association with risk	Same
Smoking	Less smoking	Prior smoking increased risk	Prior smoking not associated with risk	Different
Age at menarche	Younger age of menarche	Decreased age increase BC risk	Decreased age increases BC risk (not to the same degree as AA)	Same
HRT	No difference	Increased risk in post-menopausal	No association	Different
1st degree family member	No difference	Increased risk	Increased risk	Same
Induced abortion	No difference	No association	No association	Same
Miscarriage	No difference	No association	No association	Same

AA women have greater prevalence of certain risk factors as well as relatively higher odds of BC from risk factors. African American (AA), European White (EW), breast cancer (BC). (Dietze, Carolina, Carolina, & Seewaldt, 2017; Hall et al., 2005; Sturtz et al., 2014)

Many risk factors that have been shown to be associated, either positively or negatively, with breast cancer in European White women may have no effect or an inverse affect among African American women. These include average age of menarche, age of first child, nulliparous, hormone replacement therapy, as shown in Table 2.3 (Hall et al., 2005). Caffeine intake was found to be associated with decreased pre-menopausal breast cancers in European White but not African American women (Baker et al., 2006; Boggs et al., 2010). Multiparity was correlated with increased breast cancer risk in African American but not in European-derived White women and hormone replacement therapy was inversely related to breast cancer risk in African American women but not in European-derived White women (Hall et al., 2005).

Understanding risk factors and how they are applicable to different populations is a complex problem. The Gail or Claus models of breast cancer risk do not accurately assess breast cancer risk in African American women (Adams-Campbell, Makambi, Palmer, & Rosenberg, 2007; Adams-Campbell et al., 2009). The Gail model, originally created in 1989, was restricted to European White women because the African American sample size was too small. Since then, the model has been validated in multiple European-derived White populations but has not been validated for African American populations. Risk prediction models, such as the Gail and Clause models underestimate risk in African American women (Adams-Campbell et al., 2007; Newman, 2005). The Women's Contraceptive and Reproductive Experiences (CARE) model has been implemented to better assess risk in African American women, however, it still tends to

underestimate breast cancer risk (Lucile L Adams-Campbell et al., 2009; Gail et al., 2007). More research is therefore needed in this area.

It was originally thought that the disparity seen in African American woman compared to European White women in breast cancers was due mainly to non-biological factors such as access to health care. However, African American women had similar if not slightly higher rates of mammographic screening in 2012 (Desantis et al., 2016). While lifestyle choices and differences in known risk factors may play a role, recent studies suggest there might be inherent biologic differences within these ancestries that contribute to the disparities seen in African American breast cancer. As previously mentioned, GWAS have identified SNPs that are associated with breast cancer, and these studies have been primarily conducted on European White populations. Only 10% of the SNP index related to breast cancer are replicated in African American (Long et al., 2013). In African American women SNPs are identified at higher rates that are associated with triple negative breast cancers (Palmer et al., 2013). Therefore, African American women possess a unique genetic makeup that may make them susceptible to different cancers as seen in the epidemiological and biological data.

2.5 Breast cancer treatment

There are three main treatment modalities in breast cancer and they are: surgery, radiation, and chemotherapy. The most common treatment guidelines followed are by the American Society of Clinical Oncology (ASCO), European Society for Medical Oncology (ESMO), and the National Comprehensive Cancer Network (NCC). Treatment

regimens are highly dependent on cancer stage and to a lesser extent age, overall health, biomarkers, gene expression, and risk for recurrence. For stages 0-III, surgery is performed first, often followed with radiation therapy and neoadjuvant chemotherapy depending on the particular patient. Stage IV breast cancer has already metastasized to other parts of the body and surgery is only done as a palliative procedure. Chemotherapy is always recommended for stage IV and recurrent breast cancers. Treatment options by stage are summarized in Table 2.6.

Table 2.6 **Breast cancer treatment guidelines**.

Treatment Options	**Treatment to the Breast**	**Treatment to the Lymph Nodes**	**Chemotherapy**	**Hormonal Therapy**	**Targeted Therapy**	**Treatment to Other Parts of the Body**
STAGE 0	prophylactic total mastectomy OR lumpectomy plus radiation OR lumpectomy alone (radiation only for a limited subset of women)	none required	generally, none required	hormone-receptor-positive cancers	no current role	N/A
STAGE IA and IB	total mastectomy OR lumpectomy plus radiation	sentinel lymph node biopsy, also known OR axillary lymph node biopsy	may be given to reduce the risk of recurrence	hormone-receptor-positive cancers	targeted therapy may be used for women with cancers that have certain characteristics	N/A
STAGE IIA and IIB	total mastectomy OR lumpectomy plus radiation, in some cases following chemotherapy to shrink a large single cancer	sentinel lymph node biopsy, OR axillary lymph node biopsy AND possible radiation to supraclavicular and/or internal mammary lymph nodes	commonly recommended	hormone-receptor-positive cancers	targeted therapy may be used for people with cancers that have certain characteristics	N/A
STAGE IIIA and Operable IIIC	total mastectomy followed by radiation OR lumpectomy plus radiation following chemotherapy to shrink a large single cancer	axillary lymph node removal by traditional approach AND possible radiation to supraclavicular and/or internal mammary lymph nodes	almost always recommended	hormone-receptor-positive cancers	targeted therapy may be used for people with cancers that have certain characteristics	N/A
Stage IIIB & Inoperable IIIC	chemotherapy to shrink tumor and/or lymph nodes, followed by total mastectomy that may require removal of other nearby tissues involved with the tumor AND radiation after mastectomy	axillary lymph node removal by traditional approach AND possible radiation to supraclavicular and/or internal mammary lymph nodes	almost always recommended	hormone-receptor-positive cancers	targeted therapy may be used for people with cancers that have certain characteristics	N/A
Stage IV	surgery, radiation, or both may be used, depending on many individual factors (usually palliative)	Palliative treatment of lymph nodes	almost always recommended	hormone-receptor-positive cancers	targeted therapy may be used for people with cancers that have certain characteristics	Palliative surgery AND/OR radiation

2.5.1 Surgery

Surgery in breast cancer can be divided into two categories, breast conserving surgery and mastectomy. The overall trend over the last 30 years has been towards breast conserving surgery with a vast majority of patients undergoing this procedure (Senkus et al., 2015). Breast conserving surgery or lumpectomy involves removing the tumor while leaving as much of the breast as possible, and is always followed with radiation therapy (Darby et al., 2011). Studies show that radiation therapy reduces the recurrence rate by at least half and improves overall survival (Early Breast Cancer Trialists' Collaborative Group (EBCTCG) (Darby et al., 2011). Mastectomy is carried out when there is an inability to define clear surgical margins, when there are contraindications to radiation therapy, there is a large tumor size, or there are multiple centers of origin, or by patient choice (Association of Breast Surgery at Baso 2009, 2009; Senkus et al., 2015). Mastectomy involves removal of the entire breast. Mastectomy may avert the need for radiation therapy, however in some patient's radiation therapy is still necessary. Survival in patients with breast conserving surgery followed by radiation therapy versus mastectomy are the same (De La Cruz et al., 2016; van Maaren et al., 2016). Slightly higher rates of recurrence have been observed in patients who undergo breast conserving surgery followed by radiation therapy in the past however, currently it is at least as good as mastectomy (Fischer et al., 2002). There seems to be no difference in African American and European White women that have equal access to health care in rates of lumpectomy (Enewold et al., 2012).

2.5.2 Radiation therapy

In breast cancer radiation therapy ionizing radiation is used to eradicate cells in a specific area or in the entire breast (whole breast radiation therapy). These high energy rays destroy cells by either directly damaging the genome or via production of free radicals caused via ionization of water within the cells (Baskar, Dai, Wenlong, Yeo, & Yeoh, 2014). Radiation therapy after breast conserving surgery is always indicated, as cancer cells may be left behind. Radiation therapy tends to kill cancer cells more selectivity as their genomes are replicating (Pavlopoulou et al., 2016).

There are multiple pathways dedicated to the maintenance and repair of the genome. In breast cancer cells DNA repair pathways are often mutated and inefficient in repairing the genome. This leads to increased amounts of both double and single-stranded breaks and in turn more selective eradication of cancer cells compared to non-diseased cells with competent repair mechanisms (Mohseni-Meybodi, Mozdarani, & Mozdarani, 2009; Parshad, Tarone, Price, & Sanford, 1993; Shahidi, Mozdarani, & Bryant, 2007). Based on previous unpublished data one in our laboratory, DNA repair deficient non-tumor adjacent cells will also be killed by radiation therapy. Radiation therapy triggering of cellular adaptive responses of cancer cells allowing them to overcome cell death, remains a problem with treatment. Inherent or acquired increases in DNA repair mechanisms of cancer cells are sources of resistance to radiation therapy (Baskar et al., 2014; Toulany & Rodemann, 2013).

2.5.3 Systemic treatments

Chemotherapy

In addition to surgery and radiation therapy, systemic treatments are often used in breast cancer, these include chemotherapy, hormone therapy, and targeted therapy. The use of these treatments is highly dependent on the stage and characteristics of the tumor. Chemotherapy is always recommended in late stage breast cancer and triple negative breast cancer and can be used in stage II breast cancers upward (Table 2.8). Chemotherapy is usually given with more than one chemotherapeutic agent and the most common regimen consisting of Adriamycin and Cyclophosphamide (AC). However, cytotoxicity to the heart may be a limiting factor of this regimen and often anthracyclines (Adriamycin) may be substituted for a different class of chemotherapies (Minotti, Menna, Salvatorelli, Cairo, & Gianni, 2004). In late stage breast cancer a single chemotherapy agent such as gemcitabine is often utilized (Dawood, 2010). Chemotherapeutic agents work to eradicate cancer cells in a variety of ways that are summarized in Figure 2.6.

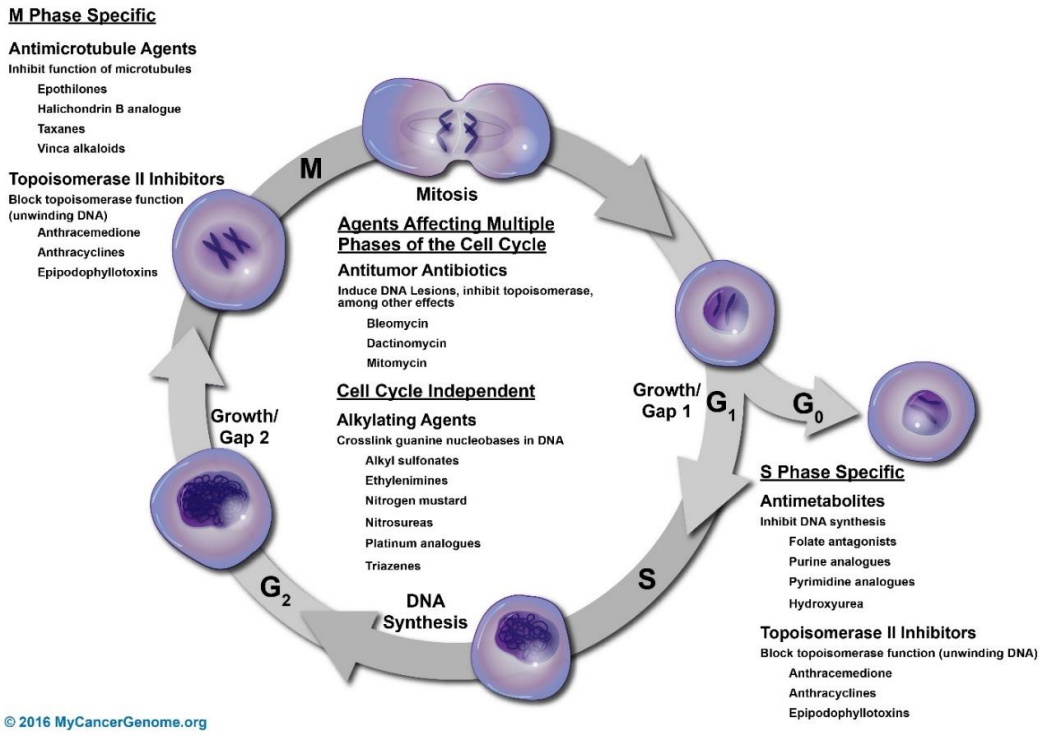


Figure 2.6. **Chemotherapeutic drug classes and mechanisms of action.** Adriamycin and cyclophosphamide are the most common chemotherapeutic agents utilized in first line treatment of breast cancer. Cyclophosphamide is a nitrogen mustard that is no cell cycle specific. Adriamycin is an anthracycline that blocks topoisomerase function. Gemcitabine is commonly used in late stage treatment as a monotherapy and is an antimetabolite that inhibits DNA synthesis (Reprinted with permission from My Cancer Genome https://www.mycancergenome.org/content/molecular-medicine/pathways/cytotoxic-chemotherapy-mechanisms-of-action, Copyright 2018 by Vanderbilt University.).

The main mechanism of action of chemotherapy drugs is that they preferentially target actively dividing cells with improperly functioning cell cycle checkpoints, which makes them more selective for cancer cells (Swift & Golsteyn, 2014). Inherent or adaptive chemotherapeutic resistance in cancer cells is a major obstacle to breast cancer treatment (Housman et al., 2014). Figure 2.7 outlines the known mechanisms of resistance to chemotherapy; as shown increased DNA repair is again an important

mechanism to be considered in chemotherapeutic treatment, because it can repair damage caused by the common breast cancer treatments (Holohan et al., 2013).

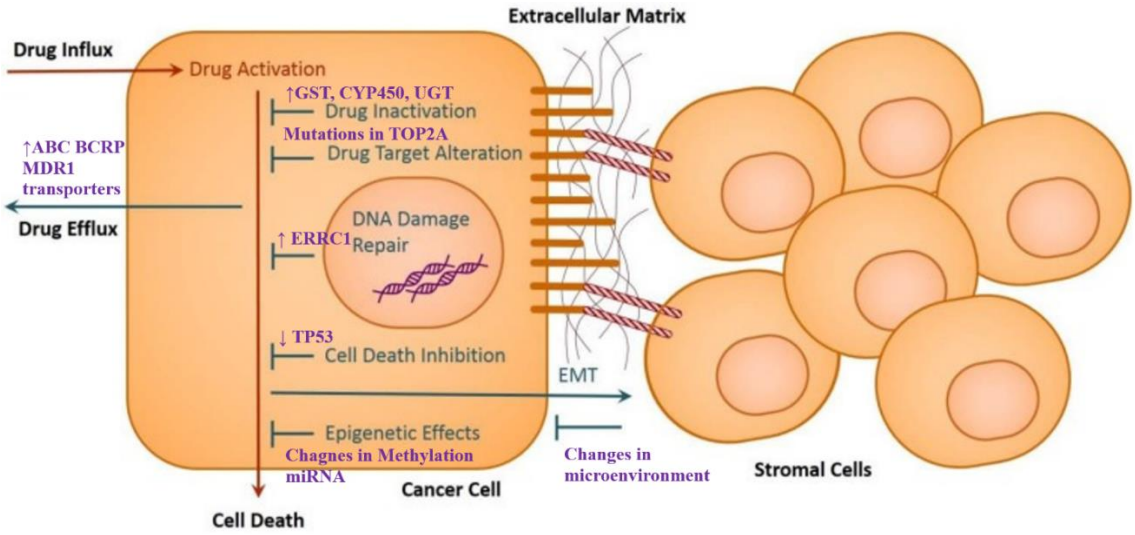


Figure 2.7 **Cancer resistance.** Drug Efflux, drug inactivation, target alterations, increased DNA repair pathways, cell death inhibition, EMT, epigenetic changes, and changes in stroma are all resistance pathways. Cancer cells often utilize multiple pathways to evade eradication. Examples of changes in these pathways are in purple. The process of EMT seems to develop an inherently more resistant and mobile (metastatic) cell.

ATP binding cassette (ABC), breast cancer resistant protein (BCRP), multidrug drug resistant protein 1 (MDR1), glutathione-S-transferase (GST), cytochrome p450 (CYP450), topoisomerase IIA (TOP2A), epithelial to mesenchymal transition (EMT)

Hormone therapy

Hormone therapy is used to interfere with estrogen and estrone (from fat) signaling in tumors that rely on estrogenic signaling for proliferation and growth. Hormone therapy is given to patients whose tumors express high amounts of estrogen

receptor, are primarily Luminal type tumor. Hormonal therapy is not effective against estrogen receptor negative cancers. Drugs used for hormone therapy fall into two main categories selective estrogen receptor modulators (SERMs) and aromatase inhibitors. SERMs work at the receptor as an antagonist at the estrogen receptor. Aromatase inhibitors work to reduce the amounts of estrogen in the body by blocking the conversion of androgen to estrogen. Aromatase inhibitors are preferred for use in post-menopausal treatment of breast cancer because they cannot stop the production of estrogen from the ovaries of pre-menopausal women (Abdulkareem & Zurmi, 2012; Kwan et al., 2017). Tamoxifen, originally developed as a contraceptive in the late 1960s, is the prototypical SERM and still widely utilized for estrogen receptor positive breast cancers (Maximov, Lee, & Jordan, 2013). Resistance to hormone therapy is common and is due to many factors, two being the activation of an altered growth signaling pathway, or the loss of hormone receptor expression. While increased levels of circulating estrogen are indeed correlated with increased risk of breast cancer via DNA repair suppression, the role of DNA repair with regards to hormone therapy resistance is unclear, if any at all (Caldon, 2014).

Targeted therapy

Breast cancer treatment has helped to revolutionize all cancer treatments with the development of targeted therapies. Patients with Her2 positive breast cancers have benefited the most from these types of treatment, which comprise 12% of all newly diagnosed breast cancers (Nadia Howlader et al., 2014). Trastuzumab is a monoclonal

antibody that specifically targets the HER2/neu protein and inhibits its activation, which in turn inhibits mitogen activated protien kinase and PI3K/Akt pathways. Both of these pathways contribute to cell growth and proliferation. Trastuzumab also has beneficial indirect effects on tumor cells. Trastuzumab increases the tumor suppressor protein PTEN levels and activity and induces cell cycle arrest via restoration of the P27 gene product (Vu & Claret, 2012). Trastuzumab also attracts immune cells to the tumor to eradicate cancer cells via an antibody-dependent cellular toxicity mechanism (Vu & Claret, 2012). While Trastuzumab has had a tremendous impact on the treatment of Her2 positive breast cancers, inherent or acquired resistance frequently develops (Gajria & Chandarlapaty, 2011; Zhang et al., 2011). Mutations in the structure of HER2 are implicated in one pathway of resistance, DNA repair and tumor suppressor proteins are implicated in other resistance mechanisms to trastuzumab (Zhang et al., 2011). Other emerging targeted therapies are being developed that use antibodies as well as small molecule inhibitors. These novel drugs not only target the HER2/neu protein but other unique proteins within cancer cells such as interleukin 5, CD275 (PDL-1), and vascular endothelial growth factor to name a few (Shepard, Phillips, Thanos, & Feldmann, 2017).

2.6 DNA repair

Maintaining the integrity of the genome is essential to life, our cells and their DNA are under constant extrinsic and intrinsic insult (Jeggo, Pearl, & Carr, 2015). DNA damage, if not repaired, results in mutations in the daughter strand and permanent alterations to the genome. Over time, this genomic instability and accumulation of

mutations lead to cancer (Helleday, Eshtad, & Nik-Zainal, 2014). The progression to cancer involves several different theories. Knudson et al. developed the two-hit theory that cancer could occur in a cell in as few as two steps and highlighted the importance of tumor suppressor genes, with each hit involving a mutation (either inherited or developed over time) (Knudson, 1971). Vogelstein et al., theorized that mutations accumulate over time in a single cell lineage to produce clonal tumors that exist as discrete pathologies (Fearon & Vogelstein, 1990). Furthermore, Vogelstein et al., showed carcinogenesis also requires activation of specific genes that can increase cell growth and proliferation, called proto-oncogenes, as well as deactivation of tumor suppressors (Fearon & Vogelstein, 1990; Lengauer, Kinzler, & Vogelstein, 1998). Eventually this theory has advanced to involve the accumulation of mutations, or alternatively, epigenetic alterations that mimic genetic mutations leading to cancer. The multiple theories of carcinogenesis highlight the importance of maintaining the genome by DNA repair pathways. Furthermore, it has been known that mutations in DNA repair pathway genes themselves contribute to genomic instability by speeding up mutational rates, and shifting cancer incidence to younger ages (Hornsby, Page, & Tomlinson, 2007; Rauth Doll, 1954).

2.6.1 Repair pathways

There are five major DNA repair pathways, base excision repair (BER), mismatch repair (MMR), nucleotide excision repair (NER), homologous recombination (HR), and non-homologous end joining (NHEJ). These DNA repair pathways are responsible for repairing specific types of damage to DNA, however, there is considerable overlap

between the types of repair performed by these pathways. The pathways are summarized in Figure 2.8. NER is the most versatile pathways, not only repairing single strand helix distorting damage, but also working with HR proteins to repair inter-strand crosslink damage and as a backup for both the MMR and BER pathways (Bret, Klein, & Moreaux, 2013; Reed, 1998).

2.6.2 Single strand repair pathways

BER corrects the non-helix-distorting single nucleotide damages to the DNA. It involves the use of specific DNA glycosylases to recognize and remove the damage, which forms an abasic site or a site that lacks a pyrimidine or purine. This site is then cleaved by endonucleases and repaired by either "short-patch" or "long-patch" BER, where the single damaged nucleotide or a sequence of 2-10 nucleotides around the damage are replaced, respectively (Zharkov, 2008). Mismatch repair, like NER, is another single strand repair pathway that happens post-replication. MMR machinery removes incorrectly incorporated nucleotides that have been incorporated during transcription, as well as removing insertion and deletion loops developed during the replication process. MMR proteins use

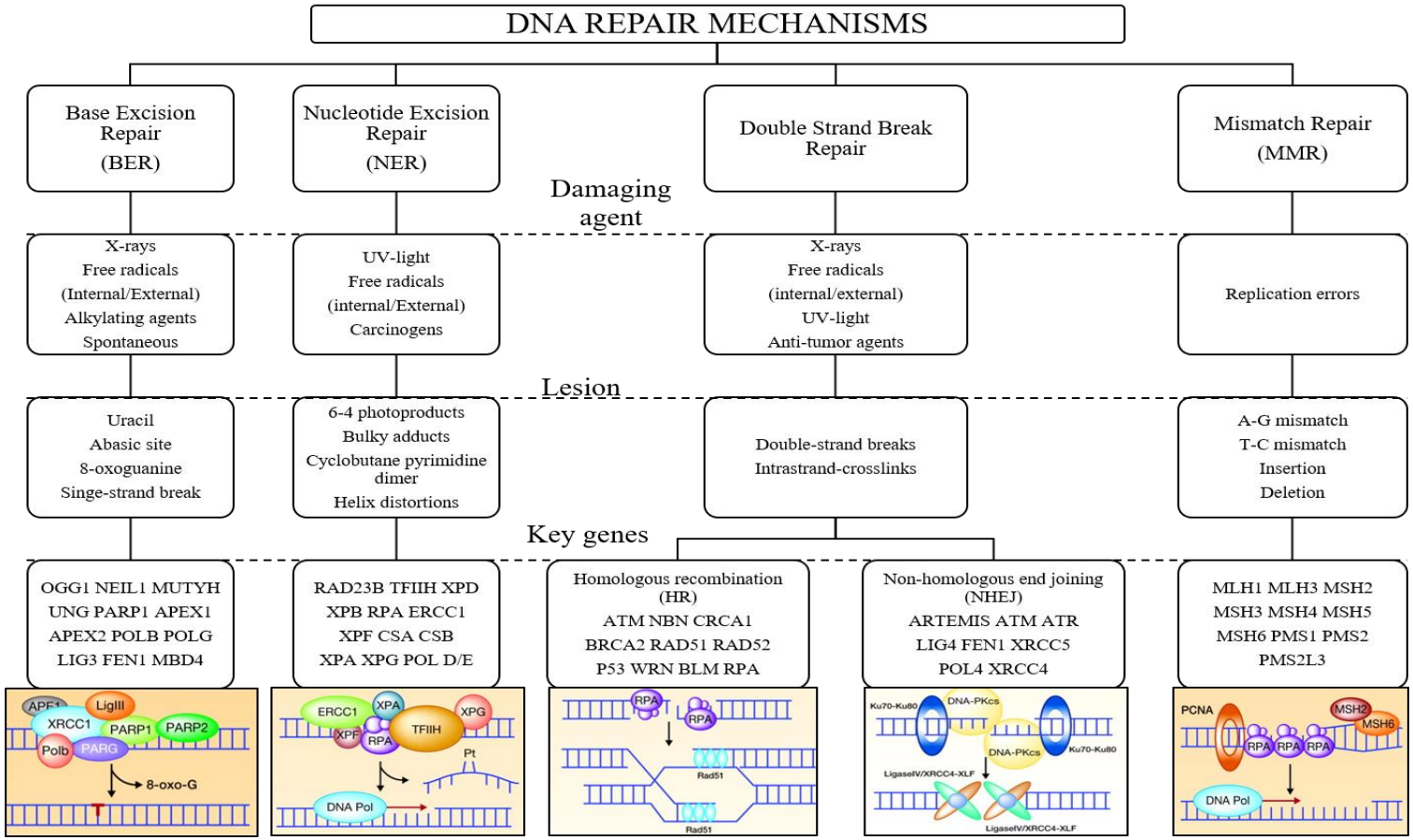


Figure 2.8 **The 5 major DNA repair pathways.** Base excision repair, nucleotide excision repair, double strand break repair, and mismatch repair are the 5 major pathways. Homologous end-joining and non-homologous end joining are two different pathways that repair double strand breaks. Adapted with permission from Jalal et al. (2011).

the parent strand of DNA as a guide to recognize, remove, and replace the incorrectly incorporated DNA base in the daughter strand.

2.6.3 Double strand repair pathways

HR is used to correct damage that lead to double strand breaks, however, it also has a role in meiosis to produce genetic variability in gamete cells. HR proteins repair these large-scale damage by utilizing the undamaged sister chromatid to accurately restore the DNA, therefore, it is mostly used during replication in S-phase and shortly after in G2 phase when sister chromatids are nearby. HR proteins involved include both ATP dependent chromatin remodeling complexes and histone-modifying enzyme to initiate repair. Then removal of the damage begins on the 5' end creating a 3' overhang that is used to search for homology with the sister chromatid. A DNA heteroduplex, or D-loop, is formed with the sister chromatid and both strands are extended accurately using the undamaged sister chromatid as a template. Finally, the D-loop is disassembled, and the two independent DNA double helixes are restored (Jasin & Rothstein, 2013).

NHEJ also repairs double strand breaks to the DNA. Because it does not utilize a homologous sister chromatid as a template, such as in HR, repair is done with much less accuracy. NHEJ is a key component of the non-DNA repair pathway, V(D)J recombination, that adds diversity to the products of immune cells.

NHEJ utilizes Ku proteins to detect double strand breaks by recognizing broken ends, then "tethers" them together. The ends are processed by removal of the damage or nucleotides by nucleases and refilled by DNA polymerases. Finally DNA ligation occurs

using specific ligases, most notably DNA ligase IV, finishing the repair (Davis & Chen, 2013; Derbyshire, Epstein, Young, Munz, & Fishel, 1994; Mahaney, Meek, & Lees-Miller, 2009; Weterings & Chen, 2008)

2.6.4 Nucleotide excision repair

NER is most commonly known for repairing DNA lesions caused by ultraviolet light (UV), however it is not limited to UV light damages. The NER pathway repairs any damage that distorts the periodicity of the DNA helix or causes bulky DNA adducts. Cyclobutane pyrimidine dimers (CPDs) and pyrimidine-(6,4)-pyrimidone products (6-4PP) caused by UV light as well as bulky adducts, and intra-strand crosslinks such as those caused by chemicals and chemotherapeutic agents are repaired by NER (Bowden, 2014; Friedberg, 2001; Reed, 1998). Furthermore, NER proteins work in conjunction with BER to repair oxidative damage and NER proteins have been found to regulate the BER pathway (Melis, van Steeg, & Luijten, 2013). Finally, the NER pathway works with HR to repair interstrand crosslinks (Minotti et al., 2004; Saffi et al., 2010; Wood, 2011).

NER has 5 major steps, (1) recognition, (2) DNA unwinding, (3) dual incision and excision, (4) strand synthesis, and (5) ligation (Figure 2.9) (Costa, 2003; Schärer, 2013). NER is divided into global genomic NER (GG-NER) and transcription coupled NER (TC-NER). TC-NER repairs DNA that is actively being transcribed and GG-NER repairs all genomic DNA in all phases of the cell cycle. The two types differ only in the first

step in which GG-NER proteins scan and find DNA lesions TC-NER is brought about by stalled RNA polymerase II (RNA polII) (Melis et al., 2013).

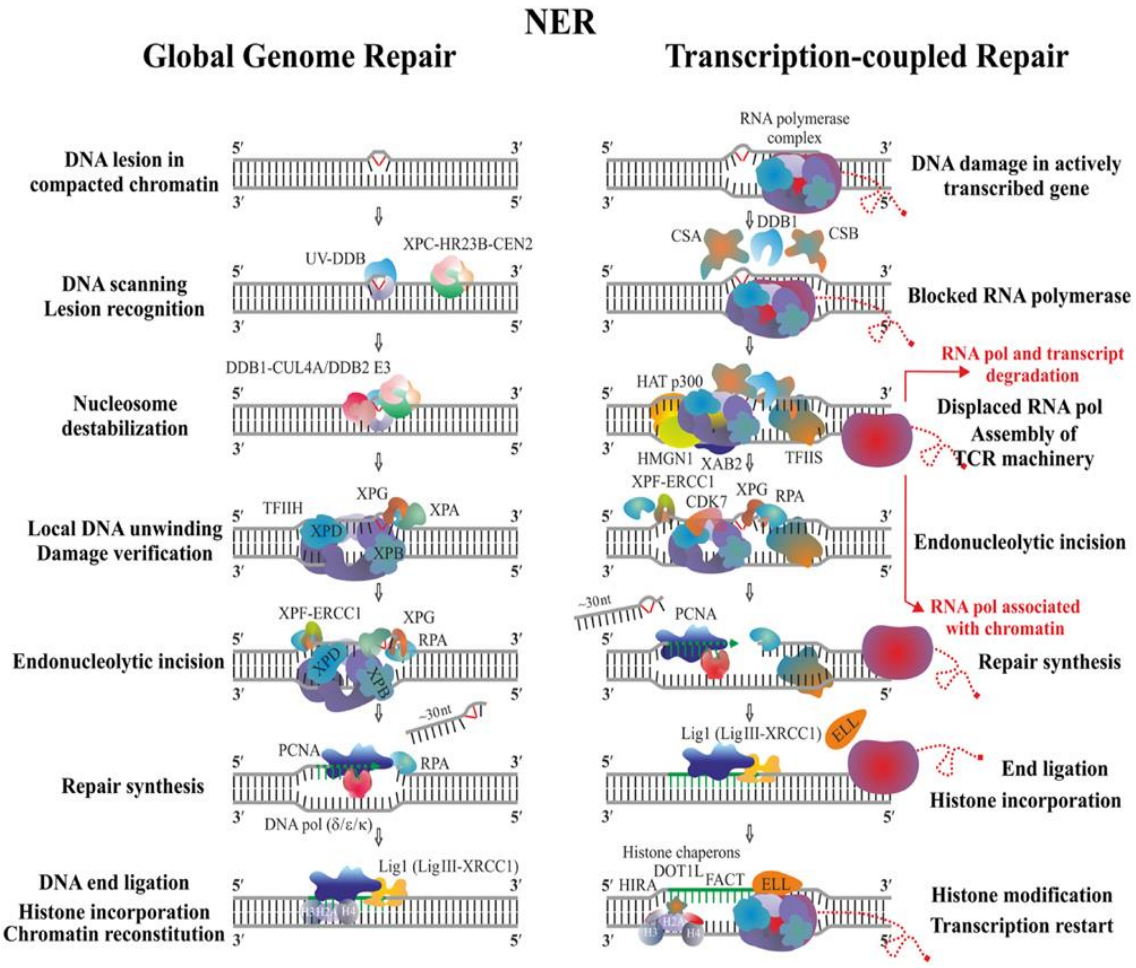


Figure 2.9 **Nucleotide excision repair pathway.** A schematic of the nucleotide excision repair pathway shows global genome repair the left and transcription coupled repair to the right. Image Copyright © 2015 Manova and Gruszka, open access under the terms of the Creative Commons Attribution License (Manova et al., 2015).

There are more than 30 proteins involved in all steps of NER and a minimum of 20 proteins are required for complete DNA repair. The first step in the NER pathway is

recognizing the damage. In TC-NER this begins with stalling of the DNA polymerase, and temporary dissociation of the polymerase. The CSB protein localizes to the lesion and recruits CSA, ring-box 1 (RBX1) and cullin 4A (CUL4A). This complex, in turn, recruits XPA binding protein 2 (XAB2) for chromatin remodeling. Transcription factor II H (TFIIH) is then brought in to begin the unwinding process. In GC-NER, recognition occurs via either the XPC/RAD23B complex or the DDB1 and DDB2 proteins that scan the genome for smaller damage. DDB1 and DDB2 recruit the XPC complex, while the XPC complex can initiate GG-NER on its own, having a greater affinity towards bulkier adducts. The XPC protein contains multiple binding domains for DNA and other proteins including TFIIH. TFIIH recruitment begins the next step of the pathway and at this point the TC and GG NER pathways converge. TFIIH forms a complex with 10 other proteins, XPB, XPD, p62, p52, p44, p34, p8, and the CDK-activating kinase (CAK) complex: MAT1, CDK7, and Cyclin H. In both TC and GC-NER this complex forms around the DNA and begins the unwinding process forming a bubble structure in the DNA, via DNA helicases XPB and XPD. XPA, RPA, and XPG are also recruited and verify that the lesion on the correct strand as well as initiating the dual incision process. RPA interacts with endonucleases ERCC1, in a complex with XPF, to make the 5' incision and XPG makes incision at the 3' end of the bubble. This results in the removal of a patch 20-30 nucleotides long and the gap is then filled by DNA polymerase δ, ε, or κ, in conjunction with proliferating cell nuclear antigen (PCNA), replication factor C (RFC), and RPA. The DNA replication machinery uses the undamaged strand as a

template to correctly restore the damaged strand of DNA. Finally, DNA ligase I in replicating cells or ligase IIIα with X-ray repair cross complementing-1 (XRCC1) in non-replicating cells seals the 3' nick to fully restore the DNA helix and sequence (R. Costa, 2003; Melis et al., 2013).

2.7 Nucleotide excision repair in cancer

Nucleotide excision repair is a double-edged sword in cancer. Nucleotide excision repair genes clearly act as tumor suppressors, however, deficient repair mechanisms in cancer etiology lead to increased genomic instability and cancer incidence at younger ages. On the other hand, overexpression of nucleotide excision repair genes and increased function of the pathway results in increased drug resistance in late stage cancer cells (Jeggo et al., 2015).

2.7.1 Nucleotide excision repair inherited diseases

There are several diseases that result from inherited mutations in genes within the nucleotide excision repair pathway including Xeroderma Pigmentosum (XP), Cockayne Syndrome (CS), and Trichothiodystrophy (TTD). XP is a rare autosomal recessive genetic disorder that results from mutations in the XP genes of the nucleotide excision repair pathway and affects 1 in 250,000 people of all ancestries (Lehmann, McGibbon, & Stefanini, 2011). There are 8 complementation groups in XP that result in deficient nucleotide excision repair function. The more deficient the nucleotide excision repair function the more severe the disease, and this is highly dependent on the specific mutation in XP. The disease is characterized by sensitivity to UV light and increased the

likelihood to develop cancers. In fact, XP patients are more than 2,000 time more likely to develop skin cancers than DNA proficient normal people (Bradford et al., 2011). This disease and it's phenotype demonstration the importance of nucleotide excision repair and its role in protection against cancer.

CS is an autosomal recessive disorder that results from mutations in either the CSB or CSA genes of the nucleotide excision repair pathway. These gene products are involved in transcription coupled nucleotide excision repair and are the root cause of the neurodegenerative disorder CS. The disease is characterized by photosensitivity, impaired development of the nervous system, and premature aging. While the disease does affect TC-NER, it does not lead to a predisposition to cancer as would be expected of DNA repair defects (Nance & Berry, 1992).

TTD is another autosomal recessive disorder that results from mutations in the nucleotide excision repair genes, XPB, XPD, and TTD (Theil, Hoeijmakers, & Vermeulen, 2014). All three of the genes are subunits of the TFIIH complex, critical for the nucleotide excision repair pathway. The disease has a variety of phenotypes including photosensitivity, ichthyosis, brittle hair and nails, intellectual impairment, decreased fertility, and short stature (Theil et al., 2014). The severity of symptoms is also highly variable with the most severe cases resulting in early mortality. The disease results in deficient nucleotide excision repair function but not elevated risks for cancer. In both TTD and CS it is likely that the blocking of RNA polymerases leads to extreme

cell toxicity and results in cell death, therefore mutations are not accumulated and there is no predisposition to cancer (de Boer & Hoeijmakers, 2000; Theil et al., 2014).

2.7.2 Nucleotide excision repair in breast cancer

Nucleotide excision repair is also implicated in breast cancer. Multiple studies have shown that that lymphocytes in breast cancer patients have deficient nucleotide excision repair compared to non-diseased controls (Kovacs, Stucki, Weber, & Müller, 1986; Ramos et al., 2004; Shi et al., 2004). Furthermore, these studies showed that decreased nucleotide excision repair capacity is associated with increased risk of developing breast cancer (Ramos et al., 2004; Shi et al., 2004). These studies, however, utilized lymphocytes as surrogates for breast tissue. Latimer et al. (2010) has shown that in stage I breast cancer explants there is an intrinsic deficiency in the nucleotide excision repair pathway. Not only were DNA expression and protein expression under-expressed when compared to normal breast cultures, but functional DNA repair capacity, as measured by the Unscheduled DNA Synthesis assay was also deficient (Latimer et al., 2010). Unscheduled DNA synthesis functional analysis further revealed that compared to the same patient's non-tumor adjacent explant the nucleotide excision repair function was also decreased in unpublished data created by our laboratory. The connection between cancer and DNA repair is evident.

2.8 Model systems

Studying DNA repair in breast cancer involves the use of different model systems. One important model are breast cancer-derived cell lines. Established breast

cancer cell lines have enriched our knowledge of breast cancer features such as proliferation, invasion, angiogenesis, metastasis, and drug resistance (Holliday & Speirs, 2011). There are currently more than 60 commercially available breast cancer cell lines, 20 of which are widely used in breast cancer research (ATCC 2017). They provide an unlimited regenerative source of breast tumor cells and possess a high degree of homogeneity within each cell line. However, there are several disadvantages to utilizing these cell lines. Most are derived from late stage metastatic breast cancer derived from pleural effusions, therefore they may not reflect qualities of the primary tumor or even the heterogeneity of the primary tumor (Burdall, Hanby, Lansdown, & Speirs, 2003; Cailleau, Olivé, & Cruciger, 1978; H D Soule, Vazguez, Long, Albert, & Brennan, 1973). Furthermore, because the cell lines are based on stage IV tumors, they may not reflect the stage I breast cancers that now represent the majority of breast tumors diagnosed in the United States (Howlader et al., 2017). Adaptation to culture of these cell lines may also cause fundamental changes in cellular and genetic characteristics not representing the primary tumor (Bahia et al., 2002). It is also clear that these cell lines continue to evolve in culture, such that genetically and functionally different sublines can be identified that have characteristics not found in the original tumor (Wenger et al., 2004). The work in this dissertation utilizers original cell lines from different stages and also from the non-diseased state, some of the well-known breast cancer cell lines derived from stage IV tumors are included as a means of comparison with the vast majority of breast cancer studies in the literature.

The Latimer laboratory has developed an organotypic tissue engineering system for the development of both non-diseased breast tissue from reduction mammoplasties as well as tissue derived from tumors. Non-diseased tissues came from a variety of sources including women of different ages (pre-, peri, and post-menopause) and ancestries. Using this method breast tumors have been successfully cultured from all stages of breast cancer including the various types.

An overview of our tissue engineering system derived from non-diseased female patients is shown in Figure 2.10. Fresh minced tissue pieces attach to a 1:1 dilution of basement membrane extract (Trevigen®) to DMEM within 24-48 hours, typically rendering the medium acidic and yellow in color. The cells are maintained in a specialized medium that had been adapted from embryonic stem cell culture called MWRI. Within 3 days, living attached cells appeared as monolayer outgrowths of multiple cell types from freshly severed 3 dimensional pieces of tissue edges. These attached cells include both epithelial and fibroblastic cells. After 10-11 days in culture a marked increase in cell populations becomes apparant, due to both cell migration and proliferation, with cells becoming visible microscopically in the areas of the dish not obscured by tissue pieces.

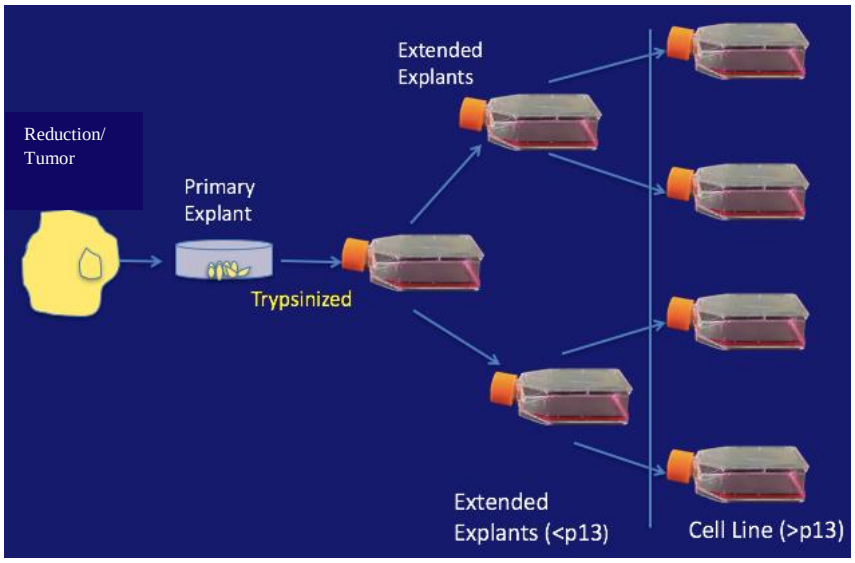


Figure 2.10 **Tissue engineering schematic**. Non-diseased and tumor cell lines created through the Latimer tissue engineering system. The primary is passaged into extended explants. After passage 13 we consider them cell lines.

The formation of epispheres *in vitro* is the first recognizable three-dimensional epithelial architecture formed in our culture system (Figures 2.11, and 2.12a, b). Epispheres develop as early as 24 hours after initial establishment of primary cultures. Epispheres, through a process of epithelial cell migration and proliferation, give rise to ductal structures over a period of 2-3 weeks in culture. With greater time in culture (2-5 weeks), linear ductal structures may form and become longer, progressively branching to form ductal networks (Figure 2.13b). Mature ducts manifest lumen containing secreted milk proteins with a diameter consistent with interlobular ducts *in vivo* (40-70 μm). Finally, the formation of intra-ductal lobular structures (Figure 2.11) and TDLUs (Figure 2.13) is also manifested in at least 40% of these long-lived cultures.

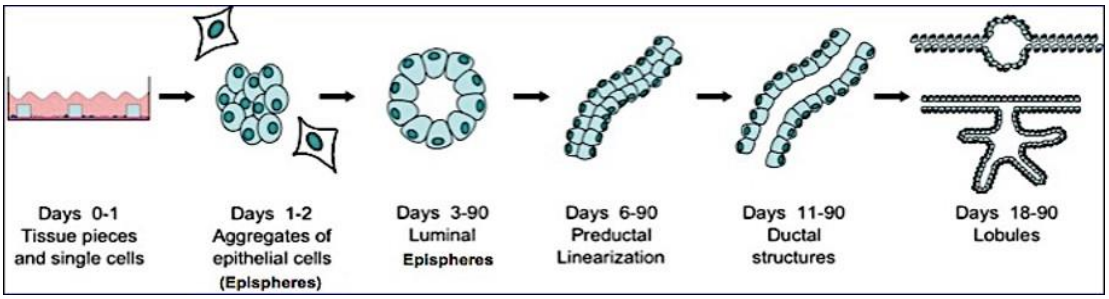


Figure 2.11 **Latimer tissue engineering system.** This timeline shows the progressive differentiation manifested by non-diseased breast reduction mammoplasties placed into primary culture

Episphere and subsequent ductal formation are active processes of cellular aggregation and recruitment rather than an artifact of the disaggregation method. Epispheres in this system are 3-dimensional clusters of 20-100 epithelial cells, with intimate contact between rounded epithelial cells and the lowest layer of cells in contact with a Matrigel substratum (Figure 2.12). Ductal structures are luminal as shown by the pattern and outline of cytokeratin-19 staining and DRAQ5-stained nuclei (Figure 2.13d, e).

One of the critical features of an organotypic culture system is the production of proteins that are related to organ function. Beta-casein is a milk protein that is rarely expressed in any primary epithelial cell culture system (Kim, Oberley, & Clifton, 1993), although it is expressed in approximately 17% of human breast tumors (Monaco, Bronzert, Tormey, Waalkes, & Lippman, 1977). In our cultures, this milk protein is expressed in the lumen of mammospheres and ductal structures (Figure 2.13f, g).

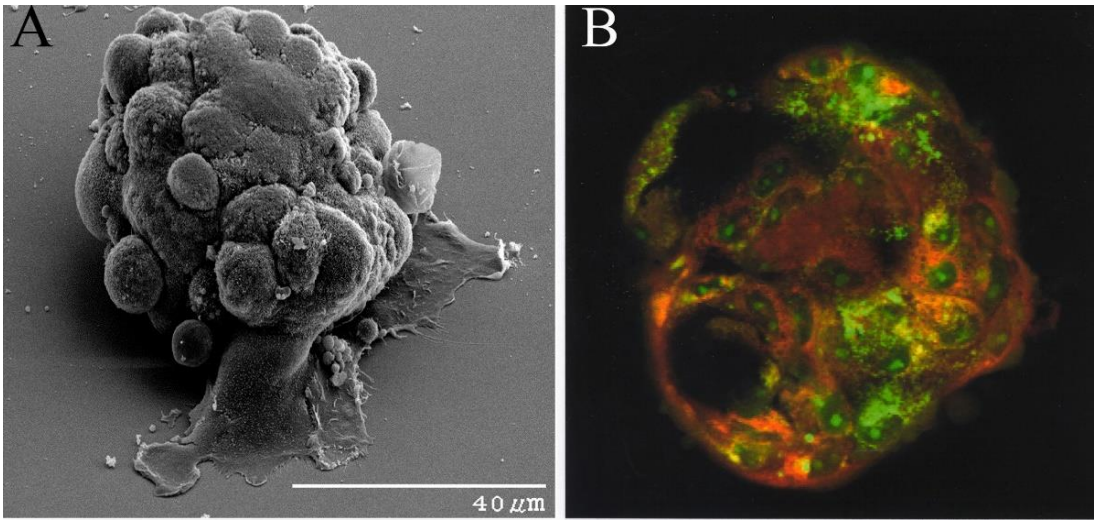


Figure 2.12 **Epispheres in culture.** (A) Scanning electron micrograph (SEM) of a normal epithelial epispheres after 2 days in culture. These cells remain in close contact via desmosomes, and retain normal epithelial tissue architecture and polarity. (B) Confocal microscopy image of a episphere, showing a view of the luminal interior of the episphere. Nucleoli are stained with acridine orange (green) and Mitochondria are stained with mitrotracker red (red).

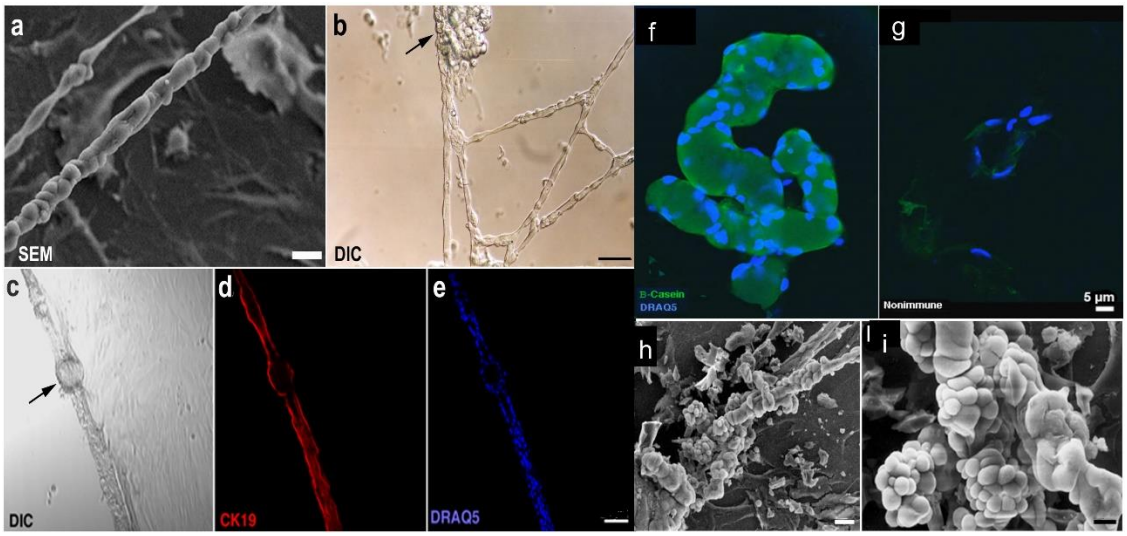


Figure 2.13. **Ductal systems in culture.** Electron micrograph (a) and DIC images (b) of duct-like structures arising from the culture of non-diseased human breast reduction epithelium, showing the elevation of the structures above the substratum and the branching nature of the ductal network. These ducts stain with CK-19 (d), and contain lumen. DRAQ5 stains the nuclei (blue) to show the luminal space between the hollow columns of cells. Functional secretion of beta casein is observed (f, g), and generation interstitial and terminal lobular structures (arrows). Higher power electron micrographs of more advanced lobular structures are given in (h) and (i).

Using this same tissue engineering system, the Latimer laboratory has also developed 60 tumor cell lines, without the use of any transforming agents. This contrasts with the most commonly used cell lines that generally use transforming agents. It is often not known how these agents can affect DNA repair, and more research is needed to assess the DNA repair in established cell lines (Burdall et al., 2003).

2.9 Nucleotide excision repair in cancer treatment

While DNA repair works to maintain a healthy genome in non-diseased cells, these same mechanisms in tumor cells often contribute to tumor resistance to genotoxic chemotherapy. Advanced stage cancer cells often show increased expression/function of DNA repair mechanism proteins to remediate damage from genotoxic insults (Helleday, Petermann, Lundin, Hodgson, & Sharma, 2008). The core of many chemotherapeutic agents is to damage the cancer cell enough to render it unable to replicate or force apoptosis. If DNA repair mechanisms remediate this damage as opposed to triggering apoptosis, the cell can escape death or continue proliferating. Cisplatin is the prototypical drug remediated by nucleotide excision repair. It causes 1,2-intrastrand cross-links between purine bases. Increased expression of nucleotide excision repair genes, most notably XPC, has been shown in many cisplatin resistant cancers (Lai et al., 2011; Rosell et al., 2003; Wang, Dombkowski, Chuang, & Xu, 2004). Furthermore, down regulation of excision repair cross-complementation group 1 (ERCC1) in prostate cancer cells has been shown to sensitize these cells to cisplatin (Cummings et al., 2006). In breast cancer treatment anthracyclines and cyclophosphamide are heavily used and

studies have shown that increased expression of nucleotide excision repair genes is also associated with chemotherapeutic resistance (Andersson et al., 1996; McHugh, Spanswick, & Hartley, 2001; Müller, Thomale, Rajewsky, & Seeber, 1998).

2.9.1 Nucleotide excision repair in cancer stem cells

There is a particular lack of information on the role of nucleotide excision repair in cancer stem cells. In fact, the literature seems to have conflicting results regarding DNA repair in cancer stem cells. Cancer stem cells are thought to be more resistant cells that can regenerate the tumor after treatment, contributing to both metastases and reoccurrences. Several studies have shown that stem cells are more resistant to chemotherapy, as well as ionizing radiation (Phillips, McBride, & Pajonk, 2006; Tanei et al., 2009; Yang et al., 2015). Other studies have shown that stem cells have shown sensitivity to the antibiotic salinomycin and, contradictorily, ionizing radiation (An et al., 2015; S.-Y. Kim et al., 2012). A major drawback of these studies has been the use of commercially available cell lines including MCF7 and MDA MB231, which have high levels of nucleotide excision repair. Only Tanei et al., (2009) used primary tumor information and they showed that tumors with high amounts of CD24- 44+ aldehyde dehydrogenase positive cells were resistant to paclitaxel and epirubicin. These studies are summarized in Table 2.6. There is a clear lack of information regarding nucleotide excision repair in cancer stem cells with virtually no relevant data in the breast cancer literature.

Reference	Insult	Model System	Stem Cell Definition	Characteristic
Phillips et al., 2006	Ionizing Radiation	MCF 7	CD24- 44+	Resistant
Yang et al., 2015	Ionizing Enhanced CHK1/2	MCF 7	CD24- 44+	Resistant
Tanei et al., 2009	Paclitaxel Epirubicin	Primary	CD24- 44+ aldehyde dehydrogenase 1	Resistant
*Pavlopoulou, et al., 2016	Review			Resistant
Sajithlal et al., 2010	Adriamycin Etoposide 5-fluorouracil Cis-platinum Methotrexate Taxol	MCF 7 MDA MB231 MDA MB453 JL BTL-12	CD24-/44+ OCT3/4 blocked	Mixed depending on cell line. All cancer stem cells were sensitive to Taxol.
Kim et al., 2012	Ionizing	MDA MB231 MDA MB453	CD24- 44+ OCT3/4 blocked	sensitive
Kai et al., 2015	LBH589 Salinomycin	HCC1937, MDA-MB-231, MCF7, and SK-BR-3	aldehyde dehydrogenase 1	Sensitized by Salinomycin
Vazquez-Martin et al., 2011	Metformin	MDA MB231, SKBR3, MDA MB468	Mammosphere formation	Sensitized by metformin

Table 2.7. **Cancer stem cells resistance and sensitivity.**
*selected review describing resistance in stem cells

2.10 Summary

This dissertation is separated into three sections. First, we discovered differences in ductal formation of non-diseased breast explants in culture between African American women and European derived white women in a unique culture system. We then set out to determine if there are differences in African American women and European derived White women breast stem cells. Differences in stem cells, either proportion or potency, could explain why we see differences in the timing of ductal formation. This is relevant because the timing of ductal formation in our culture system seems to mimic the epidemiological evidence that there is a decrease in age of thelarche. This decrease in thelarche is exaggerated in African American women and we will show that this is also seen in our unique culture system. Furthermore, a decrease in puberty is a breast cancer risk factor, therefore this puts African American women at an increased risk for breast cancer. The second section focuses on the development of a medium throughput assay using this same unique culture system. This assay will be vital for replacing outdated assays such as the E-screen in testing for chemicals that can putatively disrupt breast structure formation. Finally, the third section focuses on breast cancer stem cells. Previous laboratory data has shown that there is an increase in gene expression and function on nucleotide excision repair (Appendix A). Based on these findings, we sought to determine the nucleotide excision repair in breast cancer stem cells of early and late stage cell lines. Breast cancer stem cells are believed to be a more resistant cell in a

tumor. Establishing the nucleotide excision repair capacity in these cells may lead to future drug targets.

Chapter 3

Breast cultures from African American women manifest higher stem cell percentage and potency than those from European derived white women

3.1 Introduction

3.1.1 Breast remodeling and stem cells

The breast is a tissue that undergoes constant remodeling until death. The development of the breast, known as thelarche, signifies the start of puberty and is followed shortly by menarche (Cabrera et al., 2014). As the breast undergoes normal changes in response to milestones like puberty and lactation, or cyclical monthly changes, it requires specialized cells that can self-renew and differentiate into the luminal epithelial and myoepithelial cells that together make up the plumbing system of the breast (Tiede & Kang, 2011). Breast stem cells have been the ideal model for all stem cell research because of their sheer prevalence in the tissue (Tiede & Kang, 2011). In fact, the first cloning of a sheep, Dolly, was accomplished by using a stem cell from the mammary gland (Campbell, McWhir, Ritchie, & Wilmut, 1996).

3.1.2 Thelarche and ancestry in the U.S.

Both genetic and non-genetic can affect the onset of puberty (Cabrera et al., 2014; Mishra et al., 2017). Genetics, race, maternal body mass index, prematurity or low birth

weight, fatherlessness, environmental pollutants, and percentage body fat are all factors that influence the timing of puberty (Daniel & Balog, 2009; Day et al., 2017; Flom et al., 2017; Kelly, Zilanawala, Sacker, Hiatt, & Viner, 2017; Li et al., 2017). Studies of familial traits in monozygotic twins and close relatives have shown that only about half of the variation in the age of menarche are attributed to genetics (Morris, Jones, Schoemaker, Ashworth, & Swerdlow, 2011; Towne et al., 2005). The age of menarche has been falling since the 1800s until the present (Cabrera et al., 2014; Euling et al., 2008; Steingraber, 2007) (Figure 2.2). This decrease in age of menarche is more dramatic in African American girls and it is consistent with the falling age of thelarche (Figure 3.1). African American girls go through thelarche at 9.5 years old compared to 10.3 years old in European-derived white girls (Cabrera et al., 2014; E. Daniel & Balog, 2009; Euling et al., 2008; Steingraber, 2007).

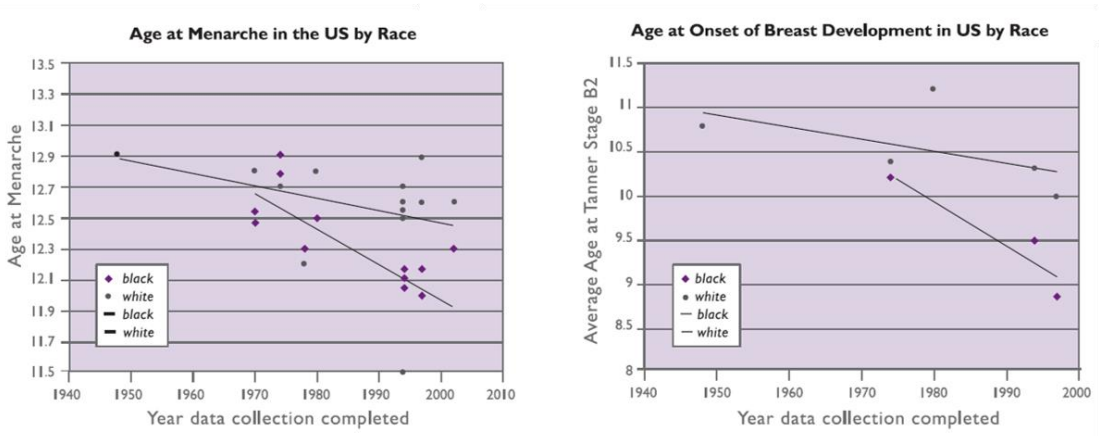


Figure 3.1. **The falling age in menarche and thelarche in the United States by ancestry.** Data from multiple sources in both the United States and Europe shows the age of both menarche and thelarche have decreased. The decline is more dramatic in African American girls. Reproduced with permission from BCPP, www.bcpp.org (T*he falling age of puberty in U.S. girls*, Steingraber, 2007)

The reason for ancestral disparity in thelarche and menarche is not well understood. African American women manifest factors that correlate with early menarche such as increased body mass index (Daniel & Balog, 2009; Spencer et al., 2013). Genetic factors have also been shown to play a role. Single nucleotide polymorphism analysis has shown a polymorphism, rs11604207, just upstream of remodeling and spacing factor 1 (*RSF1)* (Spencer et al., 2013), that correlates with decreased age of menarche. RSF1 has been implicated in both ovarian and breast cancers.

3.1.3 In vitro models of mammary differentiation

The Bissel group has developed a relevant *in vitro* culture system, which mimics *in vivo* differentiation. Using mouse mammary cells cultured under a laminin-rich basement membrane, breast alveoli with functional milk secretion has been shown (Lelièvre, Weaver, & Bissell, 1996). These alveoli do not form ductal structures but remain blind-ended circular hollow structures that die as the matrix degrades over time.

The Latimer laboratory has developed and published a novel culture system for human mammary epithelial cells that is a model of thelarche (Figure 2.12). This system allows for unusually long-term (three months or longer) establishment of normal primary cultures that begin as three-dimensional "epispheres," which are structures made up of 20-100 epithelial cells. These epispheres subsequently differentiate into complex organotypic branching ducts and lobules that demonstrate Epithelial Specific Antibody (ESA) staining, cytokeratin -18 and -19 staining, the presence of hollow lumen, polarized

epithelial nuclei, desmosomes along the lateral surfaces of the epithelial cells, and microvilli on the apical (secretory) surfaces of the polarized luminal epithelial cells. Myoepithelial cells that are also present demonstrate cytokeratin 14 staining and can be located at the basal surface of the luminal epithelial cells (Figure 3.2), or more rarely as colonies in the area around the organotypic structures. Tumor cells, in contrast, manifest a continuum of behaviors in this culture system, but completely lack the ability to form these collaborative epithelial architectures.

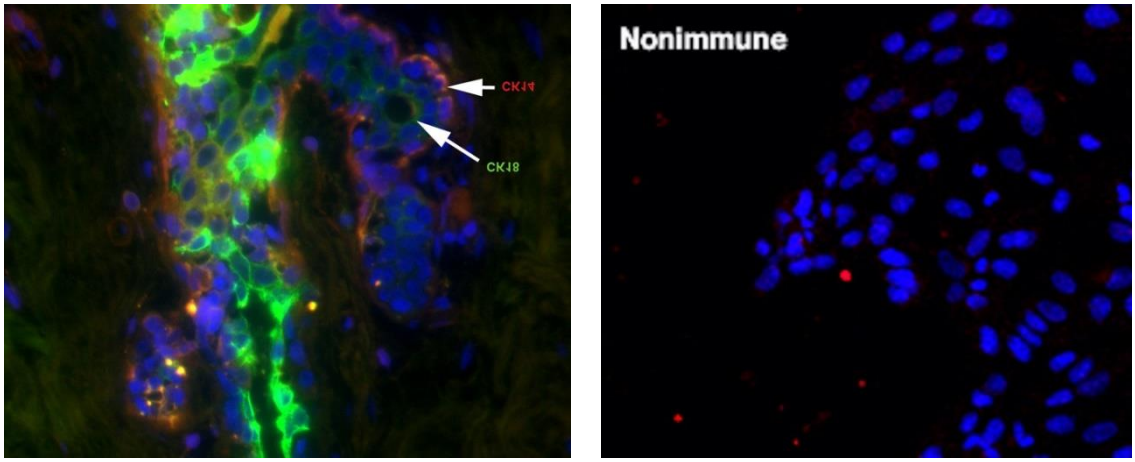


Figure 3.2 **Confocal image of breast architecture in culture.** (A) Single image from a confocal stack of a breast reduction primary culture stained with both cytokeratin 14 (Texas red conjugated CK 14) myoepithelial cells and cytokeratin 18 (FITC conjugated CK 18 green) luminal epithelial cells. The myoepithelial cells have formed a basal layer as expected under the luminal epithelial cells that have formed a lumen. Intense CK 18 staining is seen along the interior of a large ductal structure that is in part luminal (hollow) but still forming. Nuclei are chemically stained (blue) with DRAQ5.

B. Single image from a confocal stack of the Non-immune control of a similar structure from a chamber slide cultured and stained at the same time with the same explant.

3.1.4 Thelarche, menarche, and breast cancer

Thelarche and menarche, as surrogates for puberty, are well established breast cancer risk factors (Collaborative Group on Hormonal Factors in Breast cancer, 2012; Kelsey et al., 1993; Latronico et al., 2016; Mishra et al., 2017; Ritte et al., 2013). Early menarche can increase breast cancer risk by as much as 10% for each year of precocious menarche (Collaborative Group on Hormonal Factors in Breast cancer, 2012; Hsieh et al., 1990). This is particularly worrisome in the African American community, where early menarche is more common and this phenomenon may also play roles in the types of breast cancer common in African American, or in the age of onset of breast cancer, frequently pre-menopausal in African American populations.

Menarche is more often used as opposed to thelarche for age of puberty because of its relatively ease to recall. However, thelarche is the start of breast growth, the first stage of puberty. Menarche generally follows close after thelarche, however, environmental chemicals may affect them differently, and timing between thelarche and menarche may increase (Kaplowitz & Oberfield, 1999). The uncoupling of menarche and thelarche increases breast cancer risk when there is a greater than two years in between (Bodicoat et al., 2014). Timing of thelarche also affects breast cancer risk. Girls who undergo thelarche at ten years of age or younger have been shown to have an increased breast cancer risk as much as 20% higher than girls who undergo thelarche between the ages of eleven and twelve (Bodicoat et al., 2014).

In recent years, African American and European-derived white women breast cancer incidence rates have become similar (Desantis et al., 2016). However, African-American women are more likely than white women to die from the disease (Bach, 2002; Desantis et al., 2016). Breast cancer survival at five years after initial diagnosis is only 81% among African-American women compared to 92% among European-derived white women (Siegel et al., 2016). Furthermore, African American women are less likely to be diagnosed with smaller tumors (less than or equal to 2.0 cm, stages I and II) and more likely to be diagnosed with larger tumors (greater than 5.0 cm) and at later stages than white women of the same age (Desantis et al., 2016; Siegel et al., 2016). Although in the past these differences have largely been attributed to socio-economic factors, data exists to support the hypothesis that there may be intrinsic biological differences in African American breast tissue compared with white breast tissue (Wieder, Shafiq, & Adam, 2016). The first piece of evidence for this hypothesis is that thelarche occurs earlier in African American populations which invokes the idea that breast differentiation has an earlier onset. Secondly, the known hormonal risk factors for breast cancer are not accurately predicted by the Gail and Klaus models for African American women compared with European White women. Lastly, a higher percentage of African American breast cancers are seen in pre-menopausal women compared with European White women who manifest more generally as post-menopausal estrogen receptor positive breast cancers. In summary, breast development, breast cancer risk and breast cancer types are generally different between African American and European White

women. The question of socioeconomic status, access to insurance and urban versus suburban lifestyle could still be factors in the U.S. However, Nigerian women living in Nigeria have been shown to also manifest a high proportion of triple negative breast cancers (Abdulkareem & Zurmi, 2012; Adisa et al., 2012)

In this chapter we will test the hypothesis that intrinsic biological differences between African American and European White breast tissue may be a contributing factor for the differing age of thelarche using our *in vitro* model of thelarche. We are able to use an *in vitro* system to test this hypothesis because when Dr. Latimer's laboratory originally placed reduction mammoplasties into the model system, they were unaware of ancestry, BMI age, menopausal status and other patient characteristics. It was observed that some cultures formed ductal structures much earlier than other cultures. After extensive multivariate analyses, it was discovered that ancestry was one of the main factors involved in this *in vitro* disparity in ductal formation with African American samples forming the ducts before European White patients. This will be described in the following sections.

Because differentiation of cells in culture into ductal structures requires the support of breast progenitor cells, we hypothesize that there is an intrinsic difference in either the percentage of breast stem cells and/or the potency of breast stem cells between African American women and European White women. Indeed Nakashatri et al. (2015) showed that African American women have significantly higher proportions of CD24-

/44+ and PROCR+/EpCAM- cells (both are marker sets that distinguish stem cell populations in non-diseased breast) than European-derived White women (Nakshatri, Anjanappa, & Bhat-Nakshatri, 2015). Therefore, there is precedent in the literature justifying the search for differences in the size of the stem cell compartment in the breast based on ancestry. Our study differs in that we are also studying the differentiation potential of stem cells derived from African American and European-derived White subjects. Differentiation potential, also known as "stem cell potency," was determined using the CD 24-/CD44+ markers as well as CD49f+, a third marker for breast stem cells (Lloyd-Lewis, Harris, Watson, & Davis, 2017; Stingl et al., 2001).

3.2 Materials and methods

3.2.1 Non-diseased primary tissue processing

Primary human mammary epithelial cultures were established from all 48 breast reduction mammoplasty tissues obtained from the Magee-Womens Hospital of University of Pittsburgh Medical Center (UPMC). These tissues were derived from 36 pre-menopausal, 6 post-menopausal and 6 peri-menopausal (ages 45-55) and were all verified to be within the range of normal histology. 12/48 (25%) of the tissue donors were African American women matched in socioeconomic status with the white women (middle class). Magee-Womens Hospital is a tertiary care hospital that generally serves insured patients, so the question of socioeconomic status is not a confounding factor in this study.

Primary tissues were processed as described in section 2.8. Freshly obtained tissues were minced upon arrival then platted on two-chamber slides that were coated with 1:1 diluted basement membrane extract (Trevigen®) with DMEM. Minced tissue was plated in MWRI medium and incubated in a humidified cell culture incubator at 37°C and 5% CO_2. Two-chamber slides were inspected microscopically for episphere formation and ductal formation daily.

3.2.2 Multivariate analysis of primary non-diseased tissues in culture

All of the cultures formed epispheres; however, 23 (47.9%) of these cultures formed ductal structures within 11 days. Intrinsic factors were correlated with the ability to differentiate in culture. Clinical and basic demographic and medical information were obtained on all the anonymized subjects under internal review board number **0504117.** Thirteen factors were analyzed: age, gravida (pregnancy), para (live birth), ancestry, weight, body mass index, height, hormone use for contraception or hormone replacement, family history of cancer, previous lesions, time in culture, S-phase index, and menopausal status. Two multivariate analyses were performed using Minitab software. The first (n=48) using ductal formation (yes/no) as the dependent variable and the second (n=23) using time to ductal formation as a dependent variable.

3.2.3 Microarray

Microarray was completed with two African American samples, JL BRL-01 (n=1) and JL BRL-02 (n=3), and two European White samples, JL BRL-23 (n=1) and JL BRL-

14 (n=3) to assess differences in overall gene expression between ancestries in these cultures. RNA and microarray data previously generated from Latimer et al., (2010) were processed using Genespring software and analyzed in different ways. Files were normalized together using the PLIER16 algorithm (Therneau & Ballman, 2008). Results from two African American and two European White samples were averaged together, and probes representing the same gene, when available, were also averaged together. Unsupervised hierarchical clustering analysis was completed, and significance was measured using a moderated *t*-test with Benjamini-Hochberg multiple testing correction. A supervised analysis of 42 genes and markers of "stemness" was also analyzed.

3.2.4 Established non-diseased breast tissue cultures for flow cytometry

We selected 10 non-diseased breast reduction mammoplasty cultures derived from the Latimer tissue engineering system for this study (Latimer, 2002, US patent 6,383,805). Each of these cell lines was originally examined by a pathologist to confirm the absence of histological abnormalities. Five are of self-declared African American descent, five are self-declared European White, and all 10 of these are pre-menopausal samples. Patients did not significantly differ in age, height or weight; however, 2/5 African American women had given birth (were parous) compared to all nulliparous subjects in the European-derived White cohort. Samples were maintained and expanded using the Latimer culture system (Latimer et al., 2003). One African American post-menopausal patient and two European White post-menopausal patients were also

analyzed and compared separately (Table 3.2). Breast-reduction mammoplasty tissues were obtained and processed for culture as previously described (Jean J Latimer et al., 2003). Cells were incubated at 37 °C and 10% CO_2, media was replaced at least 3 times a week using MWRI.

3.2.5 Flow cytometric staining

Flow cytometry was utilized to quantify stem cell compartments based on CD24, CD44, and CD49f expression (Table 1.1). Cells were disaggregated with 0.25% trypsin containing EDTA (Hyclone), suspended in 1 mL of Hanks Buffered Saline Solution (HBSS, Hyclone) with 2% fetal bovine serum (FBS, Hyclone), and counted with a hemocytometer. Cells were washed twice with HBSS 2% FBS and resuspended in 50 uL of HBSS (Hyclone) and 2% FBS (Hyclone).

Live cells were incubated with 20 uL of FITC-conjugated mouse anti-human CD24 (Clone ML50, BD), PE rat anti-human CD49f (BD), and APC-conjugated mouse anti-human CD44 (Clone G44-26, BD) antibodies for every 10^6 cells, for 1 hour on ice, protected from light. Cells were then washed twice with HBSS. After washing, cells were resuspended in 0.5mL of HBSS 2% FBS containing propidium iodide (Sigma-Aldrich®) at a concentration of 10 ug/mL for every 10^6 cells.

Cells where then immediately analyzed on a BD Accuri™ C6 flow cytometer. To determine appropriate gating and fluorescent compensation, a fluorescent minus one (FMO) strategy was utilized for each sample. Compensation and instrument set up and

sensitivity were also verified and determined using BD CaliBRITE^{tm} FITC, PE, and APC beads (BD). Gating was determined from unstained samples and positive gates created had less than 99.99% of events in unstained samples for each antibody.

3.2.6 Assessment of stem cell compartments

To quantify stem cell compartments FCS files created on the BD Accuri™ C6 were analyzed using the latest version of the FlowJo software. Cells were first gated on forward and side scatter to select appropriate cellular population. Then cells that excluded propidium iodide were selected and gated upon. Doublets were excluded based on area versus height. CD24-/CD44+/49f+ (a more stringent definition of breast stem cells) and CD24-/44+ (the simplest definition of stem cells) populations were quantified. Stem cell proportions were determined in triplicate for each sample, then were averaged. Results from African American and European-derived White women were compared using a two-way *t*-test with significance determined at $p<0.05$ for each set of stem cell markers. Then the samples were grouped together based on menopausal status without regard to ancestry and stem cell proportions for pre-menopausal and post-menopausal were compared using a student's *t*-test. Then European-derived White pre-menopausal samples were compared to European White post-menopausal samples using a *t*-test. Finally, African American pre-menopausal women were compared to a single African American post-menopausal woman. Because significance cannot be assessed based on a single sample using a *t*-test we evaluated whether the single African American post-

menopausal sample differs from the pre-menopausal population using a *z*-test. All differences were considered significant at $p<0.05$.

3.2.7 Flow sorting and clonal plating of single stem cells

Single cell sorting was completed using the same staining technique as was used for stem cell quantification. Once the staining was complete, cells were immediately sorted using a BD FACSJazz Cell Sorter. Cells with the staining pattern: CD24-/44+/49f+ were sorted directly into 96 well plates (Corning®). The chambers of these plates were coated with a 1:1 dilution of basement membrane extract (Trevigen®) and cultured using MWRI medium supplemented with 20% conditioned media for each respective sample. Cells with the staining pattern: CD24-/44+/49f- from 5 African American and 5 European White samples were also single cell sorted. Prior experiments showed that cells grew significantly better in 20% conditioned MWRI rather than MWRI alone or MWRI with increased amounts of serum.

3.2.8 Assessment of cloning efficiency

Cells were inspected microscopically within 12 hours to verify the presence of a single cell per well (and to identify and eliminate any wells with more than one cells) then twice a week (Monday and Friday) for 4 weeks using a Zeiss Axiovert 100 inverted microscope. Images were captured under DIC optics using a Hamamatsu camera and *in vitro* software. Sorting efficiency was determined based on the number of wells out of 96 that were microscopically verified to contain a single viable cell. Cloning efficiency is

the number of wells that contained multiple cells. African American cloning efficiency was compared to European white cloning efficiency using a student's t-test ($p<0.05$).

3.2.9 Assessment of potency

Potency was determined as the proportion of cells that produced visibly different cell types based on morphology upon proliferation. Potency was assessed in two cell types CD24-/44+/49f+ (breast stem cell) and CD24-/44+/49f- (non-breast stem cell). Duplicate runs for each sample (and when possible triplicate) were averaged together. Data for single cell sorts from CD24-/44+/49- cells was also assessed from one run for each sample and averaged together for each ancestry. Sorts with low sort efficiency were excluded from calculations. JL BRL-21 CD24-/44+/49- cells had no viable cells after sorting and was not included. African American women were compared to European White women using a two-way *t*-test with significance at $p<0.05$. Stem and non-stem cell potencies were compared within each ancestry using a student's *t*-test for significance ($p<0.05$). JL BRL-02 flow cytometric and sorting data was obtained from prior work completed at UPMC.

3.3 Results

3.3.1 Primary tissue characteristics and multivariate analysis

When comparing the ability for form ducts, six significant multivariate models were found, the most significant being a 7-factor model that yielded an $R^2 = 0.51$ and $p=0.032$ (Table 3.1, Model 1A). The simplest significant model contained only two

variables that correlated with ductal formation: gravida and the S-phase index (Table 3.1, Model 6A). Gravida, or the number of times a woman has been pregnant, had a negative association with ductal formation. S-phase index, a measure of the proportion of proliferative cells in a culture, had a positive association with ductal formation.

A second multivariate analysis using the time (in days) to ductal formation as the response variable (n=23) yielded two different significant models. Model 1B yielded an $R^2 = 0.78$ ($p<0.001$) and model 2B yielded an $R^2 = 1$ ($p=0.005$), Table 3.1. In model 1B, age, previous benign lesions, and hormone use were all positively associated with time to ductal formation. Parity, however, was negatively associated with time to ductal formation. Both models involved age, previous lesions, and family history. In the second significant regression model for time to ductal formation, 2B, family history and height had positive associations with timing to ductal formation. Age, any previous lesions, gravida, S-phase index, and ethnicity were all negatively associated with time of ductal formation. In model 2B, African American decent decreased the time it took to form ductal structures in culture by a factor of 63.9. The two models shared age and previous lesions in common, but in different directions.

Table 3.1 **Multivariate models.**

Ductal formation (n=48):											
Model #										**R^2**	***P***
1A	**DF =**	2.83	- 0.515 G	+ 0.0111 SPI	- 0.0404 H	+ 0.0130 TIC	+ 0.470 PL	+ 0.290 FH	+ 0.072 HU	**51.4%**	**0.032**
2A	**DF =**	3.14	- 0.504 G	+ 0.0107 SPI	- 0.0448 H	+ 0.0126 TIC	+ 0.471 PL	+ 0.302 FH		**51.0%**	**0.016**
3A	**DF =**	2.94	- 0.473 G	+ 0.0106 SPI	- 0.0422 H	+ 0.0151 TIC	+ 0.455 PL			**49.1%**	**0.007**
4A	**DF =**	3.15	- 0.382 G	+ 0.0146 SPI	- 0.0462 H	+ 0.0122 TIC				**41.4%**	**0.008**
5A	**DF =**	3.15	- 0.425 G	+ 0.0152 SPI	- 0.0433 H					**36.4%**	**0.007**
6A	**DF =**	0.39	- 0.444 G	+ 0.0156 SPI						**29.1%**	**0.010**
Time to ductal formation (in days) (n=24):											
1B	**TDF =**	5.05	+0.8 A	-4.43 P	-20.8 FH	+17.7 PL(B)	+13.9 HU			**78.5%**	**<0.001**
2B	**TDF =**	766	-2.26 A	-19.8 G	+36.4 FH	- 27.6 PL(A)	-63.9 E	+15.6 H	- 1.82 SPI	**100.0%**	**0.005**

DF= ductal formation, TDF = time to ductal formation, A = age (years), P = para (absolute number), G = gravida (bivariate yes/no), FH = family history (non-breast), PL(B) = previous lesions (breast), PL(A) = previous lesions (any), HU = hormone use, E = ethnicity (bivariate European White/African American), H = height (inches), SPI = S-phase index (% labeled cells), TIC = time in culture (days)

Based on multiple regression model's, African American descent is associated with a decreased time to ductal formation. We therefore compared the time to ductal formation of primary cultures derived from African American women to European-derived women. We found that African American women primary explants formed ductal structures on average after 10.4 days in culture significantly earlier than European

White primary explants, which formed ductal structures at 22.61 days (p=0.028) (Figure 3.3).

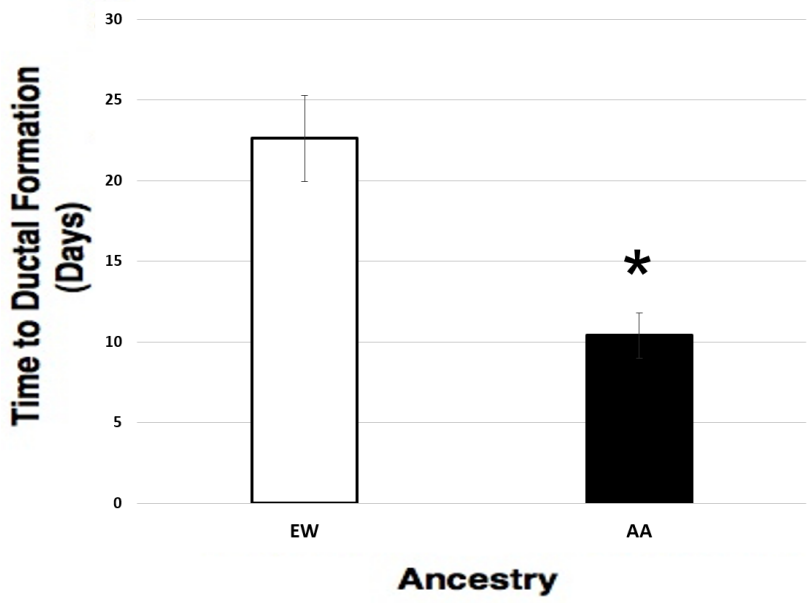


Figure 3.3. **Time to ductal formation based on ancestry.** It took European White women (n=18) 22.31 days to form ducts in culture, while African American (n=5) women's cultures formed ducts in 10.4 days (p=0.028).

3.3.2 Microarray analyses

Unsupervised and supervised analyses of gene expression from microarray using cell lines established in the Latimer laboratory (Breast Tumor Lines, BTL and Breast Reduction Lines, BRL), as well as some of the commonly used commercially available cell lines were performed. Foreskin fibroblasts (FF) were also included as non-diseased explant cultures. All 38,500 human genes on the Affymetrix chip were included for unsupervised hierarchal clustering. Two major clusters are shown: stage IV cell cancer

cell lines in the middle and far right containing HeLa and MDA MB231, MCF7 and SKBR3, and one on the left containing FF (normal) as well as several of our reduction mammoplasty cell lines (BRLs) and some stage I cell lines. BTL-8,-33,-4 are all derived from stage I breast tumors and have very subtle karyotypic abnormalities compared with the cell lines on the right (Figure 3.4). Furthermore, karyotypic analysis of cell lines shows we are studying non-transformed tissues (Figure 3.5).

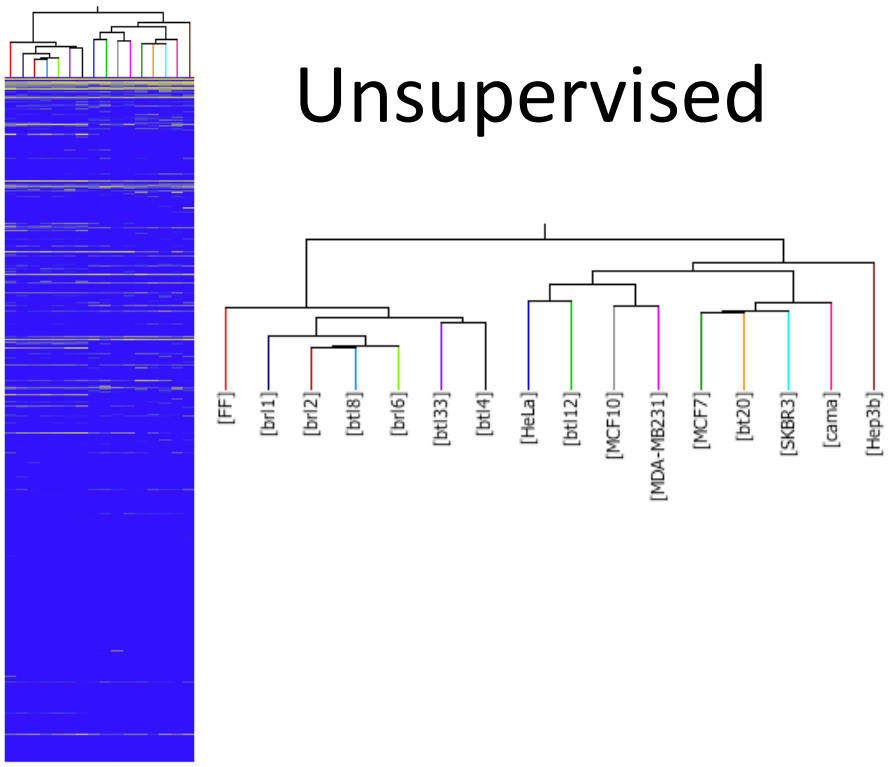


Figure 3.4. **Unsupervised hierarchical clustering of cell lines.** Breast reduction lines do not cluster with transformed late stage breast cancers.

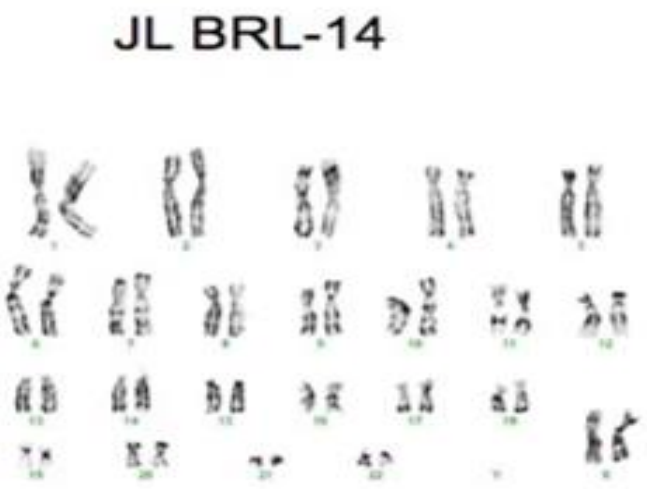


Figure 3.5 **Normal Karyotype JL BRL-14.** Representative normal karyotypes of JL BRL-14 (Cell lines not shown but also karyotyped are JL BRL-06, -23, 24)

Unsupervised analysis of microarray on two African American versus two European White women derived reduction mammoplasty cell lines showed that two genes were significantly different in unsupervised analysis of the entire chip. Interleukin 33 (IL33) was significantly under expressed in African American samples by a fold change of 10.55 (p(corr)=0.01). RP5-944M2.2 was significantly over expressed by a fold change of 3.26 in comparison to European White samples (p(corr)=0.04) (Figure 3.6)). IL33 plays a role in immune system regulation. RP5-944M2.2 is a long non-coding RNA.

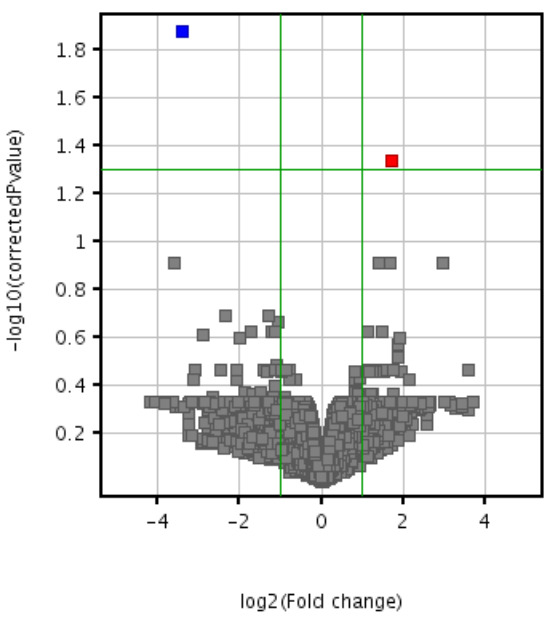


Figure 3.6 **Unsupervised microarray analysis of African American and European White reduction cell lines**. The resultant **Volcano plot is shown comparing African American (n=2) and European White (n=2) women gene expression.** Two genes are significantly different. Interleukin 33, IL33, (blue) has 10.55-fold greater gene expression in European White than African American women (corrected p=0.013). RP5-944M2.2 (red) is a long intergenic non-coding RNA that is expressed 3.26 times greater in African American than European White women (corrected p=0.046).

We also looked specifically at 42 genes that are most related to stem characters and markers. A dendrogram was created to show differences in relative expression between African American and European White women for these genes (Figure 3.7). We found that CD24 was expressed at a low level in both races but more so in African American samples. CD44 was highly expressed in both samples with relatively little differences. CD49f (ITGA6) was markedly increased in African American samples. It is important to note that there are two cell lines in each group.

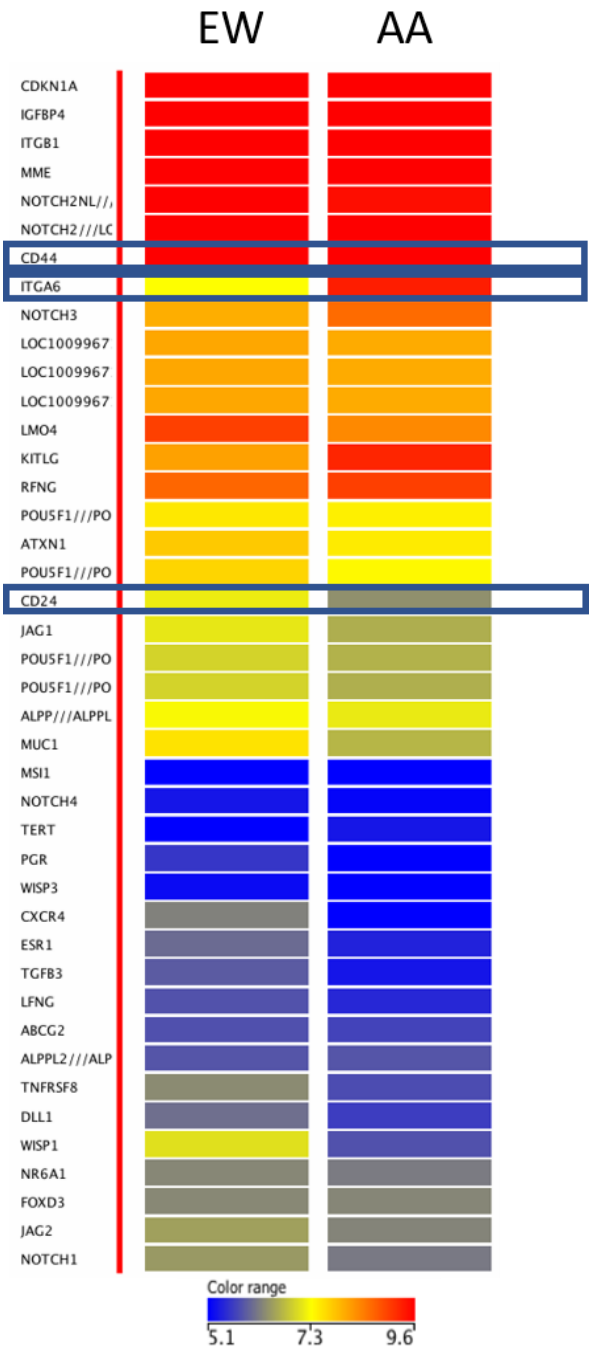


Figure 3.7 **Supervised microarray analysis of genes related to stemness.** Dendrogram comparing African American (n=2) to European White (n=2) women. Red is overexpressed and blue underexpressed, yellow is in between. Genes in blue boxes, CD24, CD44, and ITGA (CD49f) are markers used in our study for stem cells (CD24-/44+/49). African American samples showed overall slightly higher expression in stem cell markers.

3.3.3 Stem cell staining patterns of individual explants

Ten pre-menopausal and three post-menopausal cell lines were selected for stem cell analysis by flow cytometry. These cell lines are summarized in Table 3.2.

Table 3.2. **Extended explants characteristics.**

Extended explant	Ancestry	Type	Age	Grava	Para	Height (inches)	Weight (lbs.)	Karyotype	Ductal formation in 1° culture
	Pre-menopausal								
JL BRL-2	AA	R	19	0	0	59	110		Yes
JL BRL-1	AA	R	35	2	0	64	170		No
JL BRL-24	AA	R	33	1	1	65	227	46, XX	Yes
JL BRL-12	AA	R	37	2	1	68	190		No
JL BRL-15	AA	R	33	0	0	66	245		Yes
JL BRL-23	EW	R	22	0	0	66	120	46, XX	Yes
JL BRL-14	EW	R	25	0	0	62	180	46, XX	No
JL BRL-08	EW	R	26	0	0	60	135		Yes
JL BRL-18	EW	R	32	0	0	63	230		Yes
JL BRL-21	EW	R	35	UNK	UNK	67	219		Yes
	Post-menopausal								
JL BRL-36	AA	R	65	3	1	63	230		Yes
JL BRL-13	EW	R	57	2	2	62	136		Yes
JL BRL-06	EW	R	62	4	4	54	158		Yes

AA = African American, EW = European White, R = non-diseased breast reduction, UNK = unknown, Grava = gravidity, Para = parity

Flow cytometry dot plots were created for CD24/44/49f staining of non-diseased cultured breast cells in each sample. First, live cells were compared for their expression on CD24. Cells that did not show positivity in CD24 were selected (Figure 3.8A). Overall, cells in our reduction mammoplasty culture system did not show high expression of CD24. Dot plots for the CD24 negative only cells were created and the expression of

CD44 and CD49f markers were compared (Figure 3.8B). Most cells expressed CD44 markers with most cells staining positive for this marker. CD49f showed the most variation in expression with some samples having less than one percent and others with over 30% of the population expressing this marker. Overall, there were increased levels of CD44 cells in both African American and European White women and low to no cells with CD24 positivity.

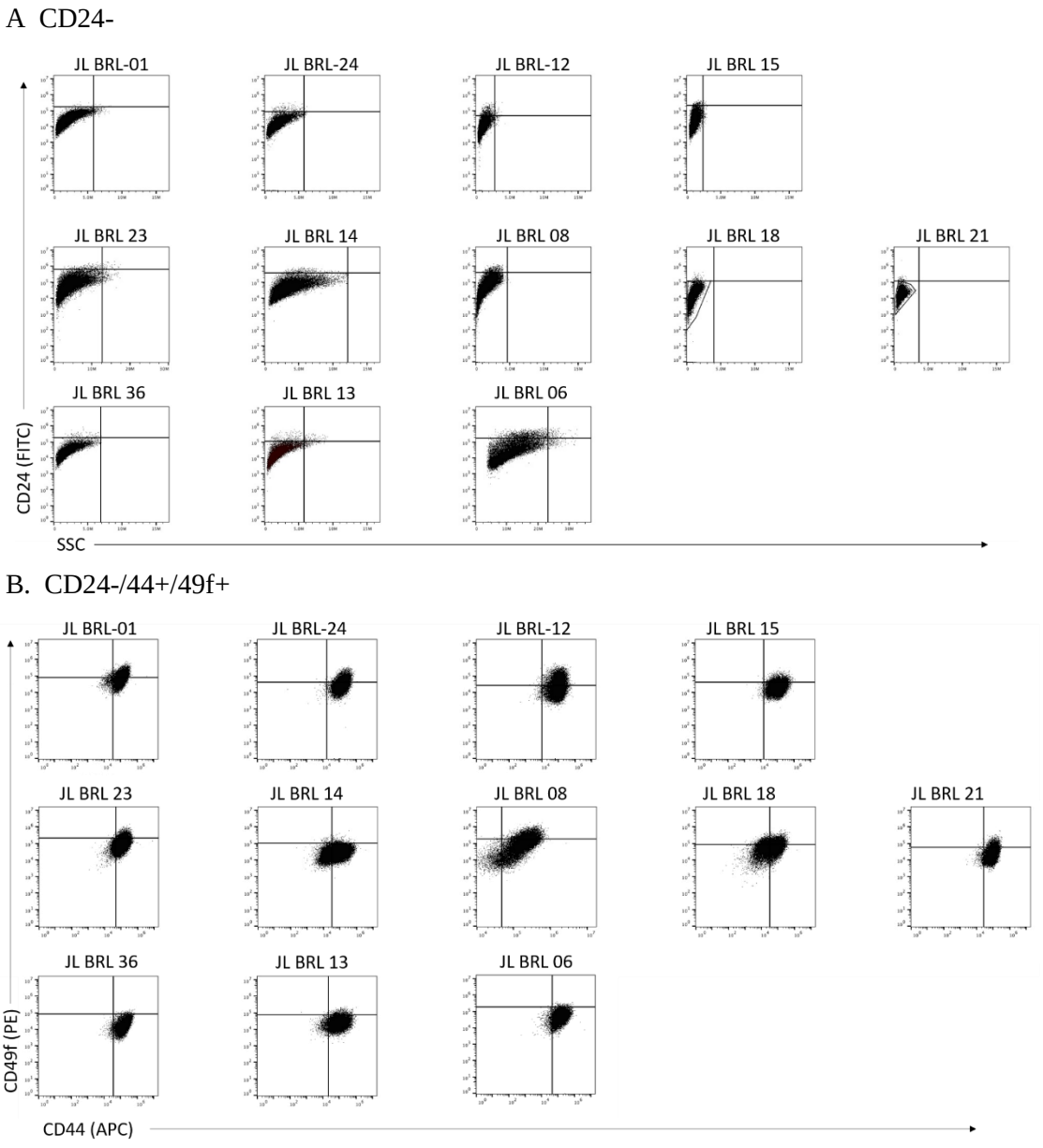
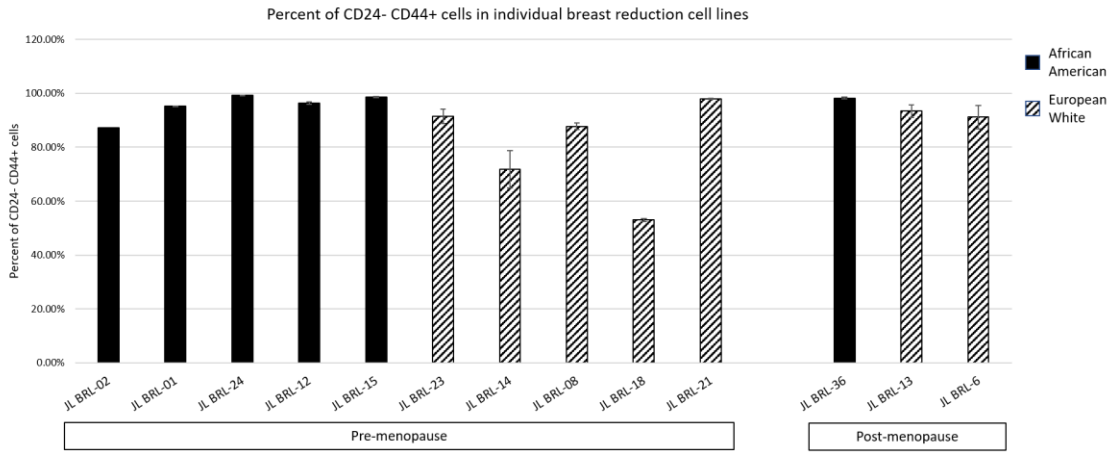


Figure 3.8 **Flow cytometry profiles of CD24/44/49f stained cells.** Individual dot plots for (top row) 4 African American, JL BRL-01, JL BRL-24, JL BRL-12, and JL BRL-15, (middle row) 5 European White, JL BRL-23, JL BRL-14, JL BRL-08, JL BRL-18, JL BRL-21, and (bottom row) 3 post-menopausal samples, JL BRL-36 (African American), JL BRL-13 (European White), and JL BRL-06 (European White). (A) Dot plots represent the cells with CD24 surface proteins versus side scatter (SSC). Cells in the lower left quadrant are CD24 negative. (B) CD44 versus CD49f markers of each individual sample, plots are gated to only include cells that are CD24-. The upper right quadrant of each plot represents cells that are positive for both CD44 and CD 49f, i.e. the stem cells. JL BRL-02 data not shown.

Samples were then quantified for the proportion of stem cells, CD24-/44+, i.e. the simplest definition of a stem cell, and CD24-/44+/49+, the more stringent definition of a stem cell (Stingl et al., 2001) in the whole population of cultured cells (Figure 3.9A and B). JL BRL-18, pre-menopausal European White-derived sample, showed the lowest proportion of these stem cells, with only 53.01% CD24-/CD44+ cells. JL BRL-24 showed the highest percent of CD24-/44+ cells at 99.34%. CD24-/44+/49f+ stem cells were lowest in JL BRL-36 (0.48%), a post-menopausal European-derived White derived sample, and highest in JL BRL-01 (33.40%), a pre-menopausal African American derived sample.

A



B

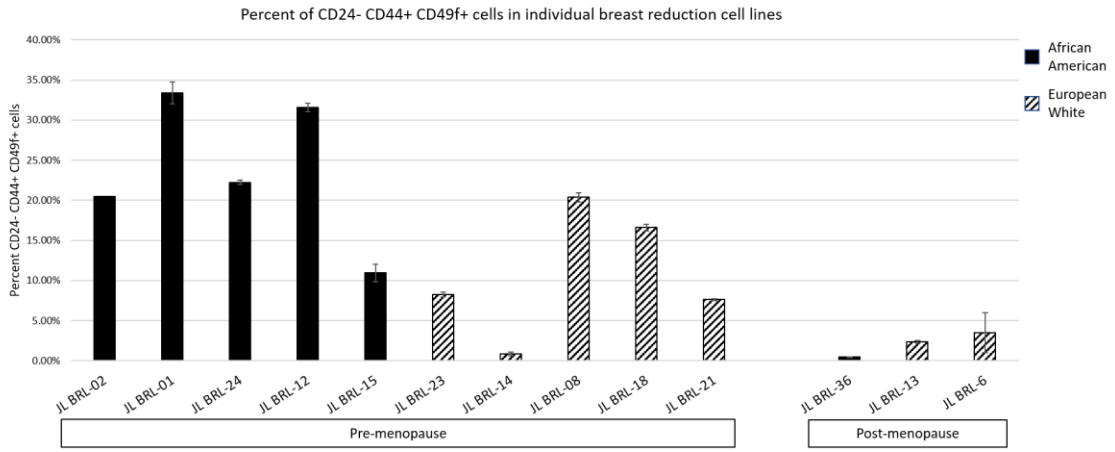


Figure 3.9 **Percent of stem cells for individual cell lines.** (A) Percent of stem cells based on CD24-/44+ expression pattern for each individual sample. (B) Percent of stem cells based on CD24-/44+/49f+ expression pattern for each individual sample.

3.3.4 Stem cell percentages in African American versus European White derived samples.

We first compared the CD24-/44+ populations between African American and European White-derived women. There was no significant difference between African American women and European White women in proportions of CD24-/44+ cells

(p=0.11). Although they did not reach significance, African American samples did overall have increased amounts of CD24-/44+ cells with an average of 95.32% compared to 80.40% in European White-derived samples (Figure 3.10). The European-derive White-derived samples showed somewhat higher interindividual variability in staining patterns for these markers.

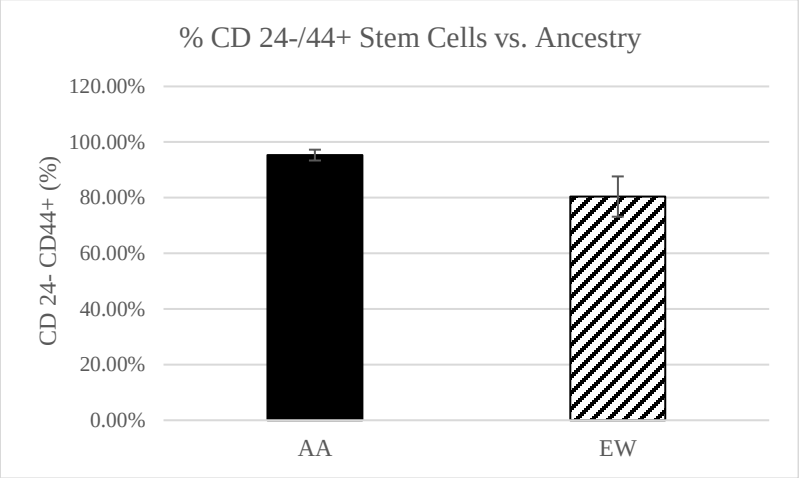


Figure 3.10. **Percentage CD24-/44+ cells based on race.** The average of CD24-/44+ cells is higher in African American samples (n = 5) at 95.32% than in European White (n = 5), 80.40%, cells, but not significant (p=0.11)

CD24/44/49f staining of non-diseased cultured breast cells showed relatively high levels of interindividual variation across both ancestries. Overall, African American women had significantly higher proportions of cells with CD24-/44+/49f+ patterns than European White women (p=0.04). African American women showed that on average 23.73% of cells stained with a CD24-/44+/49f+ pattern, whereas, European-derived White women's cells averaged only 10.73% (Figure 3.11).

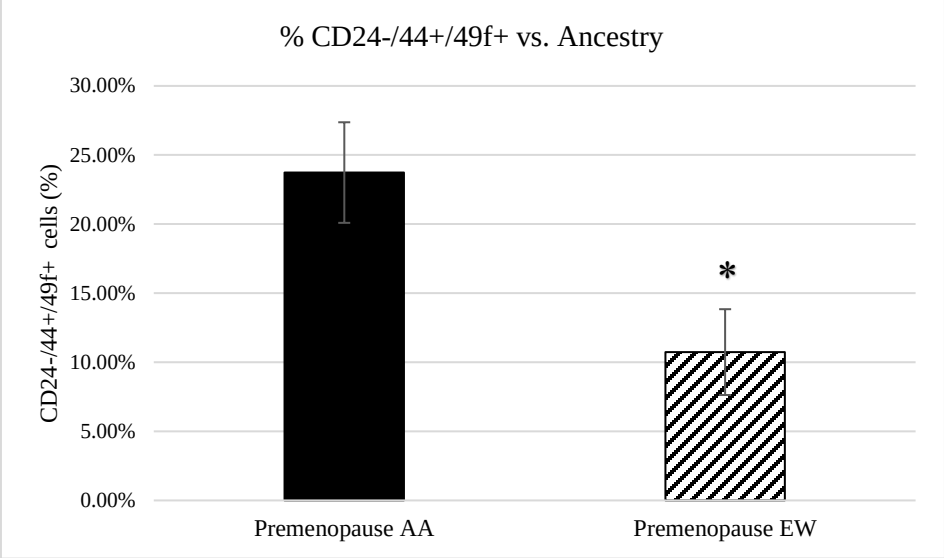
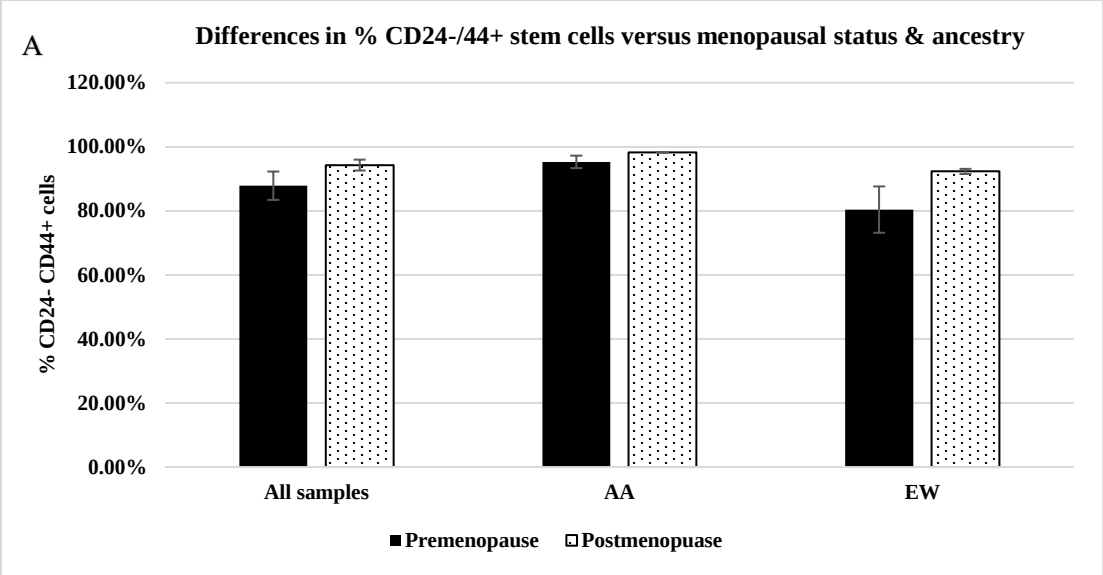


Figure 3.11 **Percentage CD24-/44+/49f cells based on race.** The average of CD24-/44+/49f+ cells is significantly higher in pre-menopausal African American (n=5) than in pre-menopausal European White cultures (p=0.02) (n=5). The average percent of African American CD24-/44+/49f+ stem cells was 23.73% and in European White it was 10.73%.

Samples from post-menopausal women also gave distinct patterns in cultured cells. Three menopausal women (one African American and two European White) were compared to the 10 pre-menopausal women. In post-menopausal women-derived cells, the average proportion of CD24-/44+ cells were 94.31% consistent with the 87.86% found in pre-menopausal samples (p=0.48). Due to their only being a single African American post-menopausal-derived sample, significance was evaluated within this ancestry using a z-test. African American post-menopausal-derived cells did not differ from pre-menopausal African American derived cells in the CD24-/44+ compartment (p=0.91). European White post-menopausal-derived cells also did not differ from

European White pre-menopausal-derived cells ($p=0.42$). Overall, there was no significant differences in patterns for CD24-/44+ percentages for these cells (Figure 3.10A). However, post-menopausal-derived cells did have significantly lower proportions of CD24-/44+/49f+ cells when compared to pre-menopausal women ($p=0.03$). When the one African American post-menopausal derived sample was compared to pre-menopausal African American derived cells there was a significant decline (based on the *z*-test) in the percentage of CD24-/44+/49f+ cells ($p<0.001$), however, a similar decline was not seen in European White-derived cells. In fact, European White women had no significant difference in pre/post-menopausal percentages of these progenitor cells ($p=0.238$) (Figure 3.12B).



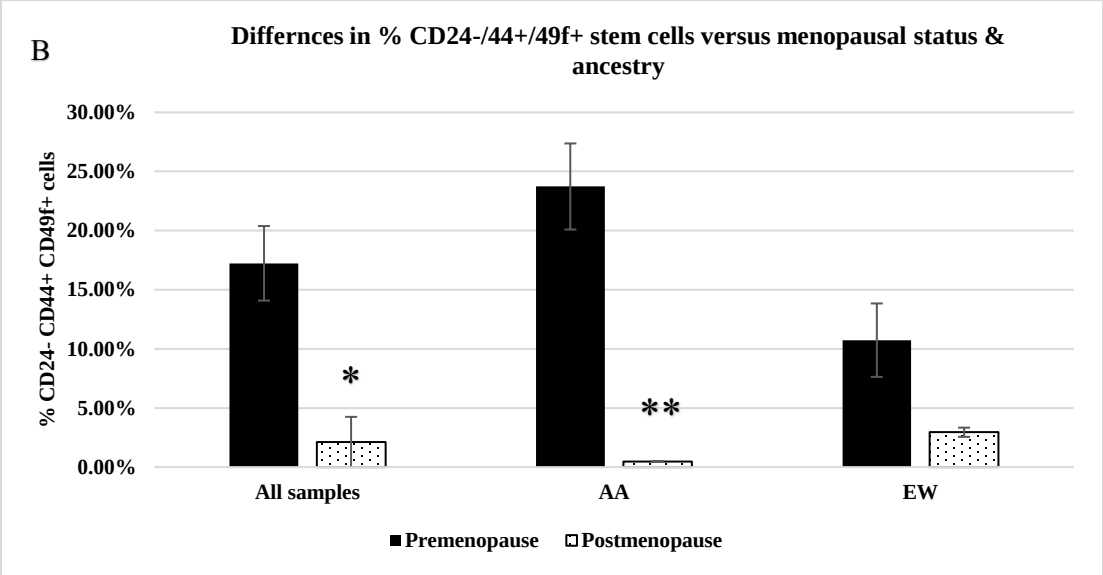


Figure 3.12 **Percentage of CD24-/44+ and 24-/44+/49f+ cells based on menopausal status and ancestry.** (A) percentages of cells that are CD24-/44+ are not significantly different regardless of ancestry or menopausal status. (B) Post menopausal cultures have significantly lower percentages of CD24-/44+/49f+ cells ($p=0.03$) ($n=3$). **The single African American sample had a significantly lower percentage of breast stem cells after menopause than African American premenopausal women, signficance is measured by z-test ($p<0.001$).

3.3.5 Stem cell potency in ancestries

When comparing cloning efficiency between African American women and European White women we found that there was no difference in the efficiency, that is cells that grew from a single viable cell after sorting, in either CD24-/44+/49f+ (p=0.252, Figure 3.13). CD24-/44+/49f- cells also showed no difference in cloning efficiency based on ancestry (p=0.240). The ability of African American and European White-derived cells exhibited no differences in their ability to grow after single cells were sorted. Therefore, potency is measured in equally proliferating colonies.

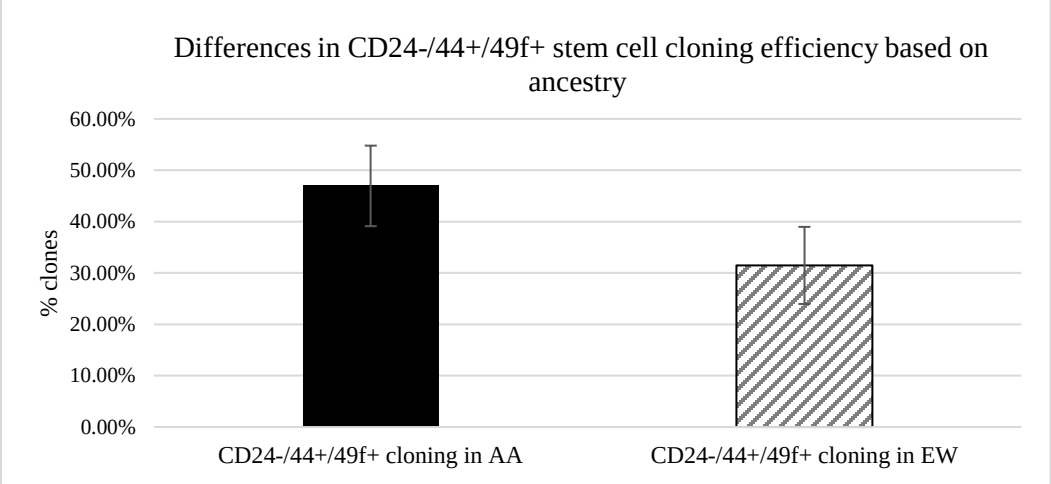
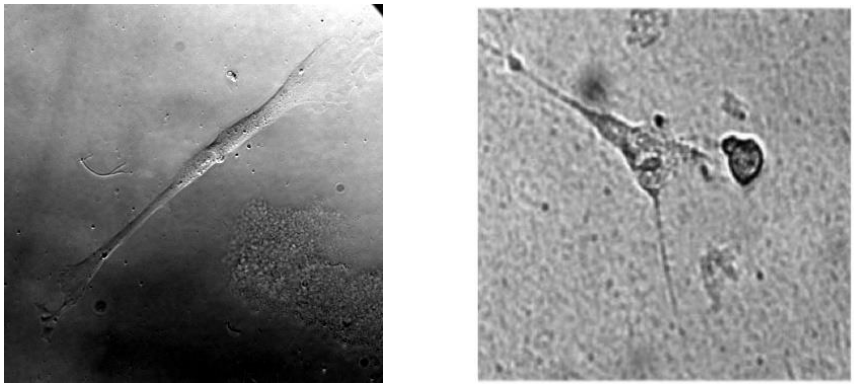


Figure 3.13 **Cloning efficiency based on ancestry.** (A) Cloning efficiency defined as the percentage of cells that attached in 96 wells that have been plated with a single cell during flow cytometric delivery, shows that there is no signficant difference in cloning efficiency based on ancestry (p=0.252).

When comparing the potency of CD24-/44+/49f+ cells, samples from African American women exhibited significantly higher proportions of bipotent cells than European White women (p=0.02). African American women showed bipotent CD24-

/44+/49f+ cells in 10.45% of the colonies compared to only 2.91% of colonies in European White women (Figure 3.14). Individual results for each cell line for stem cell percentages and potency are summarized in Table 3.3.

A



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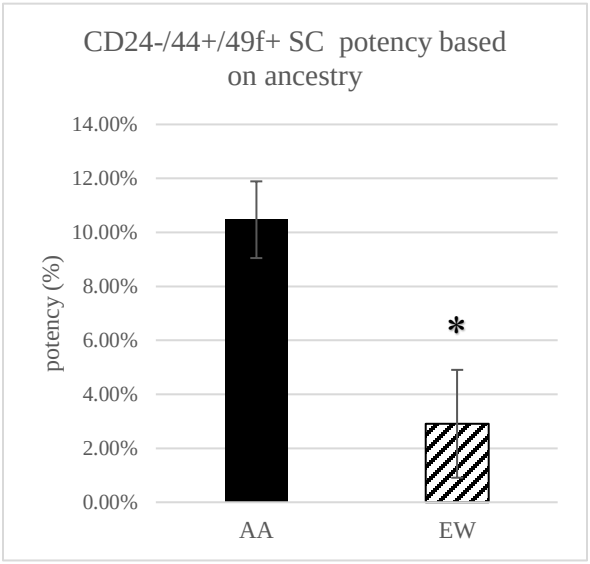


Figure 3.14 **Stem cell potency based on ancestry.** (A) DIC bright field imaging of a single fibroblastic cell at 100X (left), and a cell that has developed into two different types displaying bipotency (right), i.e. fibroblastic on the left and epithelial on the right. Both photos were taken after a single cell was originally verified in the first 12 hours in each well. (B) Among CD24-/44+/49f+ flow sorted single cells that were plated as single cells and monitored over time, African American (n=5) samples exhibited almost twice as much bipotent differentiation as European White (n=5) (p=0.04).

3.3.6 Stem cell potency in cell types.

To assess the difference in potency between CD24-/44+/49f+ (stem) and CD24-/44+/49f- (non-stem) cells we compared the two groups regardless of ancestry and we

found that potency did not significantly differ (p=0.36) based on cell type. When CD24-/44+/49f+ (stem) African American cells were compared to CD24-/44+/49f- (non-stem) African American cells, there was a significant reduction in potency (p=0.03). In contrast, there was no significant differences between European White CD24-/44+/49f+ and European White CD24-/44+/49f- cell potency. This was driven by one cell line that showed some potency in CD24-/44+/49f- cells while no others did.

Table 3.3 **Individual results for stem cell characteristics.**

Non-diseased breast epithelium donor	Age	Ancestry	Microarray gene expression pattern	Ductal Formation in 1º culture	CD24-/CD44+	CD24-/CD44+/CD49F+	Cloning after sorting of 24-/44+/ CD49f+ cells	Multicellular Phenotypes in clones (potency)
JL BRL-02	19	AA	Normal	Yes	87.14%	20.50%	ND	11.00%
JL BRL-01	35	AA	Normal	No	95.19%	33.40%	48.48%	7.14%
JL BRL-24	33	AA	Normal	Yes	99.34%	22.24%	54.35%	8.00%
JL BRL-12	37	AA	ND	No	96.33%	31.57%	63.56%	16.20%
JL BRL-15	33	AA	Normal	Yes	98.60%	10.94%	21.43%	10.00%
JL BRL-23	22	EW	Normal	Yes	91.51%	8.23%	17.90%	3.00%
JL BRL-14	25	EW	Normal	No	71.75%	0.84%	29.70%	0.00%
JL BRL-08	26	EW	ND	Yes	87.75%	20.37%	27.78%	0.00%
JL BRL-18	32	EW	ND	Yes	53.01%	16.60%	18.33%	0.00%
JL BRL-21	35	EW	ND	Yes	98.00%	7.63%	63.65%	11.54%
JL BRL-36	65	AA	Normal	Yes	98.27%	0.48%	Post-menopausal	
JL BRL-13	57	EW	ND	Yes	93.45%	2.39%		
JL BRL-06	62	EW	Normal	Yes	91.20%	3.50%		

African American = AA, European White = EW

3.4 Discussion

When comparing the global gene expression of two African American women to two European White women derived cultures we found two genes with significant differences. IL33 is part of the IL-1 cytokine family and RP5-944M2.2 is a long non-coding RNA. There is little information known about RP5-944M2.2 function as a non-coding RNA however, it's expression is increased in African American women. IL33 is a potent stimulator of both innate and adaptive immunity, it is expressed in pre-adipocytes as well as immune cells and we found it's expression to be lower in African American women. Since our cultures do not visibly contain immune cells, we have to assume we may have progenitor cells that include pre-adipocytes, which would be logical in cultured breast tissue. Supervised microarray-based gene expression patterns, showed African American gene expression was lower for CD24 and higher for CD44 and CD49f than European-derived White cultures which is consistent with the flow cytometry data on stem cell compartments.

The expression of CD24-/44+ cells is the simplest definition of a breast stem cell or a breast cancer stem cell (Sajithlal et al., 2010). In our study of non-diseased breast cultures, we found a high percentage of these cells in general. In non-diseased primary explants, Nakashatri et al. (2015), found an increase in the stem cell compartments in African American women compared to European-derived White women using CD24-/44+ to identify them; however, we did not find significant differences in our cultures for these markers (Nakshatri et al., 2015). It is possible that this is an adaptation to our

unique culture system that is highly supportive of stem cells in general. Basement membrane substrates are often used to support stem cell growth and maintain undifferentiated cells (Takahashi et al., 2007). MWRI medium is also adapted from embryonic stem cell culture and is supportive of stem cells. Therefore, it is likely that we have selected for these cells or that the expression of CD24-/44+ cells is supported by these factors.

When comparing the more stringent definition of stem cells, i.e. the proportion of CD24-/44+/49f+ staining cells from African American cultures to European-derived White cultures, we found that African American women contain significantly more of these breast stem cells. These data are consistent with a difference in earlier thelarche in the African American population and they are also consistent with the most prevalent breast cancer in the African American population is a "stem-like" cancer. The cell type of origin in breast cancers of African American patients may more frequently be a stem cell, because breast stem cells are present in higher numbers in African American than in European White breast samples.

Post-menopausal culture data suggests that European-derived White women breast stem cell proportions may not change from pre- to post-menopausal status **(**although this is based upon only two post-menopausal cultures). The African American women had a decline in the percentage of breast stem cells after menopause (although this is based on only one culture). Based on these data we postulate that African

American women have decline in stem cell percentages after menopause in their breast that is not seen in European white women. However, this would require more samples to confirm.

European-derived White women have no change in breast stem cell proportions from pre- to post-menopause in their non-diseased breast samples based on our limited post-menopausal data. There is no decline because compared to African American women, they already have considerably lower breast stem cell proportions even in their pre-menopausal breast tissue. Post-menopausal breast cancers, most commonly diagnosed in European White women, are most often ER+ with incidence rates highest in luminal type breast cancers at around 50 years old (Howlader et al., 2017). This type of cancer is not associated with "stemness".

The form of breast cancer most associated with "stemness" is triple negative breast cancer (ER-. PR-, Her2-). This type is most common in young African American women (Bauer et al., 2007). Our results show that stem cell percentages are highest in pre-menopausal African American and that African American women have higher stem cell percentages in their non-diseased breast in general. It is important to note that we only have a single sample from a post-menopausal African American patient and only two from European White patients, a limitation of this study. Therefore, post-menopausal breast stem cells from these groups should be replicated and further evaluated.

We also find that not only do African American women have higher percentages of breast stem cells in these cultures, but they are intrinsically more potent as well. This is a new finding that was not shown in Figure 3.12.

One benefit of using this culture system is that it can establish differentiated structures reminiscent of the mammary gland. We found that African American primary cultures tend to form ductal structures faster than European White cultures. This reflects what epidemiology tells us about the falling age of puberty; not only has the overall age been decreasing over the past century, but currently African American girls go through puberty at younger ages as well. This is particularly worrisome since early menarche is associated with increased breast cancer risk. Menarche is characterized by an explosion of growth and requires breast stem cells to support major changes. We now find intrinsic differences in both the proportion and potency of stem cells in tissue samples from non-diseased breasts of African American women. These differences may increase the risk for certain breast cancers, either directly by being a target for insult, or indirectly by modulating risk factors. Risk factors in African American women are still poorly understood relative to information relevant to European White women. Further characterization of the basic biology of African American breast growth and differentiation will help to better recognize risk factors affecting this minority group.

Chapter 4

In vitro model of thelarche for testing putative chemicals disruptive to breast development

4.1. Introduction

4.1.1. Brief history of the creation of breast cell lines as model systems

Tumor Cell lines

The use of breast tumor cell lines has been an invaluable tool for research dating back to the 1950s with the creation of BT-20 (Lasfargues & Ozzello, 1958), a stage IV-breast cancer-derived cell line. Tumor cell lines provide a cost efficient and unlimited supply of cells that are relatively easy to use. Since their inception, thousands of cells lines have been created and utilized to further our knowledge and understanding of many aspects of breast cancer. However, even with the creation of all these cell lines there seems to be an overwhelming use of a particular few, especially MCF7, in the breast cancer field. Originally created in 1973 by the Michigan cancer foundation, MCF7 was adapted into an estrogen response model because it was extremely sensitive to estrogen (Levenson & Jordan, 1997; Soto, Sonnenschein, Chung, & Fernandez, 1995).

Non-Diseased Cell Lines

Non-diseased breast cell lines have been considerably more difficult to create because they do not replicate immortally but manifest a version of the Hayflick

phenomenon (Hayflick, 1965). Most non-diseased breast explants/cell lines double between 10 and 20 times and then cease to replicate. As we will describe below, attempts at immortalizing these cells have been made with the carcinogen benzo[*a*]-pyrene (Martha R Stampfer, Bartley, & Rubin, 1985), more recently by stable transfection with the telomerase gene (Shay, Reddel, & Wright, 2012). These methods utilized enzymatic digestion of tissues (both normal and tumor) to the point of single cells. This disaggregation may be one of the reasons for the short lifespan of non-diseased cells in culture that rely heavily upon signaling from direct contact with other cells (juxtacrine interactions).

4.2. Cell lines used as surrogates for "normal" breast cells

4.2.1. E-SCREEN

The E-SCREEN or Estrogenic Screen assay was developed to test for environmental pollutants (Soto, 1995). The E-SCREEN assay utilizes a late stage cancer cell line that was created from a pleural effusion, MCF-7. MCF-7 has over 100 copies of the estrogen receptor gene and is exquisitely sensitive to the effects of estrogen or estrogen-like compounds (xenoestrogens). MCF-7 responds to estrogens by replicating at increased rate(Brooks, Locke, & Soule, 1973).

The E-SCREEN assay involves using serial dilutions of the test compound with 17β-estradiol is used as the positive control and the standard for comparison. After six days of growth and exposure, the relative proliferation rates are measured, and the

estrogenic activity of a compound is determined. However, there are major drawbacks of the ES-CREEN assay. Chemicals tested using this system may not be relevant to non-diseased tissue or cancer prevention. Testing how xenoestrogenic chemicals may disrupt normal cellular behavior cannot be evaluated using this and other models like it. Furthermore, the only end point measured is cell proliferation. In fact, most assays used today *in vitro*, still only use rudimentary end points such as cell death or growth rates. This is unfortunate because the disruption of development by chemicals has been shown to alter breast cancer risk.

4.2.2. MCF10A

MCF10A is a cell line produced from a non-malignant proliferative breast tissue (Herbert D Soule et al., 1990). It is characterized by epithelial cells that grow in a monolayer. It also exhibits secondary hollow spheroids when grown on MatriGel^{TM} together with collagen I, similar to what is seen in the *in vivo* breast (Debnath & Brugge, 2005; Debnath, Muthuswamy, & Brugge, 2003). MCF10A is often used as a representative for normal breast epithelial development (Qu et al., 2015).

However, recent studies suggest that the MCF10A cells have a unique expression pattern that is not seen in normal breast epithelium (Qu et al., 2015). Furthermore, unpublished data from our own laboratory has shown MCF10A expression patterns to be less like normal breast epithelium explants and more like tumor. The use of this cell line

for an abnormal early stage of breast cancer casts some doubt on its validity as a model system for non-diseased breast.

4.2.3 Transformed lines

To study toxicological effects, human and mouse mammary epithelial cells were grown in culture. Stampfer and Bartley developed a system using human non-diseased mammary epithelial cells that were transformed with benzo[*a*]pyrene (Stampfer et al., 1985). These cells do not form tumors in nude mouse but do show properties of tumor-derived cells. The original transformed line 184A1 was licensed a company called "Clonetics" and sold by the microscope slide ($400 per slide) as normal human mammary cells. Furthermore, multiple derivatives of this transformed line have been created by further transformation. These include 184A1-RF, further transformed with Raf-1, 184AaMY1-5, further transformed with c-myc, 184A1-GSE22, further transformed with GSE22, and 184A1-hTERT(12p), and further transformed with hTERT (Garbe et al., 2014; Olsen, Gardie, Yaswen, & Stampfer, 2002; Stampfer et al., 2001).

4.2.4 "Organoids" as model systems for the non-diseased breast

The growth of breast cells in 3-deminsional culture has been pioneered by Mina Bissell. Bissell, and her group have shown the importance of extracellular matrix for creating organotypic models in culture. A basement membrane purified from Engelbreth-Holm-Swarm (EHS) excreting tumors, is used to mimic breast extracellular matrix in cell culture flaks. Murine cells were embedded in the basement membrane extract in culture

and shown to differentiate into functional breast-like structures (Lee, Kenny, Lee, & Bissell, 2007). These structures were spherical with some polarization of epithelial cells, they also produced milk. This system was eventually adapted to human mammary cells then into an assay for differentiating between normal and malignant mammary cells, as malignant cells respond differently both in gene expression and morphology (Kenny et al., 2007). While functional breast like spheroid structures have been developed in the Bissel technique, none of these models differentiate into complex ductal systems.

4.3 Breast cancer etiology: from "normal" to cancer

Breast cancer is the most common type of cancer in American women after skin cancer (Howlader et al., 2017). Early puberty, expressed as age of menarche, is a well-established risk factor for breast cancer and is included in the most widely used risk assessment model (Gail, 2015; Gail et al., 1989). A one-year difference in age of menarche affects overall breast cancer risk by as much as 10% (Collaborative Group on Hormonal Factors in Breast cancer, 2012; Hsieh et al., 1990; Ritte et al., 2013). This factor has widely been interpreted as a simple estrogenic effect on proliferation in normal breast epithelium contributing to carcinogenesis, and particularly on proliferation of newly transformed cells, promoting their growth into a tumor. A second factor in the Gail model, age of first live birth, suggests another interpretation for these "hormonal" risk factors. There is evidence that there is a critical window of vulnerability for the breast between puberty and pregnancy wherein committed but not completely differentiated epithelium are susceptible to transformation (Russo, Hu, Yang, & Russo,

2000). These data suggest that breast cancer susceptibility is intricately linked to the complex development of the breast and the cycling it undergoes to prepare for puberty and pregnancy. In this way, even in the adult, the breast continues to be a developing tissue every month and at the critical milestones of puberty, pregnancy and menopause. The breast should therefore be considered from the context of developmental toxicity.

4.3.1. Developmental models of mammary epithelial cells for transformation

The mammosphere assay (Stem cells)

The laboratory of Clarke performed *in vitro* isolation of purified mouse epithelial cells via Epithelial Specific Antigen (ESA)-directed flow cytometric cell sorting followed by culture as aggregates or organoids (Smalley et al., 1999). This system showed that mouse mammary like structures can be produced *in vitro*. Further work from this laboratory by Dontu et al. (2003) led to the development of a system that involves the establishment of spherical floating aggregates of cells, or "mammospheres" (Dontu, Abdallah, et al., 2003). This technology was marketed as "the mammosphere formation assay". The assay is used to culture breast stem cells, which are important for the development of the breast, and is still widely used today. The floating mammosphere assays can investigate the subset of stem-like breast cancer cells that survive in suspension conditions and show enhanced tumorigenesis when implanted into mice (Charafe-Jauffret et al., 2009; M. Kai et al., 2015). This protocol provides a convenient *in vitro* measure of sphere-forming ability, a proxy for *in vivo* tumorigenesis.

Primary Human Breast Culture

Dairkee et al. (2003), demonstrated that bisphenol A-induced genomic changes in short-term primary non-tumor adjacent epithelial explant cultures resembled those found in aggressive breast tumors (Dairkee et al., 2008). The exposures were for seven days and the cultures senesced shortly after testing. Other than this work, development of models to test putative chemicals in non-diseased and non-transformed human tissue development is currently lacking. However, the Latimer tissue engineering system provides us with a unique ability to be able to test putative xenoestrogens and how they affect secondary structure *in vitro*.

4.3.2. Chemicals in the environment

Millions of synthetic chemicals have been developed over the last century, with ~100,000 currently in commercial or industrial use (Wilson & Schwarzman, 2009). Recent advances in combinatorial chemistry are likely to increase this number, exponentially, in near future. Toxicity testing has lagged badly behind production and has concentrated on genotoxicity, since this has been the property most clearly associated with carcinogenesis. Non-genotoxic carcinogenicity is well recognized, however, and includes estrogenic effects, but has not been widely mandated for testing. Few of these chemicals have been tested for developmental toxicity (Wilson & Schwarzman, 2009). In addition, naturally occurring substances often have significant toxic properties and these have not been extensively and systematically tested. The classical toxicological

approach to human exposures has been to analyze the chemicals, as we have proposed for agents affecting breast differentiation. Increasingly, however, a molecular epidemiological approach has been applied successfully, wherein evidence of toxic exposure is first detected in patients or the population, and then backtracked to the cause (Grant, 2001). The positive aspect of exposure-induced changes to human health is that often the effects can be ameliorated or even reversed by removing the source of the exposure.

It is clear that a fast and cheap yet accurate and informative test for substances that affect breast development could have far-reaching implications for the health and breast cancer risk of American women. We will therefore investigate the suitability of explants derived from the Latimer tissue engineering system to establish a medium-throughput assay for human non-diseased breast ductal differentiation for the testing of suspicious hormone disruptors. This chapter reflects the start of optimization of such a medium throughput assay for hormone disruptors of breast tissue.

4.4 Methods

4.4.1 Experimental design: chronic exposure of breast reduction explants

Testable Outcome: Number of Epispheres formed in the presence of the chemical or the vehicle.

Two unique cell lines were tested to develop a non-diseased model for testing putative xenoestrogens: JL BRL-6 (post-menopausal, run three times) and JL BRL-14

(pre-menopausal, run twice). Cells were plated on glass four chamber slides (Nunc) coated with basement membrane extract (Trevigen) at 3,000 cells per well in MWRI medium as previously described in (Latimer, 2002; Latimer et al., 2010). Chemicals used in testing included bisphenol A and estradiol that were dissolved in dimethyl sulfoxide (DMSO) and added to MWRI medium before each experiment.

Doses were developed using previous literature and our previous experiences with episphere formation (Dairkee et al., 2008; Welshons et al., 2003). Physiological doses of estrogen range from 0.4nM to full saturation of the estrogen receptor at 10 nM (Welshons et al., 2003).

Table 4.1 **Experimental design of chemical testing.**

Slide 1 BPA	100 nM	1,000 nM	10,000 nM	No Treatment
Slide 2 BPA (Replicate)	100 nM	1,000 nM	10,000 nM	No Treatment
Slide 3 Estradiol	4 nM	40 nM	400 nM	DMSO Control 100 nM
Slide 4 Estradiol (Replicate)	4 nM	40 nM	400 nM	DMSO Control 100 nM
Slide 5 DMSO Controls	DMSO Control 4 nM	DMSO Control 40 nM	DMSO Control 400 nM	DMSO Control 1000 nM
Slide 6 DMSO Controls (Replicate)	DMSO Control 4 nM	DMSO Control 40 nM	DMSO Control 400 nM	DMSO Control 1,000 nM
Slide 7 DMSO Controls	DMSO Control l000 nM	DMSO Control 10,000 nM	BLANK	BLANK

***Testing of JL BRL-06 also included duplicate slides of fulvestrant at doses 0.01 nM, 0.1 nM and 1 nM with matching vehicle controls, as well as diethylstilbestrol (DES) at doses 100 nM, 1,000 nM, and 10,000 nM with matching vehicle controls for each experiment. Bisphenol A (BPA) dimethyl sulfoxide (DMSO).**

Episphere formation was scored visually using a Zeiss microscope at 100X under phase microscopy. First, treatment was given 24 hours after plating, bisphenol A, estrogen, vehicle control (DMSO), and vehicle treated were all run concurrently. Each well received a chemical at a specific dose and slides were run in duplicate for each experiment (Table 4.1). Continuous treatment of cells continued until significant cell death was observed. Media containing each treatment agent was replaced every Monday and Friday. Epispheres were counted in each well by dividing the well into nine fields and counting from top to bottom (sum of nine fields equals total epispheres per well).

4.4.2. Statistical analysis

Multiple regression was utilized to test for correlations to episphere counts in our system. The chemical, dose, experiment, passage, and duration were all used as independent variables. Pearson's correlation coefficient (R^2) was used to test for correlations between episphere count and these factors. The model was considered significant at $p<0.05$). Minitab software was utilized to perform the regression analysis.

Episphere counts were plotted at each time point to show the effect of time for each dose on JL BRL-14. Counts for each time point from duplicate slides were averaged together. Each time point was compared to its vehicle control and significance was measured using t-test at $p<0.05$. Episphere counts for each dose were averaged together from all time points and plotted against dose to create dose curves. Episphere

counts were compared to vehicle control for each matching dose in duplicate experiments and significance was measured using a t-test at $p<0.05$.

4.4. Results

4.4.1 Standardization of non-diseased breast cultures for chemical testing

To assess the ability of our non-diseased cell cultures to be used as a testing model we assessed using the response of two noon-diseased cell lines generated through the Latimer tissue engineering system as previously described. We began with JL BRL-06 (post-menopausal) and JL BRL-14 (pre-menopausal) because these cell lines (>13 passages) retained the ability to form epispheres despite passaging. Multiple regression models were created for each test model. JL BRL-06 and JL BRL-14 test models both showed estrogen responses (the positive controls) that were positively correlated with episphere formation ($p=0.011$, $p<0.001$). However, JL BRL-14 was determined to be the most reproducible cell line with regard to episphere formation, as JL BRL-06 showed increased variability between experiments (Table 4.2). In testing model JL BRL-14, the adjusted coefficient of determination is 26.3%, therefore our model is able to explain 26% of the variability in episphere counts using these factors. The effect of estrogen and time were significant at $p < 0.001$ and were the strongest predictors of episphere number in this model. Time had a negative effect with a decrease of 7.9 epispheres for each increase in one unit of time. Estrogen had a positive effect with an increase of 5.1 epispheres for each unit increase of estrogen.

The vehicle control (DMSO) and bisphenol A dose were also significantly correlated, $p = 0.027$ and $p = 0.02$, respectively, but were in the opposite directions. The DMSO vehicle had an increase of 2.2 epispheres per unit increase, bisphenol A had a negative effect with a decrease of 2.33 epispheres per unit increase of bisphenol A. The constant was also significant; this was expected as we see epispheres in these cultures almost immediately. Therefore, the regression line does not intercept the origin.

The factors of slide number and experiment number were put into the model as internal controls and showed no significant correlation with episphere count, however, JL BRL-06 showed significant differences in our internal controls. This indicated that our system, using JL BTL-14 is reproducible as there was no difference in the combined experiments or slide replicates. Due to confounding factors such as batch variability of the basement membrane extract, we expect to see some variation between replicates and experiments. However, this was not significant between different experiments with JL BRL-14.

Table 4.2 **Multiple regression models of chemical exposures**

(A) JL BRL-06

Predictor	Coef	Stdev	t-ratio	p
Constant	564.550	52.220	10.810	0.000
EXPERIMENT	-38.768	6.814	-5.690	<0.001
DURATION	-2.639	1.992	-1.330	0.187
DMSO VEHICLE	-0.002	0.002	-0.960	0.339
FULVESTRANT	-25.030	22.960	-1.090	0.277
ESTROGEN	0.148	0.057	2.580	0.011
BISPHENOL A	0.005	0.003	1.750	0.083
DIETHYLSTILBESTROL	0.005	0.003	1.730	0.086
PASSAGE	-12.975	1.703	-7.620	<0.001
s = 62.90 R-sq = 34.8% R-sq(adj) = 31.7%				

(B) JL BRL-14

Predictor	Coef	Stdev	t-ratio	p
Constant	105.590	47.100	2.240	0.026
EXPERIMENT	3.705	5.798	0.640	0.523
DURATION	-1.601	0.204	-7.860	<0.001
SLIDE	1.727	3.147	0.550	0.584
DMSO	0.002	0.001	2.220	0.027
ESTROGEN	0.100	0.020	5.090	<0.001
BISPHENOL A	-0.002	0.001	-2.330	0.020
s = 35.88 R-sq = 27.7% R-sq(adj) = 26.3%				

4.4.2. Estrogen response in JL BRL-14

Continuing with JL BRL-14, the more reproducible line, we analyzed the effect over time of these chemicals. Evidence of a significant dose response to estrogen was found as early as 16 days of treatment (p=0.018). Estrogen exposure time curves showed significant increases over vehicle control in all 3 doses. At 4 nM, a significant increase in episphere count was observed at 23 days (p=0.025), while at 40 nM a significant effect

was observed at 28 days ($p=0.018$), and at 400 nM significant increases occurred at 13, 16, 20, 23, and 32 days ($p=0.029$, $p=0.015$, $p<0.001$, $p=0.48$, and $p=0.021$, respectively) (Figure 4.1A). Treatment with 100 nM of bisphenol A showed no significant difference at any time point. Significant increases in bisphenol A over vehicle was observed at 1,000 nM doses at 16, 20, 23, 32, and 37 days ($p=0.035$, $p=0.008$, $p=0.011$, $p=0.021$, and $p=0.011$, respectively) (Figure 4.1B). However, a decline in episphere formation compared to vehicle control at 28 days was observed with 10,000 nM bisphenol A ($p=0.058$). Both time courses shown are based on a single experiment. In both experiments, significant declines were seen after 44 days.

To further investigate the model, we also looked at which doses of estrogen and bisphenol A had the most significant effect over control. Both estrogen and BPA showed significant increases on episphere number over vehicle control (Figure 4.2). Estrogen at doses of 40nM and 400nM showed significant increases over vehicle controls ($p=0.036$ and $p<0.001$) (Figure 4.2A). Bisphenol A doses of 100 nM and 10,000 nM showed a significant increase in episphere number over vehicle ($p=0.002$ and $P<0.001$), the highest dose of 10,000 nM showed an overall decrease ($P<0.001$) (Figure 4.2B).

A B

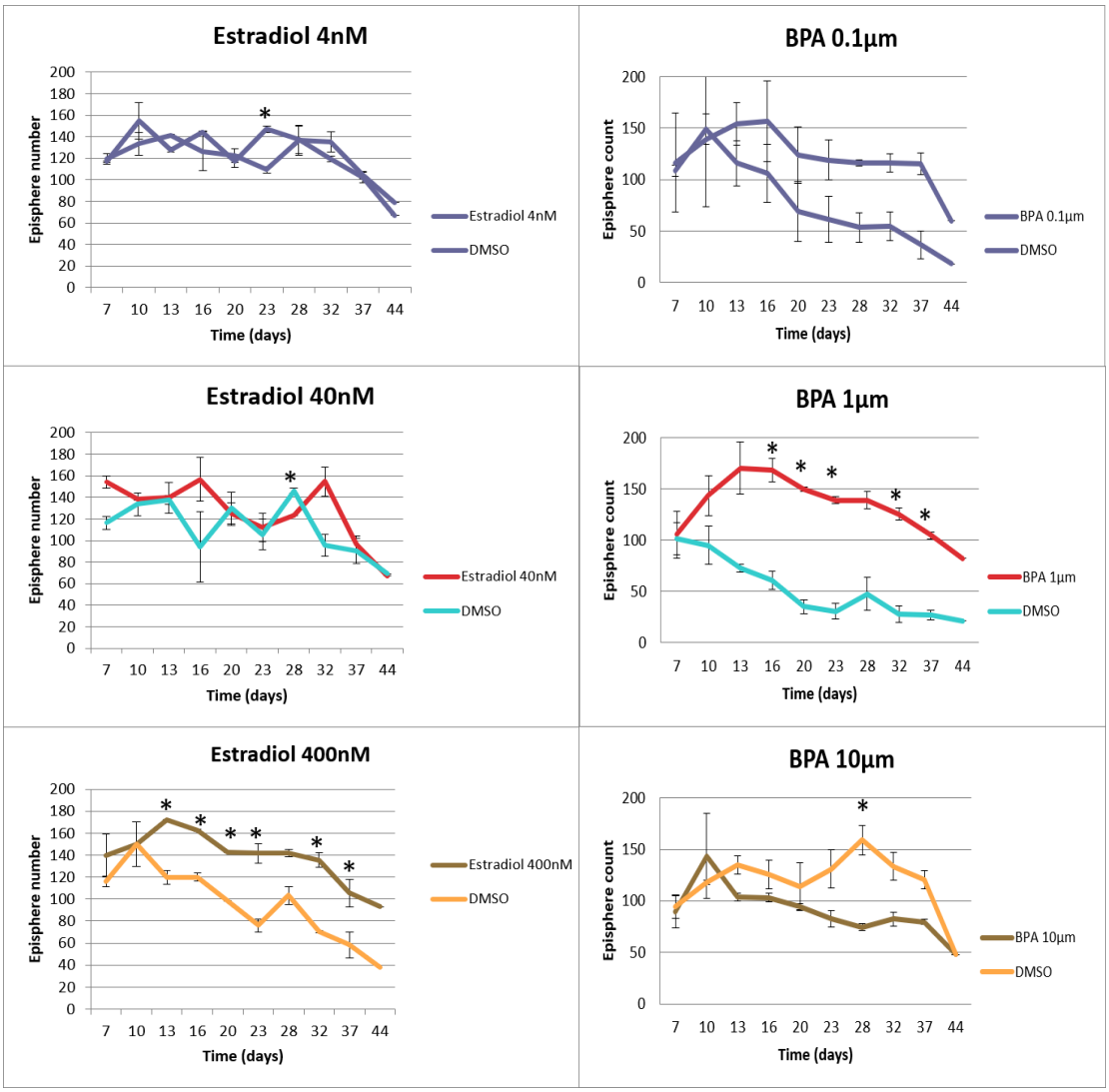


Figure 4.1. **Episphere response over time in JL BRL-14.** (A) Response to estradiol over 44 days in JL BRL-14 versus DMSO vehicle control. In 4nm of estradiol there is significant increase over DMSO control at 23 days (p=0.025). in 40 nM at 28 days (p=0.018) and in 400 nM at 13, 16, 20, 23, 32, 37 days (p=0.029, p=0.015, p<001, p=0.048, p=0.021, respectively). (B) Response to Bisphenol A over 44 days in JL BRL-14 versus DMSO vehicle control. In 0.1 µm exposure of BPA there was no significant exposure over DMSO control. In 1 µm there are significant increases at 16, 20, 23, 32, and 37 days (p=0.035, p=0.008, p=0.011, p=0.021, p=0.011). In 10 µm doses there is a significant decrease in episphere formation at 28 days (p=0.054)

A B

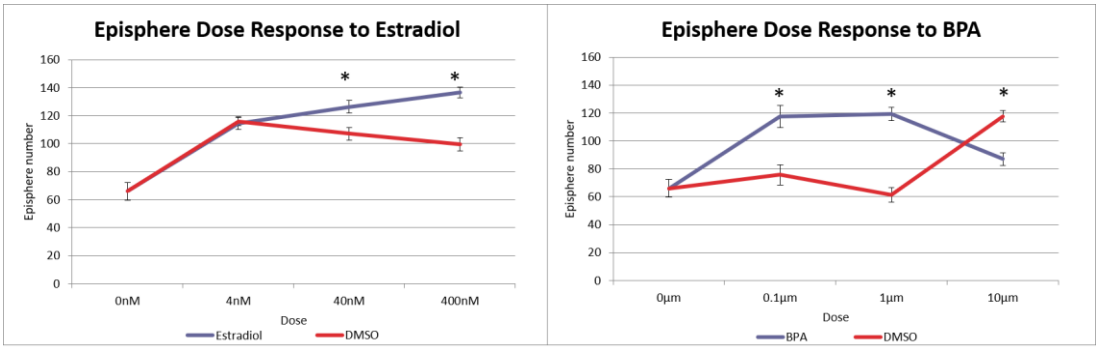


Figure 4.2. **Dose response curves for JL BRL-14 episphere formation.** (A) Dose response curves of estrogen and matched DMSO vehicle control over 44 days. Significant increases in episphere formation in medium (40 nM) and high (400 nM) exposures of estrogen over DMSO control ($p=0.036$ and $p<0.001$ respectively). (B) Dose response curves of bisphenol A and matched DMSO vehicle control. There are significant increases over control in low (0.1 µm, $p=0.002$) and medium (1 m $p<0.001$) doses. There is a significant decline in epispherses at the high dose of 10,000 nM of BPA over matched DMSO control ($p<0.001$)

4.5. Discussion

We first sought to determine the suitability of cell cultures derived using the Latimer tissue engineering system for a medium throughput assay of breast development. We scored cultures *in vitro* for the ability to form secondary structures, termed epispheres, after being exposed to estrogen and estrogen-like compounds. We first used JL BRL-06, which showed a significant positive correlation with episphere formation and estrogen exposure. However, significant differences with experiment number (between experiments) and passage were seen. JL BRL-06 is a post-menopausal derived cell line and may therefore be less able to form secondary structures in culture and therefore yield less consistent responses. Post-menopausal women may have utilized their

differentiation potential, something that may be required for continuous formation of secondary structure in passaged cells. We choose to perform chronic exposures to chemicals because the formation of epispheres may take days. To evaluate their formation, their maintenance and their disappearance would take a matter of weeks. Based on these data however, stopping the experiments at two weeks is sufficient to see the initial impact of the chemical.

We then focused on JL BRL-14, which showed no significant differences between experiments. JL BRL-14 episphere formation showed a statistically significant positive correlation with estrogen, and a significant negative correlation with bisphenol A. We therefore showed evidence that secondary structure *in vivo* can be manipulated in a dose responsive and measurable manner. While we do see a negative overall correlation with bisphenol A, the highest dose of 10,000 nM is the only dose to significantly decrease the number of epispheres. This dose may be toxic to the cells and is driving this correlation as smaller doses showed an increase in epispheres. This is an interesting point to note, as the ability of JL BRL-14 to respond differently to different doses may actually be more reflective of the *in vivo* situation.

Often, cell lines are exposed to various chemicals and apoptosis or cell death is measured. However, doses below what would be required to cause apoptosis may still be measured, and their effect quantified in this system. Dairkee et al. (2008) showed that BPA at 10^{-7} mol/L induced a pre-tumor profile in non-malignant contralateral breast

explants based on gene expression after exposure (Dairkee et al., 2008). The Dairkee group chose to use tissue that was pre-disposed to becoming malignant in order to detect possibly tumorigenic changes by expression microarray. In contrast, we developed a functional model of breast epithelial differentiation. RNA will be isolated in future studies and assessed with RNAseq rather than expression microarray because it is a more sensitive technique for gene expression that is low.

We also showed that BPA interferes with normal development of breast structure *in vitro*. *In vivo*, alterations in normal breast development such as the falling age of puberty or failure of breast epithelium to involute after lactation, are known breast cancer risk factors (Bodicoat et al., 2014; Euling et al., 2008; Radisky & Hartmann, 2009; Wyshak & Frisch, 1982). We may be able to use this system to test the ability of chemicals to affect normal breast development.

Increased lifelong exposure to estrogen is a well known cancer risk factor, especially in post-menopausal women (Burstein & Winer, 2000; Dall & Britt, 2017). Our test system allows for the measurement of the effects of putative xenoestrogens to discover how estrogenic the chemical may be in normal breast epithelium. This is especially important because assays such as the E-SCREEN utilize breast cancer derived cell lines that may not translate to an *in vivo* situation.

This test system utilizes a non-diseased breast epithelium in culture to measure secondary structure over a period of 4 weeks. This is a relatively chronic exposure as

opposed to an acute exposure. Adapting this technique to a high throughput assay may prove difficult. It would require the use of advanced cell culture as well as expensive high content platforms for imaging and analysis. The use of platforms such as the Arrayscan or Cell insight CX7 would make it feasible to adapt this system into a medium throughput assay for normal breast tissue. The Center for Collaborative Research and Nova Southeastern University now possesses a Cell Insight CX7 with automated stage, so these experiments will be considerably easier to perform now than they were.

Chapter 5

The role of nucleotide excision repair in breast cancer stem cells.

5.1 Introduction

Before 2000, cancer researchers and clinicians were taught that solid tumors were de-differentiated mature cells that had undergone accumulated somatic mutation (Knudson, 1971). Tumor cells were closer to the embryo than to the mature tissue-specific cell types from which they came. Tumor cells were originally considered to each be capable of forming another tumor by metastasizing to a new site in the body. Around the year 2000, the cancer stem cell theory became popular (Bonnet & Dick, 1997; Lim et al., 2009; Miller et al., 1989). That theory cast into doubt whether each cell in a tumor could actually form another tumor because it was hypothesized that only the "cancer stem cell" was capable of doing this. The cancer stem cell theory has been partially proven but remains controversial because it has more recently been shown that non-stem cells in a tumor can become cancer stem cells, i.e. de-differentiate, when they are under duress from hypoxia, radiation, chemotherapy, etc. (Mani et al., 2008; Morel et al., 2008; Owens & Naylor, 2013).

5.1.1 Cancer stem cells

Tumors are heterogeneous. Within each tumor resides a subset of cells called cancer stem cells. Cancer stem cells share many common characteristics with embryonic

stem cells. In the field of breast cancer, work by Al-Hajj et al. (2003) confirmed the presence of these cells. They showed that CD24-/44+ cancer stem cells can repopulate a tumor when grown and subsequently passaged in non-obese diabetic/severe combined immunodeficient mice (NOD/SCID) (Al-Hajj, Wicha, Benito-Hernandez, Morrison, & Clarke, 2003). This part of the cancer stem cell theory is true. That is these cells are capable of being passaged in immunodeficient mice and creating a new tumor, whereas, non-stem cells may grow initially in the first mouse but are not capable of being passaged to another.

5.1.2 Cancer stem cell isolation and detection

While many definitions of breast cancer stem cells have been proposed, the gold standard to verify "stemness" remains the ability to be passaged in NOD/SCID mice (Al-Hajj et al., 2003; K. Kai, Arima, Kamiya, & Saya, 2010; Owens & Naylor, 2013). However, other methods have been discovered and utilized to detect and isolate cancer and non-cancer stem cells. The mammosphere formation assay developed by Dontu et al. (2003) demonstrated that normal stem cells can be grown in suspension as spherical structures (Dontu, Abdallah, et al., 2003). When plated on bacteriological plastic round dishes, stem cells will form spherical clusters that are suspended in culture because they cannot attach to bacteriological plastic (which is not positively charged as is a cell culture plate). While originally developed from terotocarcinoma culture, this assay has also been used to describe and purify breast cancer stem cells, which also form spherical clusters on non-adherent dishes (Dontu, Abdallah, et al., 2003).

Breast cancer stem cells are most often detected and isolated by the proteins they express. By far the most common definition of breast cancer stem cells are cells that are CD24 negative and CD44 positive (CD24-/44+) (Kai et al., 2010; Phillips et al., 2006; Tannishtha, Morrison, Clarke, & Weissman, 2001; Yin & Glass, 2011). However, many proteins have been investigated and are utilized for the detection of breast cancer stem cells (Table 1.1). Functional markers of stem cells have also been developed based on key characteristics of stem cells. One such marker, aldehyde dehydrogenase, is an enzyme that has increased activity in cancer stem cells (Croker et al., 2009). This observation has led to the development of the aldefluor assay which is used to detect and select for increased activity of the aldehyde dehydrogenase ($ALDH^{+}$) in cancer stem cells (Ginestier et al., 2007). PKH26 is a membrane dye that is retained in breast cancer stem cells. PHK26+ cells have also been utilized to detect and isolate breast cancer stem cells (Owens & Naylor, 2013; Pece et al., 2010). These markers are used in conjunction with flow cytometric methods, to flow sort and isolate stems based on their presence or absence.

Breast cancer stem cells can also be prevented from differentiating by transfection with of OCT3/4 promoter. Cancer stem cells transfected with the OCT3/4 promoter showed all the characteristics of cancer stem cells and could be passaged in NOD/SCID mice (Sajithlal et al., 2010). Dr. Latimer's laboratory in concert with Dr. Prochownic at the University of Pittsburgh showed this with one of her advanced stage cell lines JL BTL-12, as well as several conventional breast cancer cell lines. Breast cancer stem cells

developed in this manner have also been used to test for mechanisms of ionizing radiation resistance in cancer stem cells (S.-Y. Kim et al., 2012). The exact mechanism of this differentiation "blockage" is currently unknown.

5.1.3 Cancer stem cell mechanisms of resistance

One proposed hallmark of breast cancer stem cells is that they are inherently more resistant to treatment (Chang et al., 2015). Some of the same characteristics or proteins that are taken advantage of in detection of stem cells directly contribute to their treatment resistance. Aldehyde dehydrogenase is an enzyme that converts aldehydes to less toxic carboxylic acids. In this way cancer stem cells are capable of detoxifying chemotherapeutic agents such as cyclophosphamide (Abdullah & Chow, 2013; Croker et al., 2009; Januchowski, Wojtowicz, & Zabel, 2013). Associations with ABC transporters and ALDH+ cells indicate they may function cooperatively to increase drug resistance in these cells (Januchowski et al., 2013).

Cell death pathways are also implicated in cancer stem cell resistance. BCL2, FLIP and BCL-Xl are all anti-apoptotic genes and have been shown to have increased expression in cancer stem cells (Abdullah & Chow, 2013; G. Liu et al., 2006; Pavlopoulou et al., 2016; Safa, 2016). Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), a gene that promotes inflammation and inhibition of apoptosis plays a role in breast cancer stem cell survival (Safa, 2016).

The activity of checkpoint kinases (CHK1/2) have been shown to contribute to ionizing radiation resistance in breast cancer stem cells. CHK1/2 is a serine/threonine protein kinase that performs the rate-limiting step in cell cycle arrest in response to DNA damage (Yang et al., 2015). Multiple studies have shown association and increased expression in DNA repair genes including EGFR, MGMT, ATR, ATM, CDKN2A, PDGFRA, RAD51, and BRCA1 in breast cancer stem cells (Cabarcas, Mathews, & Farrar, 2011; Eyler & Rich, 2009; Y. Liu et al., 2017; Maugeri-Saccà, Bartucci, & De Maria, 2012; Sciuscio et al., 2011; Verhaak et al., 2010).

More specifically single strand break repair has also been shown to be functionally increased in breast cancer stem cells. MCF7 mammospheres have shown increased activity in response to 4 Gy of radiation, using the comet assay, when compared to non-stem monolayers of MCF7 (Karimi-Busheri, Rasouli-Nia, Mackey, & Weinfeld, 2010). Also, protein expression was increased in Apurinic-Apyrimidinic Endonuclease 1 (APE1), a multifunctional gene involved in DNA base excision repair pathway.

5.1.4 Conflicting evidence in therapy resistance for cancer stem cells.

Even though there are multiple mechanisms of treatment resistance in cancer stem cells, conflicting results have also been shown. MCF7 stem cells defined by CD24-/44+ surface marker patterns have been shown to be resistant to ionizing radiation (Phillips et al., 2006; Yang et al., 2015). However, Kim et al. (2012) showed that MDA MB231 and

MD MB453 CD24-/CD44+ and OCT3/4 blocked stem cells were sensitive to ionizing radiation than cancer non-stem cells(Kim et al., 2012). These contradictory outcomes are unresolved.

Chemotherapy resistance to paclitaxel and epirubicin was associated with breast cancer stem cells in ALDH1+ tumors. However, this association was not found in histologically stained CD24-/44+ breasts tumors (Tanei et al., 2009). One study showed that MCF7 CD24-/44+ OCT3/4 blocked stem cells were resistant to adriamycin, etoposide, 5-flourouracil, cis-platinum, and methotrexate, however, they were sensitive to Taxol (Sajithlal et al., 2010). MCF7, MDA MB231, MDA MB453, and JL BTL-12 CD24-/44+ OCT3/4 blocked stem cells showed differing responses to chemotherapeutic agents depending on the cell line (Sajithlal et al., 2010). MDA MB453 cancer stem cells were resistant to 5-fluorouracil, MDA MB231 and JL BTL-12 were resistant to adriamycin as well as 5-flurouracil.

In different studies, both the antibiotic salinomycin and the diabetic drug metformin seemed to sensitize cancer stem cells to cytotoxic agents. Metformin seemed to work synergistically with herceptin to inhibit self-renewal, in a mechanism that is not been defined (Vazquez-Martin, Oliveras-Ferraros, Del Barco, Martin-Castillo, & Menendez, 2011). Salinomycin, in conjunction with histone deacetylase inhibitors, seems to sensitize cancer cells via induction of apoptosis and cell cycle arrest (Kai et al., 2015).

5.1.5 DNA repair in breast cancer stem cells

No specific functional analyses for the 5 major DNA repair pathways have been performed on breast cancer stem cells. Many of the mechanisms of resistance in cancer stem cells seem to be shared, however, there is a great deal of variability in response (Table 2.6). Cancer stem cell surface markers/isolation method, as well as the biological origin of the cancer stem cell are likely to play a role in this variability. Conflicting results in breast cancer stem cell resistance highlights our lack of understanding of cancer stem cells. Many DNA repair genes have been implicated in cancer stem cell resistance, however, functional assays have not been tested (Karimi-Busheri et al., 2010; Maugeri-Saccà et al., 2012).

Nucleotide excision repair function has not been specifically tested in cancer stem cell and the corresponding non-stem cells of tumor cell lines. This is surprising as nucleotide excision repair plays a role in both chemotherapeutic resistance as well as ionizing radiation (Kelley, Logsdon, & Fishel, 2014; Martin, Hamilton, & Schilder, 2008; Michailidou et al., 2015; Stordal & Davey, 2007). Furthermore, no information is available on the stage specificity in cancer stem cell behavior. We therefore propose that increased nucleotide excision repair capacity measured by the unscheduled DNA repair assay will be displayed in breast cancer stem cells.

5.2 Methods

5.2.1 Cell culture

Three cell lines were used for this experiment MDA MB231 (ATCC), JL BTL-12, and JL BTL29. MDA MB231 is a triple negative stage IV breast cancer cell line obtained from a 51-year-old patient pleural effusion. The JL BTL cell lines were created using the Latimer tissue engineering system (US Patent 6,383,805). JL BTL-12 is a stage III breast tumor cell line obtained from a 35-40 year-old patient with luminal A breast tumor. JL BTL-29 is a stage II hormone negative breast tumor cell line obtained from a 35-40 year-old patient (Table 5.1). Both JL BTL cell lines were chemo naïve, MDA MB231 (ATCC) is a chemotherapy resistant cell line. BTL cell lines were grown and maintained in MWRI medium at 37 °C with 5% $C0_2$, as described in previous sections. MDA MB231 was grown in DMEM with 10% FCS with the same incubator conditions.

Table 5.1 **Cell line characteristics for stem cell analysis**

Cell line/culture explants	Stage and site of isolation	Patient age and race	Molecular characteristics	
			ER	PR
MDA-MB-231	IV, pleural effusion	51, Caucasian	-	-
JL BTL-12	III, primary breast tumor	35-40, Caucasian	+	+
JL BTL-29	II, primary breast tumor	35-40, Caucasian		

5.2.2 Stem cell staining

Cells were disaggregated with 0.25% trypsin containing EDTA (Hyclone), suspended in 1mL of Hanks Buffered Saline Solution (HBSS, Hyclone) with 2% fetal

bovine serum (FBS, Hyclone), and counted with a hemocytometer. Cells were washed twice with HBSS containing 2% FBS and resuspended in 50 uL of HBSS (Hyclone) with 2% FBS (Hyclone).

Live cells were incubated with 20 uL of FITC conjugated mouse Anti-human CD24 (Clone ML50, BD) and APC conjugated mouse Anti-human CD44 (Clone G44-26, BD) antibodies for every 10^6 cells, for 1 hour on ice, and protected from light. Cells were then washed twice with HBSS. After washing, cells were resuspended in 0.5 mL of HBSS 2% containing FBS and propidium iodide (Sigma-Aldrich®) at a concentration of 10 ug/mL for every 10^6 cells.

5.2.3 Cell sorting and optimization of sample preparation

Before each sort, compensation, instrument set up and sensitivity were verified and determined using BD CaliBRITE^{tm}, FITC, PE, and APC beads (BD, Cat No. 340486). Gating was determined from unstained samples and positive gates created had less than 99.99% of events in unstained samples for each antibody.

Once the staining was completed, cells were immediately sorted using the BD FACSJazz Cell Sorter. Cells with the staining pattern: CD24-/44+ (stem) and CD23-/44- (non-stem) cells were sorted directly into separate 5 mL polypropylene tubes. Tubes were prefilled with 4 mL of warm MWRI medium. Cells were then moved to 2-well slides at 1 mL per well (Nunc). The chambers of these slides had previously been coated with a 100% basement membrane extract (Trevigen®, Cat#3432-005-01) for both JL

BTL-12 and JL BTL-29. MDA MB231 cells were plated directly on the glass slides. Cells were incubated at 37 °C with 5% CO_2 for 24 hours, then were ready to be analyzed by the unscheduled DNA synthesis assay. Preliminary experiments done with a 48 hour incubation before beginning the unscheduled DNA synthesis resulted in bimodal peaks in grain counts of nuclei. This finding is evidence that multiple cell types, most likely due to differentiation of cells during that time or possible contamination of other cell types maybe causing the bimodal peaks. We also found that diluted 1:1 basement membrane extract (Trevigen®) was not sufficient for supporting attachment of cancer stem cell and non-stem cells after sorting. We therefore decreased the incubation time to 24 hours prior to beginning the unscheduled DNA synthesis assay and coated 2-chamber slides with undiluted basement membrane extract (Trevigen®).

5.2.4 Unscheduled DNA synthesis assay

The unscheduled DNA synthesis assay was performed as previously described (Latimer et al 2003; 2010). Cancer stem cells, non-stem cells and unsorted parental cell lines were all ran concurrently. Control foreskin fibroblast cells were plated 24-48 hours prior to experiments onto glass 2 chamber slides (Nunc).

On the day of the unscheduled DNA synthesis assay, all medium was removed, and slides were placed into an irradiation chamber with the well closest to the ground glass protected from UV. The other chamber was exposed to a total of 14 Joules/m^2 of 254 nm UVC irradiation. Immediately following irradiation, cells were incubated in

DMEM supplemented with 10% FBS and 10 μCi/ml [H^3] methyl-thymidine (PerkinElmer Life Sciences®) for two hours in a Forma Series II Water Jacketed CO_2 incubator that is dedicated to radioactive treated cultures at 37 ˚C in 5% CO_2. Labeling was followed by a two hour chase with DMEM medium supplemented with 10% FBS and 10mM thymidine (Sigma-Aldrich®). Slides were then washed with 1X sodium citrate (Sigma-Aldrich®) in PBS, then fixed in 33% acetic acid (Fisher Scientific®) in ethanol (Sigma-Aldrich®) for 15 minutes, then 70% ethanol in distilled water for 15 minutes. Slides were left overnight to dry, then dipped in a photographic emulsion (Caresteam®). Slides were then sealed in light tight slide boxes wrapped in foil to protect them from light and left in complete darkness for 11 days at 4 ˚C. After 11 days, two tester slides, that had been packaged separately, were developed first in Kodak® developer then in Kodak® fixer. If adequate exposure of emulsion had occurred in controls (50 grains per nucleus for FF), the remaining slides were then developed and stained with Giemsa (Sigma-Aldrich®), rinsed, and counted. Counting was done on Zeiss Axioskop microscope at 1,000X with oil immersion lens.

Quantification of silver grains on 100 nuclei from unirradiated and irradiated chambers was done by trained laboratory personnel and local background was subtracted from each nucleus. Cells that had greater than 80 grains per nuclei were considered to be in S-phase and were excluded. The mean grains per nucleus was calculated for both sides of the slide. Then the unirradiated mean was subtracted from the irradiated mean, to remove background. The remaining value was divided by the same value calculated for

the positive control Foreskin Fibroblast cells. Duplicate slides were compared. Total number of cells in S-phase per field were also recorded to calculate the S-phase index of each cell line.

5.2.5 Statistics

The average DNA repair capacity was obtained from the unscheduled DNA synthesis assay. cancer stem cells were compared to both non-stem and the parent line within each cell line. Non-stem cells were also compared to the parent line. Significance was determined using a two-way *t*-test ($p<0.05$).

5.3 Results

The unscheduled DNA synthesis assay was used to determine the nucleotide excision repair capacity of three cell lines. Table 5.1 summarizes the cell line characteristics. All three cell lines had similar stem cell percentages. MDA MB231 had the highest percent of CD24-/44+ cells and JL BTL12 had the lowest percent of CD24-/44+ cells. When a third marker, CD49f, was added we still saw very few differences between the three cell lines. For the sake of sorting speed, we used only CD24-/44+. JL BTL-12 had the highest percent of cells that are CD24-/44+/49f+ and JL BTL-29 has the lowest (Table 5.2).

Table 5.2 **Cell line stem cell percentages in cell lines.**

Cell line/culture explants	Percent CD24-/44+	Percent CD24-/44+/49f+
MDA-MB-231	95.99%	80.69%
JL BTL-12	85.76%	82.55%
JL BTL-29	95.73%	76.89%

Individual dot plots for each cell line to calculate percentages are provided in Figure 5.1. Overall, all three cell lines stained strongly for the CD44 antibody with little variation in any cell line. Unstained cells are provided in each plot for reference. These cell lines are high in stem cell percentages for both CD24-/44+ and CD24-/44+/49f+ cells.

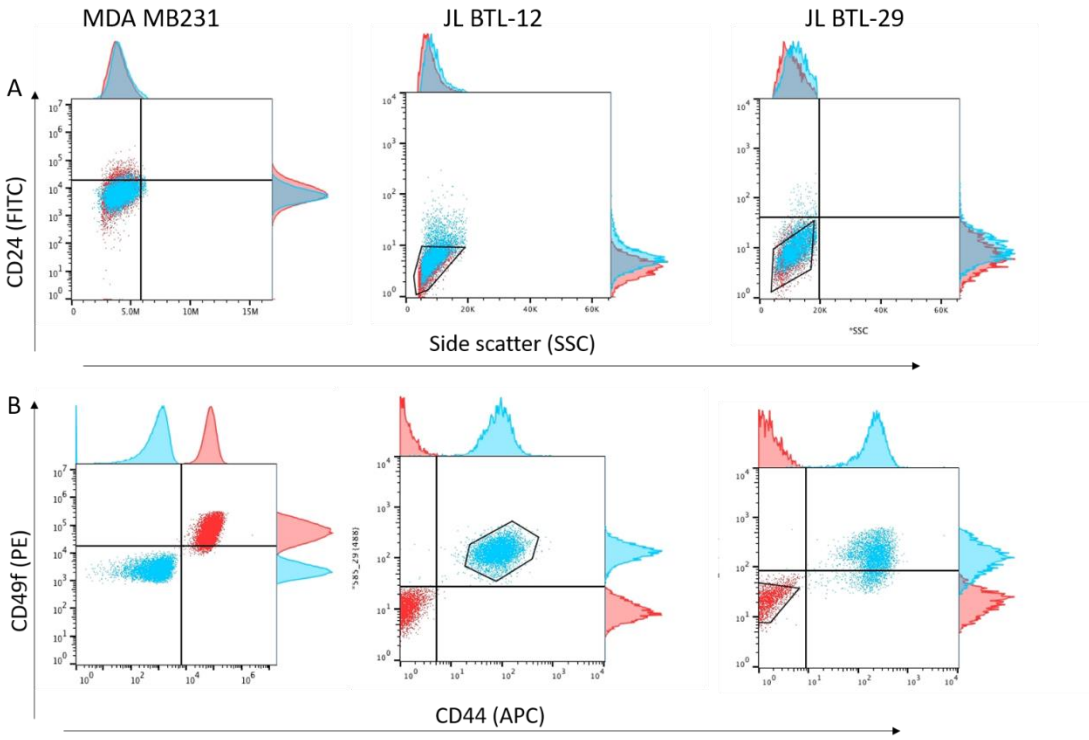


Figure 5.1 **Cancer cell lines stem cell staining patterns.** Stained cells for CD24, CD44, and CD49f (blue) are shown with accompanying unstained cells (red) for each cell line. (A) Dot plot represents CD24 vs. side scatter, increases on the y-axis of stained cells over unstained indicate cells positive for CD24. Overall there is very little expression of CD24 across all cell lines (bottom left quadrant). (B) Stained cells for CD44 vs. CD49f show increases over unstained population for both markers. Cells in top right quadrant are positive for both markers.

5.3.1 Nucleotide excision repair capacity in stem and non-stem cells

The nucleotide excision repair capacities based on flow sorted stem cells (CD24-/44+) and non-stem cells (CD24-/44-) showed differences. The original parent line was not sorted and is used to compare stem cells to the overall heterogeneous population of cells in each cell line. MDA MB231 demonstrated a very high nucleotide excision repair capacity, 106.69% of foreskin fibroblasts. The nucleotide excision repair capacity in the stem cells of MDA MB231 was similarly high, 117.13% of FF, and the non-stem nucleotide excision repair capacity was considerably lower, 64.15% of FFs. JL BTL-12, the nucleotide excision repair capacity of the parent cell line was 48.12% of FFs. The stem line was again similar, 48.60% of FFs, however the non-stem cell lines were lower with only 26.24% of FFs. Finally, in JL BTL-29 the nucleotide excision repair capacity in the parent line was 50.63% of FFs. The stem cell compartment has a nucleotide excision repair capacity that was 44.68% of FFs, and the non-stem was slightly lower at 37.70% of FFs. Overall, the trend shows that breast cancer stem cells were considerably higher in nucleotide excision repair capacity than the remaining cells in the cell line, i.e. the cancer non-stem cells, as measured by the functional unscheduled DNA synthesis assay. The fact that the parental lines were similar to the cancer stem cells in repair capacity reflects the high percentage of cancer stem cells in these cell lines. These results are summarized in Figure 5.2.

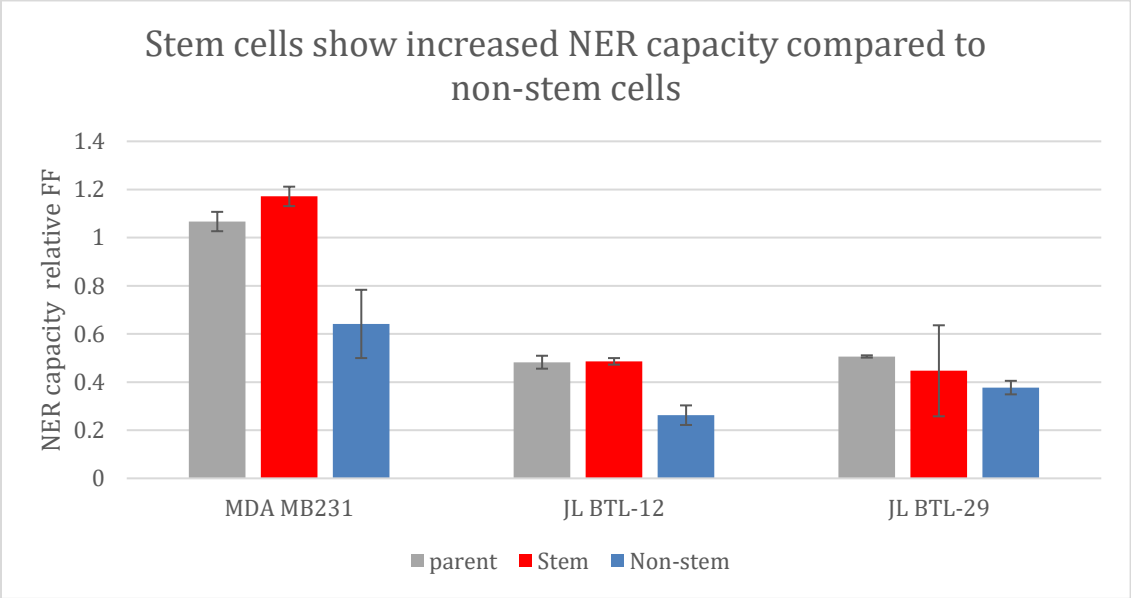


Figure 5.2 **Nucleotide excision repair capacity of stem and non-stem cells.** CD24-/44+ cells are considered cancer stem cells and CD24-/44- cells are considered non-stem cells. DNA Nucleotide Excision Repair in the cancer stem cells (RED) is higher than in the non-stem cells (BLUE) from each cell line. The parental line (GRAY) represents the unsorted original cell line. MDA MB231 is a stage IV cell line that is chemotherapy resistant and it manifests the highest DNA repair. JL BTL-12 is a stage III cell line that is chemotherapy naïve. Both show a similar pattern in the cancer stem cells having higher repair than the non-stem cells. JL BTL-29 is a stage II cell line that is chemotherapy naïve and the significance is unclear without further experiments. Both of the chemotherapy naïve cell lines are on the whole are lower in DNA repair than MDA MB231. nucleotide excision repair capacities are based on duplicate slides from a single experiment. Standard error is calculated between slides.

5.4 Discussion

Based on a single experiment, we did not see significant changes in nucleotide excision repair capacity in cancer stem versus cancer non-stem cells. However, we do see that in all three cell lines breast cancer stem cells have increased nucleotide excision repair capacity compared to the cancer non-stem cells. More experiments are needed to confirm these results and measure significance; a second experiment is currently being

analyzed. Functional assay of nucleotide excision repair has never been shown on cancer stem cells probably because they differentiate very rapidly, and it is a challenge to sort them, get them to attach in culture and run the unscheduled DNA synthesis biochemical assay while they are still stem cells.

MDA MB231 is a highly aggressive tumor that displays high levels of stem cells as shown both here. Furthermore, MDA MB231 was derived from a patient that had undergone genotoxic chemotherapy, therefore it is likely that selection for more resistant cells occurred *in vivo*. JL BTL-12 is also derived from a late stage cancer; however, it is chemotherapy naïve. Interestingly, its cancer stem cells are lower in nucleotide excision repair capcity than MDA MB231; the lack of chemotherapy induction may play a role in this.

JL BTL-29 is also a chemotherapy naïve cell line but it was derived from a stage II (early stage) tumor. Therefore, it is not surprising we see little differences in the parent and breast cancer stem cell nucleotide excision repair capacity as the majority of cells in the parent unsorted population are already very stem like.

Comparisons of the nucleotide excision repair capacities in breast cancer stem cells compared to breast cancer non-stem tells gave interesting results. The trend in all three cell lines shows that nucleotide excision repair capacity of non-stem cells is lower than stem cells from the same cell lines. The difference was most dramatic in stem cells

from MDA MB231 and JL BTL 12. Both cell lines showed that stem cells had almost twice the repair capacity of non-stem cells.

Nucleotide excision repair has been shown to be a mechanism of tumor resistance (Ferry, Hamilton, & Johnson, 2000; Martin et al., 2008; Rosell et al., 2003). Increased nucleotide excision repair capacity in stem cells would be very much in line with a majority of literature, as most studies have shown cancer stem cells to be generally resistant to toxic injury (K. Kai et al., 2010). One contradictory study provided evidence that breast cancer stem cells can actually be more sensitive to ionizing radiation (S.-Y. Kim et al., 2012). This study, while intriguing, used OCT3/4 "blocked cells" in addition to CD24/44 markers. The introduction of the OCT3/4 into cells is known to make dramatic changes in cells (Rizzino, 2009). Our study uses no such transforming agent, furthermore we see the same trend across stages II-IV. Future experiments will include stage I. This may indicate that there is in intrinsic difference in nucleotide excision repair in cancer stem cells. Furthermore, while chemotherapeutic induction of nucleotide excision repair was a possibility, our JL BTL cell lines still show a difference in breast cancer stem cell nucleotide excision repair over the rest of the cells in the cell line. This indicates that chemotherapeutic selection may not be responsible for these differences.

The cell lines used here remain heterogeneous, with at least two distinct cell types in regard to DNA repair. It is interesting to note that the increased variability in JL BTL-29 cells may be hints at there being more than one type of stem cell or progenitor in this

population. Indeed, the breast has multiple progenitor cells (Hwang-Verslues et al., 2009). Further molecular analysis and sorting based on more and/or different markers may shed light on this observation.

Most obvious is the need for further confirmation of these findings, and repeated experiments have already been completed and are currently being processed by our laboratory. While only based within a single experiment the trend continues across all three cell lines. Upon confirmation of these findings, not only would we support the theory of resistant more aggressive tumor we also intend to find the molecular basis by which stem cells increase their resistance.

Chapter 6

Overall implications and future directions

6.1 Exploring our *in vitro* model system for the factors that control it

We investigated possible epidemiological factors associated with how well primary cultures from non-diseased breast explants formed secondary breast ductal structures in culture. We found that African American women developed these structures, on average, in half the time of European white women. Based on this observation, we compared gene expression, stem cell compartment size and stem cell potency between breast explant cultres from African American and European White women

We found that African American reduction mammoplasty cell lines had increased proportions of stem cells, defined as CD24-/44+/49f+ cells relative to European White, and that these cells were also more capable of bi-potency than those from European White derived cell lines. Although preliminary, we also found that in samples from European White women stem cell compartments are more consistent with those seen in post-menopausal derived cell lines. However, this aspect of the study is limited by the lack of available post-menopausal cell lines. The present results rely on a very small number for post-menopause analysis, one African American cell line and two European White cell lines. Another limitation of this study is the that we have not determined the percent admixture in the self-declared African American and European-derived White

subjects. We have additional samples that may reflect patients that have more European White lineage than African American lineage, so all cell lines need to be validated for admixture.

We know that there are "windows of vulnerability," for genetic mutations, in breast development that coincide with breast developmental phases (Martinson, Lyons, Giles, Borges, & Schedin, 2013). During these windows, women are more sensitive to genotoxic agents and this may affect their risk of developing breast cancer (Martinson et al., 2013; Russo & Russo, 2011). African American women's stem cell characteristics are different then European-derived White women and they develop ductal structures in culture, differently, as well. African American women go through thelarche, one of the "vulnerability" phases, at much younger ages than European derived white woman. They may therefore respond at different times and differently to factors that influence breast cancer (Bodicoat et al., 2014; Cabrera et al., 2014; Martinson et al., 2013).

We have developed an *in vitro* model of thelarche where we can test various chemicals and how they affect breast architecture. The Latimer tissue engineering system is a model of thelarche when it spontaneously undergoes ductal development, in which breast stem cells play a role. Our first attempts at using this system as a model for the thelarche utilized the simple characteristic of formation of epispheres. Episphere formation is an active process that has been documented in time lapse movies in the laboratory.

We have shown that the development of JL BRL-14 epispheres is responsive to estrogen and BPA in a dose dependent manner. With further validation and use of other estrogenic and antiestrogenic compounds this early work lays the foundation for the development of a medium throughput assay. The use of high throughput instruments such as the Thermofisher Cellinsight^{TM} will allow us to turn this assay into a medium throughput assay. Additional work is already under way, and it is our hope that we can test additional chemicals for their putative role in hormone disruption of breast differentiation. RNA sequencing will be used to determine the gene expression changes and potential mutation that are present as a result of these exposures. In addition, pre-disposed cultures that are already on the path to cancer can be used instead of reduction mammoplasties in order to see visible steps towards cancer.

Episphere formation from women of different ancestries could also be tested when the system is optimized. The two cell lines tested were both European White, but they had the capacity to form architecture over later passages. Only one was reproducible and another approach to chemical testing might be to use only one for all chemicals in the tradition of the ESCREEN.

An interesting experiment would compare nucleotide excision repair function of normal stem cells in African American and European-derived White women to their respective non-stem cell populations with the unscheduled DNA synthesis assay. Then, using the Latimer tissue engineering system, we would expose the derived cells to

mutagenic chemicals. This would give us valuable information. First, can non-diseased stem cells be transformed in this system to malignancy? Then, do African American and European-derived White women explants respond differently to insult? If malignant cells are formed, do we see differences in the malignances of European White and African American women, i.e. the resultant breast cancer subtype? This can be taken further by testing the resulting transformed cells by again sorting and measuring the nucleotide excision repair capacity after malignancy, in the parent, cancer stem cells, and cancer non-stem cells. A major obstacle in this experiment would be maintaining the stem cell state during the transformation process. Historically, it has been shown that transformation assays are extremely difficult to make work *in vitro* and the long period of time necessary for exposure to cause enough somatic mutations would cause the stem cells to differentiate (Tralau & Luch, 2012).

6.2 Cancer stem cells and treatment resistance

While breast cancer etiology through exposure represents one part of this study, progression and treatment resistance represents the other. We have shown that there is an intrinsic deficiency in both nucleotide excision repair function and nucleotide excision repair gene expression in sporadic stage I breast cancer (Latimer et al., 2010). However, more recently, we have shown that there is an increase in nucleotide excision repair in late stage breast cancer cell lines (Appendix A). Only cancer stem cells from late stage-derived cancer cell lines have been studied in the literature, therefore, our use of a stage II cell line is novel. It may be that early stage breast cancers have cancer stem cells with

very little intrinsic treatment resistance. This has never been explored in spite of the fact that the majority of breast tumors diagnosed in the US and Europe are stage I.

In order to control differentiation, groups have transfected cancer stem cells with constructs that limit differentiation (Kim et al., 2012). The study found the opposite result with ionizing radiation as we found with UV radiation. They also used the less specific comet assay, as opposed to specific repair remediated by nucleotide excision repair in the unscheduled DNA synthesis assay. To limit differentiation, an OCT3/4 transfection was used in MDA MB231 and MDA MB453 cells. We also used MDA MB231 in addition to two JL BTL cell lines, however these cells were not transformed. The addition of the OCT3/4 promoter may explain some discrepancies between our results.

Not only do commercially available, chemotherapy resistant cell lines show increased nucleotide excision repair function, but the chemotherapy naïve stage III cell line JL BTL-12 does as well (Appendix A). These finding suggests that increased nucleotide excision repair capacity may be an intrinsic quality of the progression of late stage breast tumors, and not necessarily only the result of *in vivo* selection for chemotherapy resistance or adaptation to cell culture. However, the chemotherapy resistant cell line MDA MB231 is overall higher in repair than the chemotherapy naive JL BTL12 or JL BTL29 (Appendix A). Suggesting that chemotherapy can cause selection of even higher treatment resistance than the cancer stem cells intrinsically

possess. Analysis of the cancer stem cells of additional cell lines will clarify this.

It is possible that cancer stem cells are the driving force behind the increased nucleotide excision repair capacity observed in the cell lines derived from advanced stage tumors, but it has been shown that the non-stem cells can convert to cancer stem cells upon treatment and with hypoxia *in vivo* (Ansieau, 2013). While based on only a single experiment, this is the first-time nucleotide excision repair specific function was assessed in breast cancer stem cells. We have already completed a second experiment and included an additional stage I cell line, JL BTL-33. The nucleotide excision repair capacities are being calculated and will be added to this data upon completion.

While targeting cancer stem cells for therapeutic treatment may seem ideal it has proven to be elusive. This is due to the epithelial to mesenchymal transition pathway, whereby epithelial cells lose their polarity and adhesion and become migratory (Ansieau, 2013). In cancer, epithelial to mesenchymal transition is highly associated with cancer stem cells and a mechanism of metastasis and increased resistance. Furthermore, under certain stressful conditions, i.e. hypoxia, radiation, or chemotherapy, non-stem cells can convert to cancer stem cells (Lee et al., 2016). This makes them extremely difficult to target. It may be possible that by targeting nucleotide excision repair genes we can lower repair in cancer stem cells to be similar to the rest of the tumor. In theory this should sensitize cells to treatment.

6.3 Conclusion

Although we are limited in the number of cell lines we determined that African American derived cell lines had increased percentages and potency in their breast stem cells compared with European derived White cell lines. The literature indeed has shown evidence of increased breast proportions in breast stem cells of African American women we now also show differences in behavior of these cells. This dissertation then lays the foundation for development of a medium throughput assay using a unique culture system. While this work is preliminary we have shown that structures termed epispheres can be measured and are responsive to estrogen and other chemicals. While this relied on microscopic analysis of chamber slides, the use of high-throughput instruments will now be implemented. This assay will be vital for determining the effect of chemicals on breast development. Finally, we showed that breast cancer stem cells are increased in nucleotide excision repair capacity over breast cancer non-stem cells. Again, this work is preliminary and is still being completed, however, we see that in to late stage breast cancer cell lines there is increased DNA repair capacity in cancer stem cells that may not exist in early stages. This is novel and explains the increased resistance seen in these cell lines and late stage breast tumors in general. Prior work by our lab also shows that nucleotide excision repair is modifiable (Appendix A), therefore, decreasing this mechanism will allow for increased sensitivity to already existing chemotherapy. Targeting nucleotide excision repair in breast cancer stem cells is a vital pharmacological point of intervention in breast cancer treatment.

Appendix

Appendix A

The following section is a study completed in a joint effort with prior members of the Latimer laboratory and myself. This section is an excerpt from a thesis that has been submitted for publishing by Homood As Sobeai titled "DNA Repair Capacity in Commercially Available Breast Cancer Cell Lines Compared to Primary Early Breast Tumor Cultures" 2017. The subsequent manuscript is being submitted for publication to BMC genomics by the following authors: Jennifer M. Johnson[1] , Homood As Sobeai[2], Nancy Lalanne[3], Omar Ibrahim[2], Stephen G. Grant[4], Sharon L. Wenger[5], Jean J. Latimer[2]; [1]Thomas Jefferson University Hospital, Philadelphia, PA; [2]Nova Southeastern University College of Pharmacy, Fort Lauderdale, FL [3]Case Western Reserve University, Cleveland, OH; [4]Nova Southeastern University College of Osteopathic Medicine and public health, Fort Lauderdale, FL [5]West Virginia University, Morgantown, WV. Work in this section completed by myself includes microarray of breast cancer cell lines. This work is pertinent to my dissertation in that it established nucleotide excision repair as increasing in both function and gene expression with increasing stages of breast cancer. This work sets the foundation of our hypothesis that breast cancer stem cells are increased in nucleotide excision repair function.

Elevated DNA nucleotide excision repair capacity in established breast cancer-derived cell lines relative to primary breast tumor cultures

A1.1 Introduction

Established stage IV breast cancer cell lines are the most common models used to study breast cancer. There are several reasons behind their popularity in breast cancer research. They are easy to handle, provide an unlimited regenerative source of breast tumor cells, and possess a high degree of homogeneity that allows researchers, under well-designed experimental conditions, to generate reproducible and reliable data. The use of these cell lines has enriched our knowledge of breast cancer features such as proliferation, invasion, angiogenesis, metastasis, and drug resistance (Holliday & Speirs, 2011). Utilizing such knowledge in preclinical and clinical settings has yielded breakthrough discoveries that have improved patient outcomes over the last 50 years (Lee, Oesterreich, & Davidson, 2015).

There are more than 60 human breast cancer cell lines that are commercially available, 20 of which are widely used in breast cancer research (American Type Culture Collection, 2017). There are many concerns regarding using these cell lines to study breast cancer. First, the vast majority of these cell lines have been derived from metastatic breast cancer cells that are isolated from the pleural effusion of stage IV breast cancer patients (Cailleau et al., 1978; H D Soule et al., 1973). Metastatic cell lines might

not reflect the cancer cells that are present in the primary tumor. Second, these cell lines are stage IV tumor cells which are not true representatives of the majority of breast tumors that are diagnosed in the clinic now, which are stage I tumors (Howlader et al., 2013). Lastly, these cell lines may have changed some of their fundamental cellular and genetic characteristics due to culture condition adaptation. For instance, a number of MCF-7 cell lines that were obtained from different laboratories have been shown to have significant biological and genomic parameters such as growth rate, hormonal response, and karyotype, despite the fact that they all had the same origin, suggesting that MCF-7 is a highly mutable cell line capable of altering fundamental cellular and molecular characteristic that might not reflect the original tumor from which this cell line has been derived (Bahia et al., 2002; Wenger et al., 2004). Thus, researchers should constantly characterize the established cell lines they are using to ensure that they maintain the cellular and genetic features necessary to make them appropriate and representative.

Our laboratory has developed a novel tissue engineering culture system to overcome the flaws of the established breast cancer cell lines. Using this culture system, numerous breast tumor primary cultures that represent all stages and molecular subtypes of breast cancer have been successfully maintained and established novel cell lines without the use of human telomerase reverse transcriptase or exogenously transforming agents. This system provides the laboratory with a set of invaluable research models that can be used to study a variety of cellular and molecular characteristics associated with breast cancer etiology and progression (Latimer, 2000; Latimer et al., 2003).

Genomic instability is a hallmark of human cancers that can be caused by the loss of efficient DNA repair function (Vogelstein & Kinzler, 2004). We have shown that sporadic stage I breast tumor primary cultures exhibited a significant loss of nucleotide excision repair (NER) function compared to non-diseased breast tissue primary cultures (Latimer et al., 2010). Gene expression analyses of the 20 NER canonical genes using RNase protection assay showed that 19 out of 20 genes were significantly downregulated in stage I breast tumor explants compared to non-diseased breast epithelial tissue explants. These data suggest that loss of NER plays an intrinsic role in breast cancer etiology.

In the present study, we extended our investigation of NER function and gene expression to a series of widely used commercially available breast cancer cell lines to determine whether the loss of NER function that was observed in stage I tumors is maintained in these cell lines. We hypothesized that the well-established, commonly used breast cancer cell lines that have been derived from late stage breast tumors that survived chemotherapy treatment have been clonally selected for an aggressive tumor population that has acquired high DNA repair capacity during tumor progression.

We believe our study is clinically relevant since it examines whether the widely used commercially available breast cancer cell lines are true representatives of the majority of newly diagnosed breast cancer cases, stage I tumors, for an essential genomic characteristic, NER function, that is associated with genomic instability and breast

carcinogenesis. Also, our study provides a new potential NER targeted therapeutic approach that might be used with genotoxic agents to avoid chemotherapy resistance, especially breast cancer patients who at a high risk for being chemoresistant.

A1.2 Materials and methods

A1.2.1 Established breast cancer cell lines and culture conditions

We selected five established breast cancer cell lines, MCF-7, CAMA-1, SK-BR-3, BT-20, and MDA-MB-231, and a variant of MCF-7, MCF-7/LY2 (Table A1.1). The selected cell lines were chosen based on their popularity as models in breast cancer research and because they represent different molecular subtypes. MCF-7 is the most commonly used breast cancer cell line, due to its estrogen receptor expression profile (H D Soule et al., 1973). MCF-7 strongly expresses the estrogen receptor and is used to represent luminal breast tumors, which represent the majority of U.S. breast tumor cases. MCF-7/LY2 is a stable variant of MCF-7 that is resistant to the antiestrogenic compound LY117018 (Bronzert, Greene, & Lippman, 1985). We included this cell line in our study to investigate whether antiestrogen resistance impacts NER function. CAMA-1 is another estrogen receptor positive cell line that is used to study luminal tumors (Fogh, Wright, & Loveless, 1977). SK-BR-3 strongly expresses HER2 and is thought to be representative of HER2-enriched tumors (Trempe, 1976). MDA-MB-231 is the most widely used triple negative breast cancer cell line that is used to represent basal type tumors, especially highly metastatic claudin-low tumors (Cailleau et al., 1978). BT-20 is the oldest breast cancer cell line, and was derived directly from a primary breast tumor,

unlike the majority of breast cancer cell lines, which were derived from lung pleural effusion (Lasfargues & Ozzllo, 1958). BT-20 is also a triple negative cell line used to study basal type tumors.

All the selected cell lines were obtained from the American Type Culture Collection (Rockville, MD). MCF-7, MCF-7/LY2, and MDA-MB-231 were cultured in Dulbecco's Modified Eagle Medium (DMEM) (HyClone™) (Cat# SH30243.01) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (HyClone®) (Cat# SH30084.03) and 1% penicillin/streptomycin (Corning®) (#Ref 30-002-cl). CAMA-1, SK-BR-3, BT-20 were cultured in Roswell Park Memorial Institute medium (Corning®) (#Ref 10-040-CV) supplemented with 10% heat-inactivated FBS and 1% penicillin/streptomycin. All cell lines were cultured in uncoated 25 cm^2 flasks (Corning®) (Cat# 430639) and maintained in Forma Series II Water Jacketed CO_2 Incubator (Thermo Electron Corporation®) at 37°C in 5% CO_2. Cells were plated on two-chamber slides (Lab-Tek®) (Cat# 177380) one or two days prior to the unscheduled DNA synthesis assay.

A1.2.2 the primary culture and cell line of a stage III breast tumor JL BTL-12 and culture conditions

The study included a chemotherapy naïve advanced stage (stage III) breast tumor primary culture, JL BTL-12, which was established in our laboratory. JL BLT-12 shares much of the gene expression profile shown by the commercially available breast cancer

cell lines. We included JL BTL-12 in our study to exclude any artifacts that might be due to our unique way of performing culture and establishing cell lines that might influence gene expression. JL BTL-12 was obtained and handled in the same way as the non-diseased tissues and stage I tumors to establish explants and a cell line (Latimer et al., 2010).

Upon delivery to the laboratory, the tissues were washed with phosphate buffer saline (PBS) (Corning®) (#Ref 21-030-CV) supplemented with 3% Antibiotic-Antimycotic (Cellgro®) (Cat# 30-004-Cl) containing penicillin, streptomycin, and amphotericin B. Tissue was then minced into small pieces in MWRIα medium. MWRIα medium was developed in our laboratory. The novel medium consists of Dulbecco's DMEM, 10% heat-inactivated FBS, 10% heat-inactivated newborn calf serum (HyClone®) (Cat# SH39118.84), 2.5% whole embryo culture rat serum (Harlan Sprague Dawley®) (Cat# 4520), 5% nucleosides comprised of adenosine (Sigma-Aldrich®) (Cat# A4036), thymidine (Sigma-Aldrich®) (Cat# T1895), guanosine (Sigma-Aldrich®) (Cat# G6264), cytidine (Sigma-Aldrich®) (Cat# C4654), and uridine (Sigma-Aldrich®) (Cat# U3003), 5% non-essential amino acid (Corning®) (#Ref 25-025-Cl), and 0.0035% β-mercaptoethanol (Sigma-Aldrich®) (Cat# M3148). The tissue pieces were plated on two-chamber slides that were coated with basement membrane extracts (Trevigen®) (Cat# 3432-005-01). The primary cultures were grown in MWRIα medium and maintained in the cell culture incubator at 37ºC in 5% CO_2 for 7-10 days prior to the unscheduled DNA synthesis assay.

Table A1.1 **Clinical and molecular characteristics of the selected established cell lines and our laboratory culture explants that were included in the study.**

Cell line/culture explants	Stage and site of isolation	Patient age and race	Molecular subtype	Molecular characteristics			
				ER	PR	HER2	P53
MCF-7 (H D Soule et al., 1973)	IV, pleural effusion	69, Caucasian	Luminal-A	+++	+	-	WT
MCF-7/LY2 (Bronzert et al., 1985)	A stable variant of MCF-7 that is resistant to the potent antiestrogen LY117018. Used as a model for antiestrogen resistance		Luminal-B	+	-	-	NA
CAMA-1 (Fogh et al., 1977)	IV, pleural effusion	51, Caucasian	Luminal-B	+	-	-	Mut
Cell line/culture explants	Stage and site of isolation	Patient age and race	Molecular subtype	Molecular characteristics			
				ER	PR	HER2	P53
SK-BR-3 (Trempe, 1976)	IV, pleural effusion	43, Caucasian	HER2-enriched	-	-	+++	Mut
BT-20 (Lasfargues & Ozzllo, 1958)	IV, primary breast tumor	74, Caucasian	Basal	-	-	-	Mut
MDA-MB-231 (Cailleau et al., 1978)	IV, pleural effusion	51, Caucasian	Claudin-low	-	-	-	Mut
JL BTL-12 (Sajithlal et al., 2010)	III, primary breast tumor	35-40, Caucasian	Luminal-A	+	+	-	NA
JL BTL-8 (Latimer et al., 2010)	I, primary breast tumor	50-55, Caucasian	Luminal	+	+	NA	NA
JL BRL-6 (Latimer et al., 2010)	Non-diseased breast epithelial tissues	60-65, Caucasian	Normal breast tissues				

A1.2.3 Unscheduled DNA synthesis assay

NER function was determined using the autoradiographic unscheduled DNA synthesis assay. Newborn foreskin fibroblast explants (less than 10 passages), which

exhibit a high level of NER function, along with MDA-MB-231 were used as positive standards in every experiment. Newborn foreskin fibroblasts and MDA-MB-231 were plated on four slides each, two days prior to beginning the experiments. Four slides of each established cell line were analyzed in three independent experiments. At least two slides of each primary culture or explant that was derived from non-diseased breast epithelial tissues or stage I breast tumors were run in one experiment. The stage III breast tumor primary culture was run in an experiment and the cell line that has been derived from this stage III breast tumor primary culture (JL BTL-12) was run in another experiment.

The unscheduled DNA synthesis assay was performed using a UV delivery system that is specifically designed for this functional assay (Steier & Cleaver, 1969). The delivery system has three UV germicidal bulbs that are placed at a distance of three feet (91.4 cm) from a turntable platform where the experimental chamber slides are placed. A six-inch diameter photographic shutter that is electronically controlled opens to deliver a precisely timed dose of UV radiation. UV light bulbs were warmed for an hour before performing the experiment and the UV fluence rate was checked before irradiating the chamber slides to ensure delivering an adequate UV radiation dose. The optimal UV fluence is one Joule/m^2.

The unscheduled DNA synthesis assay was performed as previously described (Latimer et al 2003; 2010). All experimental slides were fed with fresh culture medium

one hour before the beginning of the experiment. Then, the chamber farthest from the ground glass end of each slide was irradiated with UV light at a wavelength of 254 nm for 14 seconds in the absence of the culture medium, for a total UV radiation dose of 14 Joules/m^2. The left chamber of each slide was shielded from the UV radiation, serving as the unirradiated control for each slide.

After UV exposure, all the chamber slides were incubated with DMEM supplemented with 10% FBS and 10 μCi/ml [H^3] methyl-thymidine (PerkinElmer Life Sciences®) (#Part NET027W001MC) for 2 hours in a Forma Series II Water Jacketed CO_2 Incubator that is dedicated to radioactive treated cultures at 37 ^oC in 5% CO_2. Labeling medium was then replaced with unlabeled chasing DMEM medium supplemented with 10% FBS and 10 mM non-radioactive thymidine (Sigma-Aldrich®) (Cat# T1895) and incubated for another two hours to release unincorporated radioactive thymidine from the nucleotide pools inside the cell nuclei.

After incubation in the chasing medium, the slides were washed with 1X sodium citrate (Sigma-Aldrich®) (Cat# 1613859) in PBS, then fixed in 33% acetic acid (Fisher Scientific®) (Cat# A38-500) in ethanol (Sigma-Aldrich®) (Cat# E7023) and 70% ethanol in distal water for 15 minutes each. Slides were rinsed in 4% perchloric acid (Fisher Scientific®) (Cat# A228) overnight at 4 ^oC. Slides were dried the following day, then dipped in a photographic emulsion (Caresteam®) (Cat# 8895666) and packaged in tightly

sealed slide boxes in a dark room. Finally, the emulsion was exposed in complete darkness for 11-14 days at 4 ^{o}C.

The length of the emulsion exposure was determined in each experiment using two slides of each experimental control, foreskin fibroblasts and MDA-MB-231. These slides were called "tester slides" and they were dipped and packaged in a separate slide box. After 11 days following the dipping process, selected tester slides were developed in (Kodak®) D-19 developer (Cat# 1464593), fixed in (Kodak®) fixer (Cat# 1971746), then Giemsa stained (Sigma-Aldrich®) (Cat# G9641). The incorporated radioactive thymidine in the nuclei expose the photographic emulsion directly over them and ultimately appear as silver grains that can be seen and quantified at 1000X magnification under oil emersion using a Zeiss Axioskop microspore. If the silver grain average counts per nucleus were 50 or more per nucleus in foreskin fibroblast cells on the tester slides, then the rest of the slides in the same experiment were developed. If the grain counts were below 50, the experimental slides were left to expose for another day before developing.

Grain counting

After developing the emulsion on experimental slides, nuclei were stained with Giemsa and then slides were dried overnight. The slides were ready to count the next day. The silver grains were counted over 100 non-S phase nuclei on both unirradiated and irradiated sides at 1000X magnification under oil emersion using the Zeiss Axioskop

microscope. S-phase nuclei were not counted because incorporated radioactive thymidines in these nuclei reflect DNA replication, not DNA repair. S-phase nuclei were easy to distinguish by their high grain counts, usually at least 10-fold higher than non-S phase nuclei. Local background grain counts were evaluated for each microscopic field, over an area approximately the same size as adjacent nuclei.

Unscheduled DNA synthesis statistical analysis

The grain count in the local background of each field was subtracted from each nuclear grain count in that field (local background). The average grain count per nucleus was quantified for each side chamber, unirradiated and irradiated. The final grain count that indicates NER function was calculated by subtracting the unirradiated average grain count per nucleus from the irradiated average grain count per nucleus. Data from all of the slides for the same cell line or primary culture were pooled together and expressed as a proportion of the final grain count of the concurrently processed and analyzed foreskin fibroblast control slides. The relative NER values for the same cell line or primary culture compared to foreskin fibroblasts were averaged over all the independent experiments and normalized to the average relative NER values of the 23 primary cultures of non-diseased breast epithelial tissues.

Normalized NER values for each of the established cell lines and JL BTL-12 were compared to the normalized NER values of both non-diseased breast epithelial tissues and stage I tumor populations using the statistical z-test. Also, all the established cell

lines and JL BTL-12 were compared as a late stage group to both non-diseased breast epithelial tissues group using two-tailed unpaired student's t-test. For all statistical tests, a *P* value < .05 was considered significant.

S-phase index analysis

Besides NER function, cell proliferation (replication) was assessed in each unscheduled DNA synthesis experiment. Cell proliferation was evaluated by calculating the S-phase index, which is the percentage of S-phase nuclei in all counted unirradiated fields. S-phase indices were calculated for the established breast cancer cell lines and JL BTL-12 to investigate the effect of proliferation rates on NER function in these cell lines. The association between s-phase indices and NER capacities was examined using a simple linear regression model. *P* values < .05 was considered statistically significant.

A1.2.4 Total RNA isolation

A representative non-diseased breast epithelial tissue explant (JL BRL-6), a representative stage I tumor explant (JL BTL-8), JL BTL-12, MCF-7, CAMA-1, SK-BR-3, MDA-MB-231, and BT-20 were subjected for further molecular analysis, to quantify expression levels for 20 canonical NER genes using a microarray technique.

Total RNA was harvested from three biological replicates of JL BRL-6, JL BTL-8, and JL BTL-12 and at least one sample of the five-established cell line using the RNeasy mini kit (Qiagen) (Cat# 74104) following the manufacturer's protocol. Cells were seeded and maintained in 100 mm petri dishes (Corning®) (Cat# 430167). RNA

isolation was performed when the cells in these petri dishes reached 80% confluence. RLT (700 uL) lysing buffer (supplied) was added to the petri dishes. Cell lysate was scraped using a cell culture scraper (Fisher Scientific®) (Cat# 8100241), then homogenized by passing 15 times through an RNase-free 20-gauge needle fitted to an RNase-free 5 mL syringe (Becton Dickinson®) (Cat# 309635). An equal volume of 70% ethanol was added to the cell lysate and mixed well. The total volume was transferred to an RNeasy spin column placed in a two ml collection tube (supplied) then centrifuged for 30 seconds at a speed of 12,000 revolutions per minute (RPM). The eluant solution in the collection tube was discarded. RW1 (350 uL) washing buffer (supplied) was added to the spin column and centrifuged for 30 seconds at 12,000 RPM. The eluant was discarded. DNase (10 μL) mixed with 70 μl RDD buffer (Qiagen®) (Cat# 79254) was added directly to the spin column membrane and incubated for 30 minutes at room temperature to digest DNA. RW (350 μL) washing buffer was added to the spin column and centrifuged for 30 seconds at 12,000 RPM. The eluant was discarded. RPE (500 μL) washing buffer (supplied) was added to the spin column and centrifuged for 30 seconds at 12,000 RPM. The eluant was discarded. The spin column was placed in an autoclaved, RNase free 1.5 mL tube (eppendorf®) (Cat# 22363204). RNA-free water (50 μL) (supplied) was added directly to the spin column and then centrifuged for one minute at 12,000 RPM to elute the RNA. The RNA eluant was added to the spin column and centrifuged again for a minute at 12,000 RPM to increase the RNA yield. The RNA samples are kept on ice at this point.

The total RNA concentration for all samples was determined by measuring the sample absorbance at 260 nm in an Ultrospec 2100 pro spectrophotometer (Amersham Biosciences®). RNA purity was obtained by calculating the ratio of the sample reading at 260 nm to 280 nm. Pure RNA sample has a ratio of 1.8-2 (Matlock, 2012). Samples were flash frozen in cold ethanol (with dry ice added) and stored in a – 80 °C freezer (Van Waters & Rogers®) (model# 40086A).

A1.2.5 Microarray gene expression analysis

Two µg of total RNA from each sample was used to perform microarray gene expression, which was done at the Clinical Genomic Facility of University of Pittsburgh Cancer Institute by using the Affymetrix Human Genome U133 plus two chip. Five publicly available Gene Expression Omnibus (GEO) datasets: GSE41445 (Groth & Politz, 2012), GSE20713 (Dedeurwaerder et al., 2011), GSE34211 (Hook et al., 2012), GSE12777 (Hoeflich et at., 2009), GSE36133 (Barretina et al., 2012), representing the five established cell lines were utilized to complete the microarray analysis (Table A1.2). Raw data for each sample was downloaded as .CEL files and then processed using GeneSpring software (Agilent, Inc). The probe logarithmic intensity error (PLIER16) algorithm was applied to normalize the sample files. Gene expression data on 51 probes representing the 20 NER canonical genes were extracted. Data on multiple probes for a single gene, when available, were averaged.

Table A1.2 **Source and the number of microarray .CEL files that were processed in this study**.

Culture explants/ cell lines	Our laboratory	GSE41445 (Groth & Politz, 2012)	GSE20713 (Dedeurwaerder et al., 2011)	GSE34211 (Hook et al., 2012)	GSE12777 (Hoeflich et al., 2009)	GSE36133 (Barretina et al., 2012)	Total
JL BRL-6	3	-	-	-	-	-	3
JL BTL-8	3	-	-	-	-	-	3
JL BTL-12	3	-	-	-	-	-	3
SK-BR-3	1	-	1	2	1	1	6
CAMA-1	1	-	-	2	1	1	5
MCF-7	3	3	1	2	1	1	11
BT-20	1	-	-	2	1	1	5
MDA-MB-231	2	3	-	2	1	1	9

Gene expression statistical analysis

NER gene expression was expressed relative to the average of NER gene expression in the normal breast epithelial biological replicates of JL BRL-6. We performed a one-tailed unpaired Student's *t* test in order to identify individual NER genes that were significantly increased in expression in the established cell lines and JL BTL-12 compared to JL BRL-6 and JL BTL-8. A P value $< .05$ was considered statistically significant.

Gene expression clustering analysis

Using hierarchical clustering, samples were divided into clusters and sub-clusters forming a tree-shaped structure (dendrogram) based upon their gene expression profile. The distance to the horizontal lines reflects how similar two samples within the clusters are with respect to their gene expression patterns. The larger the distance, the less similar or related these samples are to each other.

Supervised hierarchical clustering analysis based on the 20 NER canonical genes of JL BRL6, JL BTL-8, JL BTL-12, and the five established cell lines was performed using GeneSpring software (Agilent, Inc). Multiple probes for a single gene, when available, were averaged. Euclidean algorithm and the average linkage method were used to generate the dendrogram.

A1.2.6 RPA3 silencing experiments in late stage high-DNA repairing breast cancer cell lines (cell line selection and experimental design)

Three high-DNA repairing cell lines, MDA-MB-231, MCF-7, and JL BTL-12, were selected for RPA3 loss of function experiments. MDA-MB-231 and MCF-7 were selected because they are the most commonly used established breast cancer cell lines in breast cancer research and represent two completely different molecular types of breast cancer. JL BTL-12 was selected to assure the any results that are obtained using MDA-MB-231 and MCF-7 are attributed to intrinsic characteristics of late stage breast cancer tumors and not to artificial phenomena in these long-standing established cell lines.

RPA3 gene expression was silenced using the small interfering RNA technique (siRNA) (Dykxhoorn, Novina, & Sharp, 2003). Four transfection treatment groups were assigned for each cell line: blank, mock-treated, negative control, and RPA3 siRNA transfected. The blank sample was maintained under the optimal culture conditions in which the cell line is handled without any treatment. This sample was included in the study to ensure all optimal cell culture conditions during the experiments were maintained. The mock sample was transfected with only the transfection vehicle, lipofectamine® RNAiMAX (Life technologies®) (Cat# 13778150). The negative control sample was transfected with the transfection complex that is composed of lipofectamine and fluorescein isothiocyanate (FITC)-tagged scrambled RNA (BLOCK-iT® Fluorescent Oligo) (Life Technologies®) (Cat# 2013). The fluorescent-scrambled RNA duplex mimics RPA3 siRNA duplex structure but does not have any functional effect on all

human gene transcripts. Both mock and negative control samples were included in the study to exclude experimental artifacts that might affect RPA3 gene expression and NER function. The RPA3 siRNA treated sample was transfected with the RPA3 siRNA transfection complex, consisting of lipofectamine and a predesigned, commercially available RPA3 siRNA duplex (Invitrogen®) (Cat# 4392420) (ID# s12133) that was used to assess silencing of RPA3 gene expression and the impact on NER function. Three independent transfection experiments were performed for each cell line.

A1.2.7 Lipofectamine transfection

Four RPA3 siRNA doses 20, 40, 60, and 80 pmole were used to optimize transfection conditions for each cell line. The 60 pmole dose was selected to be used in this study for two reasons: this dose had a higher transfection efficiency in the three cell lines compared to other doses, and this dose was used in the miR-145 experiment, which allowed us to perform comparative gene expression and NER function analyses between RPA3 siRNA and miR-145 experiments.

Two hundred fifty thousand cells of each of MDA-MB-231 and MCF-7 were plated separately in 60 mm petri dishes (Corning®) (Cat# 430166) for each treatment group 36 hours prior to transfection. Two hundred fifty thousand cells of JL BTL-12 were plated in 60 mm petri dishes for each treatment group 24 hours prior to transfection. This number of cells was found to allow the cultures to reach 40-50% confluence by the time of transfection.

On the transfection day, transfection complex for each sample was prepared as the following: 60 pmole of FITC-tagged scrambled RNA (negative control), and RPA3 siRNA were diluted with 250 μL of Opti-MEM® (Life technologies®) (Cat# 31985070), which is serum- and antibiotics-free culture medium. Ten μL of lipofectamine was diluted in 250 μL of Opti-MEM. The diluted FITC-tagged scrambled RNA and RPA3 siRNA were mixed with the diluted lipofetamine and incubated for 10 minutes for each negative control and RPA3 siRNA sample, respectively. The diluted lipofectamine was mixed with 250 μL of Opti-MEM™ and incubated for 10 minutes for each mock sample. Five hundred μL of the final transfection complex medium mixed with 3.5 mL of Opti-MEM and incubated with cells in each assigned treatment group for 6 hours in the cell culture incubator at 37 ^{o}C in 5% CO_2. The Opti-MEM medium was replaced with regular culture medium, 10% FBS in DMEM for MDA-MB-231 and MCF-7 and MWRI for JL BTL-12 after a rinse with PBS then maintained in the cell culture incubator.

A1.2.8 Transfection efficiency

Transfection efficiency of the FITC-tagged negative control transfected samples was evaluated using fluorescent Olympus® IX51 microscope 24 hours after transfection in each experiment. Bright field and dark field images of five representative 20X microscopic fields were captured by a Hamamatsu® digital camera (model# C848-03G02) using MetaMorph® software 7.7.4.0v (Molecular Devices, Inc.). The total number of cells in each field was counted on the bright field images while the number of transfected cells was counted on the corresponding dark field images using ImageJ 1.47v software

(National Institute of Health). Transfection efficacy was calculated by dividing the number of transfected (fluorescent) cells by the number of total cells in each field. Transfection efficiency for each experiment was obtained by averaging the transfection efficiency values of the five representative fields.

A1.2.9 Total RNA isolation (RPA3 siRNA experiments)

Total RNA was harvested from the treatment groups using the RNeasy mini kit (Qiagen) 48 hours after transfection. The extraction process and RNA concentration and purity measurement of the samples were performed as described in section A1.2.4 in this chapter. The samples were flash frozen and stored in a – 80 ^{o}C freezer.

A1.2.10 Northern RNA gels

The harvested RNA samples were run in mini-northern RNA gels (1.5% agarose, 6.3 % formaldehyde) to evaluate RNA quality and adjust the total RNA concentration that was initially measured using the spectrophotometer. Ribosomal RNA bands were densitometrically scanned. RNA concentration was adjusted with these ribosomal bands using an MDA-MB-231 control RNA sample that was run in triplicate on each northern gel.

One μg of total RNA was diluted in 10 μL in diethyl dicarbonate (DEPC) H_2O (Cellgro®) (Cat# 46-000-CM) to obtain 100 ng/μL final concentration. Each diluted RNA sample was mixed with 15 μL of denaturing buffer that consisted of 64% deionized formamide (Sigma-Aldrich®) (Cat# 11814320001), 37% formaldehyde (Amresco®) (Cat#

M134), 0.01% of 100x northern gel buffer stock solution (1 M Na_2HPO_2) then denatured at 65^{o}C for 10 minutes using Matercycler EP Gradient S (Eppendorf®). Then, 2.5 µL of RNA loading dye, which is comprised of 90% orange G (Sigma-Aldrich®) (Cat# O3756) and 10% bromophenol blue (Sigma-Aldrich®) (Cat# B5525) dyes, was mixed with the denatured samples.

The samples were loaded in a 44 mm 1.5% agarose gel that was prepared as follows: 1.125 gm of agarose (Lonza®) (Cat# 50070) was dissolved in 59.29 mL of DEPC H_2O then heated until it reached a boiling temperature. The agarose solution was cooled to 80^{o}C then mixed with 12.75 mL of 37% formaldehyde, 0.74 mL of the 100X northern gel buffer stock solution, 2.22 mL of DEPC H_2O to obtain the final northern gel concentration (1.5% agarose, 6.3 % formaldehyde). The final agarose solution was cooled to 60 $^{\circ}$C then poured into an RNA gel-casting tray. The agarose solution was allowed to cool for 30 minutes in order to allow agarose polymerization. The gel was transferred to a northern gel electrophoresis box, then immersed in 100 mM Na_2HPO_2 buffer. The gel was run at 120 volts for two hours.

After electrophoresis, the gel was rinsed with distilled H_2O three times to dilute the formaldehyde, then incubated with distilled H_2O containing ethidium bromide (Sigma-Aldrich®) (Cat# E1510) for 30 minutes, covered with foil, under a constant low speed shaking. The gel then de-stained by washing with distilled H_2O for 30 minutes under a constant moderate speed shaking four times. The gel was exposed to UV light at

365 nm in AlphaImager MINI gel imaging system (Cell Biosciences, Inc.) and several images of the RNA gel were captured at multiple exposure times using Chameleson® digital camera (model# CMLN-13S2M) and AlphaImager MINI software (Cell Bioscineces, Inc.).

Total integrated intensities of S28 and S18 RNA ribosomal bands of MDA-MB-231 control replicates and experimental samples were measured using the ImageJ software. A local background below each band was assessed and subtracted from the total integrated intensity value of that band. Background subtracted values of S28 and S18 bands were combined for each sample to obtain a final intensity value. RNA samples concentrations were adjusted based on the final intensity values of these samples relative to the final intensity value average of control replicates. RNA quality was examined using the ratio of S28 band intensity to S18. A good quality RNA sample has a ratio of 1.8-2.2 (Ausubel et al., 2001).

A1.2.11 Reverse transcriptase polymerase chain reaction

The efficacy of RPA3 siRNA transfection on *RPA3* gene expression was evaluated in three independent experiments for each cell line using reverse transcriptase polymerase chain reaction (RT-PCR). Two μg of total RNA of each sample was converted to a copy DNA transcript (cDNA) using the high capacity cDNA reverse transcription kit (Life technologies®) (Cat# 4368814) as follows: 10 μL of 2X reverse transcription master mix was prepared by mixing 2 μL of 10X RT buffer, 0.8 μL of 25X

deoxynucleoside triphosphate (dNTP) mix of the four DNA monomeric units deoxyadenosine triphosphate, deoxythymidine triphosphate, deoxyguanosine triphosphate, and deoxycytidine triphosphate, 1 μL of MultiScribe® reverse transcriptase, and 4.2 μL of DEPC H_2O. The 2X reverse transcription master mix was then added to 2 μg of total RNA of each sample that was diluted in 10 μL of DEPC H_2O to obtain a 100 ng/μL final concentration. RNA was converted to cDNA in three thermal steps: 25 ˚C for 10 minutes, 37 ˚C for 120 minutes, then 85 ˚C for 5 minutes using Matercycler EP Gradient S (Eppendorf®).

A predesigned RPA3 Taqman® gene expression assay (Invitrogen®) (Cat# 448892) (ID# Hs01047933_g1) was used to quantify RPA3 gene expression in the samples. A predesigned GAPDH Taqman® gene expression assay (Invitrogen®) (Cat# 4453320) (ID# Hs02758991_g1) was used to quantify the housekeeping gene GAPDH that was used to normalize RPA3 gene expression data. 200 ng of three technical replicates were run for each sample. An RT-PCR reaction volume of 20 uL was prepared for each technical replicate by mixing one μL of 20X Taqman gene expression assay, 10 μL of 2X Taqman gene expression master mix (Applied Biosystems®) (Cat# 4369016), two μL of cDNA (200 ng), and seven μL of DEPC H_2O. The 2X Tagman gene expression master mix contains AmpliTaq Gold™ DNA polymerase, uracil-DNA glycosylate, dNTPs, ROX™ passive reference, and optimized buffer components. 10% access volume was considered to compensate for volume loss from pipetting. The 20 uL RT-PCR reaction volume of each replicate was transferred to an RT-PCR 96-well plate,

then sealed with an adhesive cover to prevent cross contamination among wells. The well plate was loaded into StepOnePlus Real times PCR system (Applied Biosystems®). The PCR amplification process was run using three thermal steps 50 °C for two minutes, 9 °C for 10 minutes, then 40 cycles of 95 ºC for 15 seconds followed by 60 °C for one minute.

The average of GAPDH cycle threshold (C_T) value was subtracted from the average of RPA3 C_T value of the technical replicates to obtain a ΔC_T value for each sample. Then, the ΔC_T value of the reference group (mock sample) was subtracted from ΔC_T values of negative control and RPA3 siRNA transfected samples to obtain logarithmic relative gene expression values ($\Delta\Delta C_T$). $\Delta\Delta C_T$ values were exponentially transformed using the equation $2^{-\Delta\Delta C_T}$ to obtain relative fold change expression values. The significance of *RPA3* gene expression reduction in the RPA3 siRNA treated samples were evaluated statistically compared to *RPA3* gene expression in the mock and negative control samples in the three independent experiments for each cell line using one tailed, paired student's t test. A *P* value < .05 was considered statistically significant.

A1.2.12 Unscheduled DNA synthesis assay (RPA3 siRNA)

The impact of silencing RPA3 gene expression on NER function was assessed in the most transfectable cell line, MDA-MB-231, that had the most significant RPA3 gene expression suppression. Forty thousand cells were plated in each chamber of a two-chamber slide 36 hours prior to transfection. Slides were transfected with either negative

control or RPA3 siRNA with a dose of 12.6 pmole. Three replicate slides were used for each treatment group. Transfection efficiency was examined 24 hours after transfection as described in section A1.2.2. Unscheduled DNA synthesis analysis was performed 48 hours after transfection as described in section A1.2.1.

The final grain counts of the RPA3 siRNA transfected slides were pooled together and expressed as a percentage of the pooled final grain counts of the negative control slides. One tailed, paired student's t test was used in order to test for the significance of silencing RPA3 on NER function suppression in the RPA3 siRNA treated cells compared to in the negative control cells. A P value < .05 was considered statistically significant.

S-phase indices of both negative control and RPA3 siRNA transfected cells were calculated to assess the impact of silencing RPA3 gene expression on replication (proliferation). S-phase indices were evaluated by calculating the S-phase cell percentage on all counted microscopic fields on the irradiated sides. One tailed, paired student's t test was used in order to evaluate the significance of silencing RPA3 on cell proliferation inhibition statistically in the RPA3 siRNA treated slides compared to the negative control slides. A P value < .05 was considered statistically significant.

A1.3 Results

A1.3.1 NER function in the established breast cancer cell Lines and JL BTL-12

We evaluated NER function in six widely used commercially available breast cancer derived cell lines and a chemotherapy naïve primary culture of late stage breast

cancer (stage III) JL BTL-12, and compared to NER function in primary cultures of 23 non-diseased breast epithelial tissues and 19 tissues with stage I invasive breast tumors stage I breast tumors that have been previously published (Latimer et al., 2010). All repair capacity was expressed as a percentage of non-diseased breast tissue (derived from 23 primary cultures of breast reduction mammoplasties). Our goal was to determine whether these established breast cancer cell lines reflect the same DNA repair capacity as stage I breast tumor cells, which is the most diagnosed breast cancer stage, in the context of NER capacity.

The six established cell lines and JL BLT-12, as a late stage breast cancer group, exhibited a significantly higher NER function by 2.35 times ($P < .001$) and 5.15 times ($P < .001$) compared to the non-diseased breast epithelial tissue and stage I tumor groups, respectively (Table A1.3).

JL BTL-12, CAMA1, MCF-7, MCF-7/LY2, BT-20, and MDA-MB-231 had a significant increase in NER capacity by 1.60 ($P = .019$), 1.68 ($P = .002$), 2.20 ($P < .001$), 2.71 ($P < .001$), 3.40 ($P < .001$), and 3.63 times ($P < .001$) compared to the population of non-diseased breast epithelial primary cultures using one-tailed z tests, respectively (Table A1.3). SK-BR-3 had 1.27 times increase in NER function over the average of non-diseased breast epithelial tissues but this result did not reach statistical significance ($P = .087$) (Figure A1.1; Table A1.4).

When compared to the primary cultures of stage I breast tumors, SK-BR-3, JL BTL-12, CAMA1, MCF-7, MCF-7/LY2, BT-20, and MDA-MB-231 had a statistically significantly increased NER function by 2.79 ($P < .001$), 3.49 ($P < .001$), 3.67 ($P < .001$), 4.81 ($P < .001$), 5.93 ($P < .001$), 7.44 ($P < .001$), and 7.94 times ($P < .001$), respectively (Figure A1.1; Table A1.4).

These data suggest that the established breast cancer cell lines consistently manifested higher NER capacity over both normal breast epithelial and stage I tumor populations, and that these cell lines do not resemble stage I tumor cultures in terms of NER function. The primary culture of the stage III breast tumor primary culture and established cell line JL BTL-12 showed NER function similar to the established cell lines. This result indicates that the increase in NER function that was observed in the established cell lines and JL BTL-12 was attributable to molecular characteristics of late stage breast tumor and was not due to intrinsic differences in culture conditions.

Table A1.3 **NER capacity results of one-tailed unpaired student t test values of the late stage breast tumor group compared to non-diseased breast epithelial tissue and stage I breast tumor groups.**

Group	NER Capacity	Standard Error	T Test vs. Non-Diseased Group	T Test vs. Stage I Group
Non-diseased breast epithelial tissue	1	0.084		
Stage I breast tumor	0.46	0.045	< .001	
Late Stage tumor	2.35	0.347	< .001	< .001

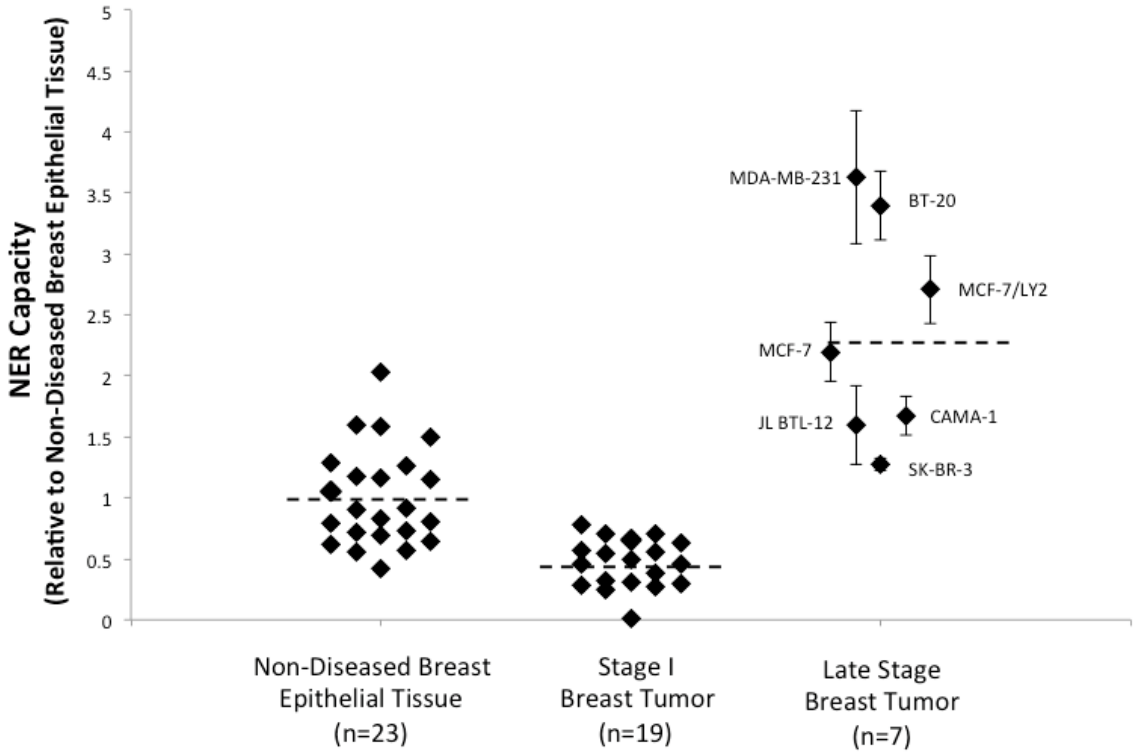


Figure A1.1 **NER function in the established breast cancer cell lines and primary cultures.** Established cell lines and JL BTL-12 (n=7) compared to the primary cultures of non-diseased breast epithelial tissues (n=23) and stage I breast tumors (n=19). JL BTL-12, CAMA-1, MCF-7, MCF-7/LY2, BT-20, and MDA-MB-231 had significantly increased NER capacities relative to both non-diseased breast epithelial tissues and stage I breast tumors. The increase in NER function of SK-BR-3 was statistically significant in relative to stage I breast tumors but fell in the range of NER values of non-diseased breast epithelial tissues. NER capacity was presented relative to the average of NER function in non-diseased breast epithelial tissue group. The dotted line in each group represents the mean. Standard error from at least three independent experiments is shown in the established cell lines.

Table A1.4 **NER capacities, standard errors, and one-tailed z test values of the established cell lines and JL BTL-12 compared to non-diseased breast epithelial and stage I breast tumor primary cultures.**

Cell line/ Primary culture	Relative to non-diseased breast epithelial tissues			Relative to stage I breast tumors		
	NER capacity	Standard Error	Z test	NER capacity	Standard Error	Z test
SK-BR-3	1.27	0.055	.087	2.79	0.121	< .001
JL BTL-12	1.60	0.324	.019	3.49	0.709	< .001
CAMA-1	1.68	0.162	.002	3.67	0.355	< .001
MCF-7	2.20	0.245	< .001	4.81	0.537	< .001
MCF-7/LY2	2.71	0.279	< .001	5.93	0.611	< .001
BT-20	3.39	0.284	< .001	7.44	0.621	< .001
MDA-MB-231	3.63	0.546	< .001	7.94	1.196	< .001

The association between molecular characteristics and NER function in the late stage breast cancer cell lines and primary culture was evaluated. The triple negative breast cancer cell lines BT-20 and MDA-MB-231, as a group, had a statistically significant higher NER capacity by 1.72 times (P = .020) compared to the luminal type breast tumors group consisting of JL BTL-12, CAMA-1, and MCF-7, and MCF-7/LY2 (Table A1.5). This suggests that triple negative breast tumors might exhibit a highly efficient NER function that might play a role in the tumor aggressiveness and chemotherapy resistance that have been observed in such tumors. Although the antiestrogen resistant cell line MCF-7/LY2 had an increase in NER function over its parent by 23%, the antiestrogen sensitive cell line MCF-7, this increase was not statistically significant (P =

.231). These data suggest that increased NER capacity might not be involved in antiestrogen resistance. Hormonal therapy resistance is associated with tumor aggressiveness and cancer progression (Viedma-RodríGuez et al., 2014).

Table A1.5 **NER capacities, standard errors, and two-tailed unpaired student's t test value of the late stage luminal type breast cancer cell line subgroup compared to triple negative breast cancer cell line subgroup.**

Molecular subgroup	NER capacity (relative to non-diseased tissues)	Standard Error	T Test
Luminal type	2.04	0.258	.020
Triple negative	3.51	0.114	

A1.3.2 S-phase indices in the established breast Cancer cell lines and JL BTL-12

S-phase indices were calculated for the established breast cancer cell lines and JL BTL-12 to investigate the possible effect of proliferation rates on NER function in these cell lines (Table A1.6). The association between S-phase indices and NER capacities were examined by a simple linear regression model (Figure A1.2). The model was not statistically significant (P = .274) and yielded a low coefficient of determination value (R^2 = 0.232). These data suggest that proliferation and NER function are independent biological mechanisms in these breast cancer cell lines. These results are consistent with a similar analysis performed on stage I breast cancer primary cultures. No association was found between proliferation and NER repair capacity (Latimer et al., 2010).

Table A1.6 **S-phase indices and NER function in the commercially available cell lines and JL BTL-12.**

Cell line/ primary culture	S-Phase Index (%)	NER capacity (relative to non-diseased breast tissues)
SK-BR-3	42	1.27
JL BTL-12	34	1.60
CAMA-1	36	1.68
MCF-7	27	2.20
MCF-7/LY2	39	2.71
BT-20	35	3.39
MDA-MB-231	27	3.63

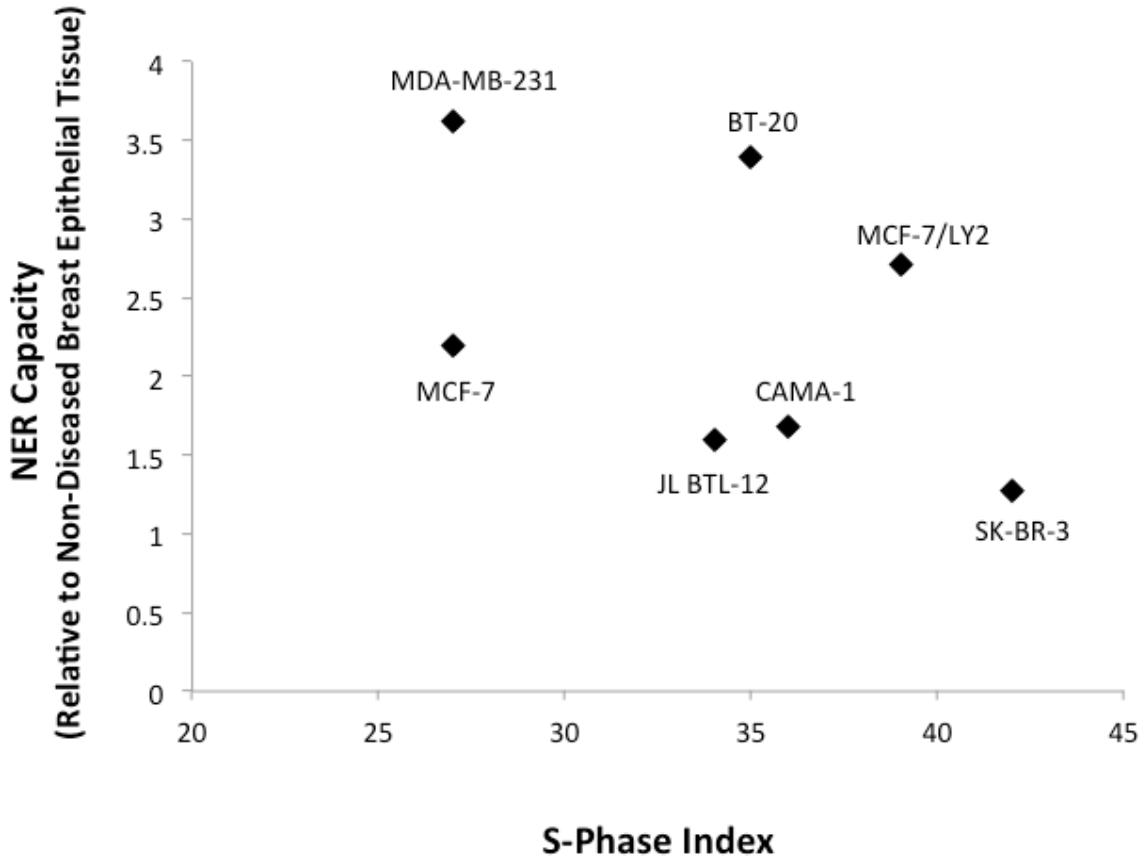


Figure A1.2 **S-phase indices in relation to NER function in the commercially available cell lines and JL BTL-12.** No significant association between S-phase indices and NER capacities in the late stage cell lines and JL BTL-12 was found (P = .274) (R^2 = 0.232). NER values were expressed relative to the average of NER function in non-diseased breast epithelial tissues.

A1.3.3 NER gene expression in the established breast cancer cell lines and JL BTL-12

Gene expression analysis of the 20 canonical NER genes was performed using expression microarray analysis on the Affymetrix Human Genome U133 plus 2 chip. A previous study in the Latimer laboratory on stage I breast cultures showed there was a

strong correlation between NER functional capacity and gene expression (Latimer et al., 2010). It was therefore hypothesized that overexpression of NER genes would be associated with the increased NER function that was observed in the late stage breast tumor cell lines represented by the established cell lines and JL BTL-12 when compared to the non-diseased breast tissues and stage I tumors. The non-diseased breast epithelial tissue explant JL BRL-6 and the stage I breast tumor explant JL BTL-8 were used in this molecular analysis as representatives of non-diseased breast epithelial tissues and stage I breast tumors, respectively.

NER gene expression of late stage breast cancer compared to the non-diseased breast epithelial tissue explant JL BRL-6

As shown in Figure A1.3, microarray analysis demonstrated that the NER pathway was generally overexpressed in the established cell lines and JL BTL-12 compared to JL BRL-6. Fourteen out of 20 NER canonical genes were upregulated in the HER2-enriched cell line SK-BR-3 in comparison to JL BRL-6. Five of these genes were individually significant. The luminal type stage III breast tumor explant JL BTL-12 that was derived in our laboratory had 13 genes overexpressed seven of which reached statistical significance. The luminal type cell line CAMA-1 had 14 overexpressed genes, 6 genes were individually significant. The most widely used luminal cell line MCF-7 also had 14 genes upregulated. Eight out of these 14 genes were statistically significant. The oldest triple negative, basal type commercially available cell line BT-20 had 13 genes with increased gene expressions, six genes were individually significant. The triple

negative, claudin-low MDA-MB-231, which exhibited the highest NER function among the established cell lines, had 12 NER gene overexpressed six of which reached a statistical significance

Taken together, three genes, *XPA*, *GTF2H4*, and *RPA3*, were significantly overexpressed in all the cell lines relative to JL BRL-6. These data suggest that these three genes may play a major role increasing NER function in late stage breast cancer in advanced breast tumor cell lines. Two additional upregulated genes, *ERCC3* and *RPA1*, reached significance in both of the triple negative cell lines BT-20 and MDA-MB-231. These cell lines had the highest increase in NER function among the established cell lines, suggesting these two genes might additionally and/or specifically contribute to induction of NER capacity in this aggressive type of breast cancer.

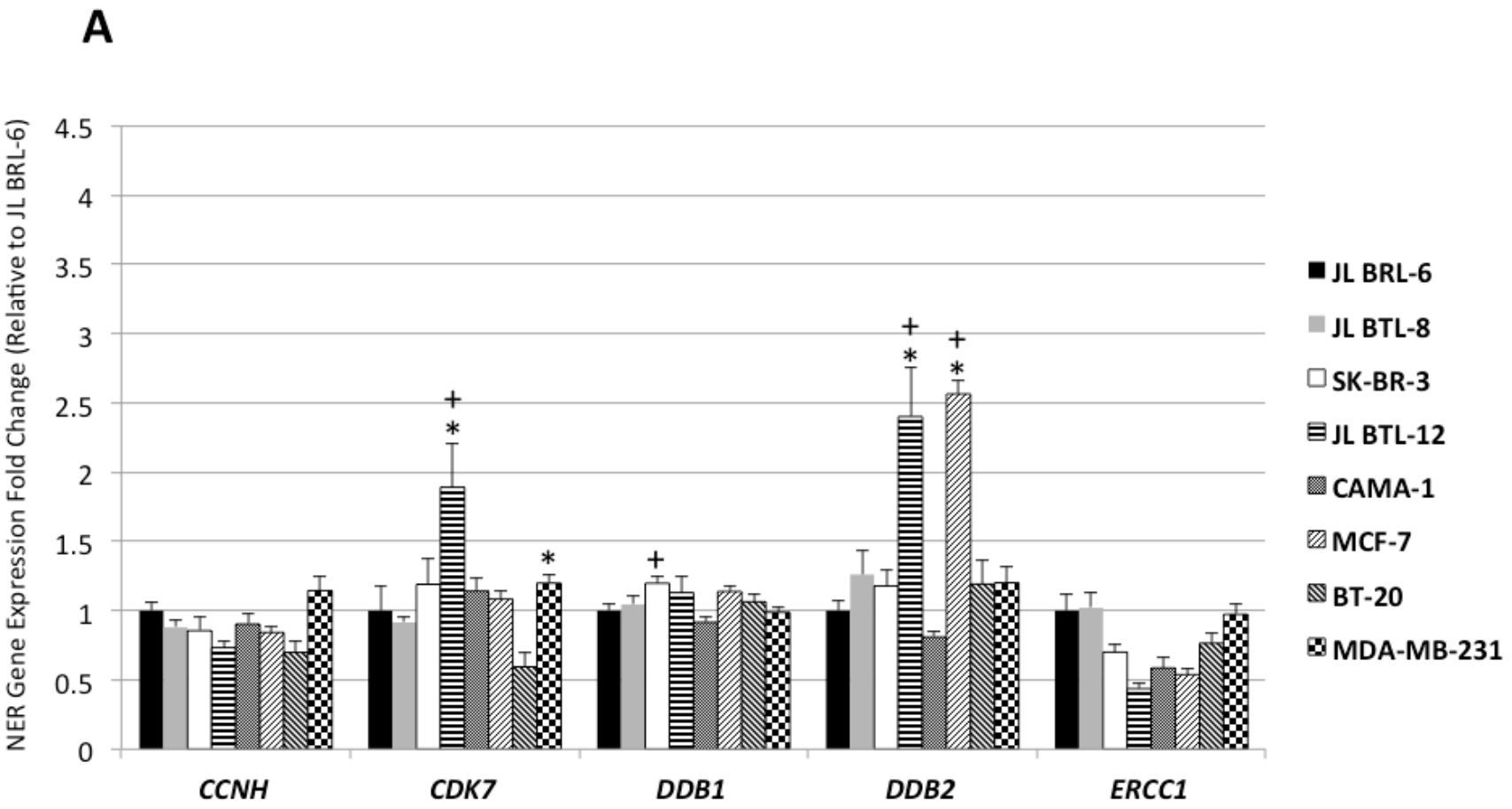
NER gene expression of late stage breast cancer compared to the stage I tumor explant JL BTL-8

As also shown in Figure A1.3, the majority of the 20 NER canonical genes were found to be upregulated in the established cell lines and JL BTL-12, ranging from 13 to 16 overexpressed genes, when compared to the representative stage I tumor JL BTL-8. In SK-BR-3, 16 genes were unregulated seven of which were significant. A total of 14 NER genes showed an increase in expression in JL BTL-12 and seven genes were individually significant. CAMA-1 showed 16 overexpressed genes eight of which were individually significant. The expression in 15 genes was increased in MCF-7 and 11 of

these genes reached significance. BT-20 had 15 genes that were found to be overexpressed, seven of which were individually significant. Lastly, MDA-MB-231 had 13 upregulated genes and the upregulation of nine of these were statistically significant.

Taken together, four genes, *ERCC3*, *GTF2H4*, *RPA3*, and *RPA2*, were significantly overexpressed in all five cell lines and JL BTL-12 compared to the stage I tumor explant JL BTL-8. The upregulation of two of these genes, *GTF2H4* and *RPA3*, in the established cell lines and JL BTL-12 were found persistently significant when compared to both JL BRL-6 and JL BTL-8. These data suggest that *GTF2H4* and *RPA3* may play an important role in the increased NER capacity that late stage tumors exhibit relative to non-diseased breast and stage I breast cancer. In addition, later stage disease may acquire the high expression of these genes and they may assist late stage disease in resisting genotoxic chemotherapy agents.

RPA3 was the most overexpressed NER gene that was statistically significant in the commercial cell lines and JL BTL-12 compared to both JL BRL-6 and JL BTL-8. Theoretically, RPA3 might be a potential therapeutic target to suppress NER function, and therefore, decrease DNA repair-induced chemotherapy resistance. To test this hypothesis, we designed a series of *RPA3* loss-of-function experiments to examine the impact on NER function of loss of *RPA3* gene activity.


A
NER Gene Expression Fold Change (Relative to JL BRL-6)
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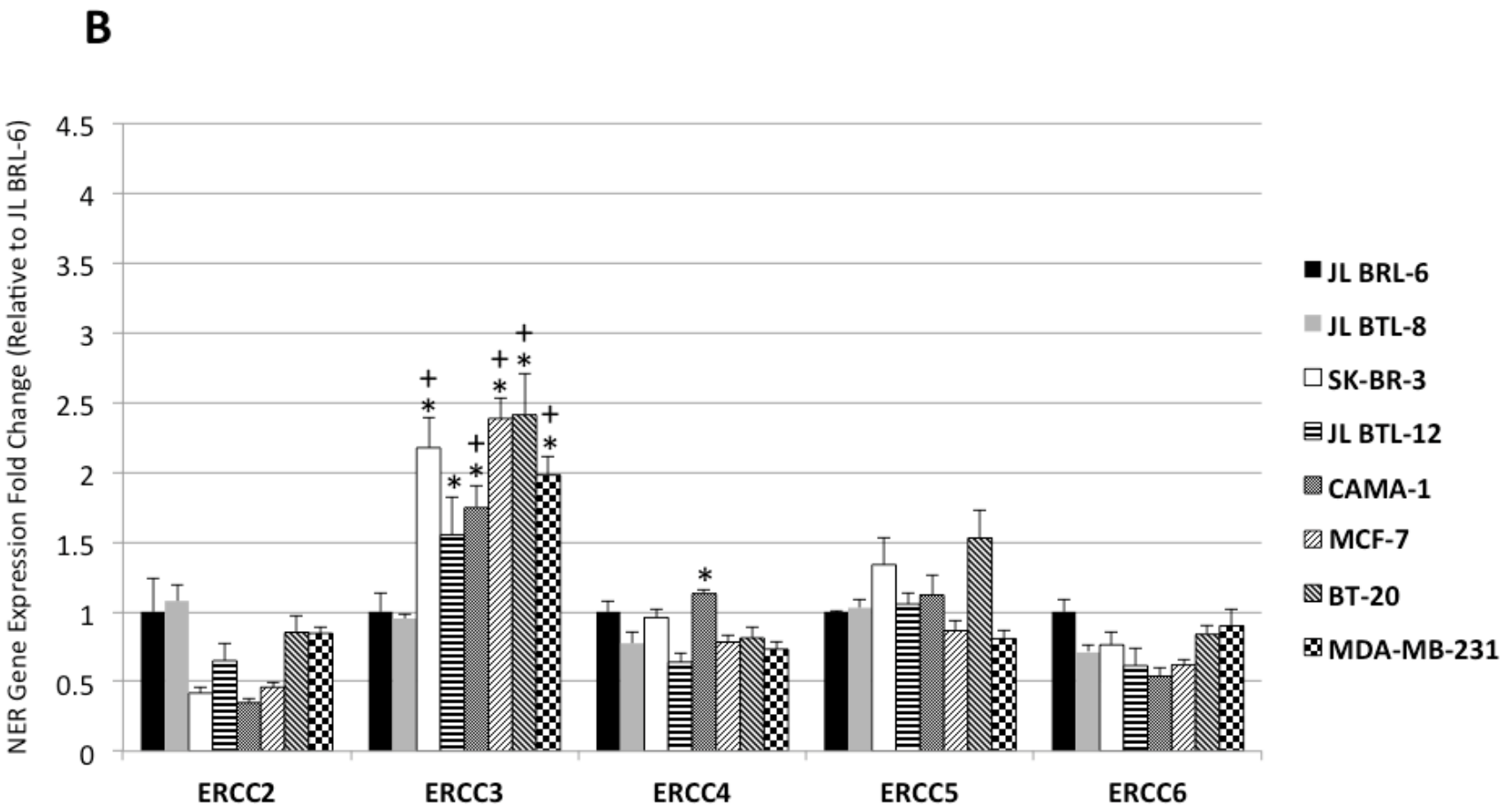
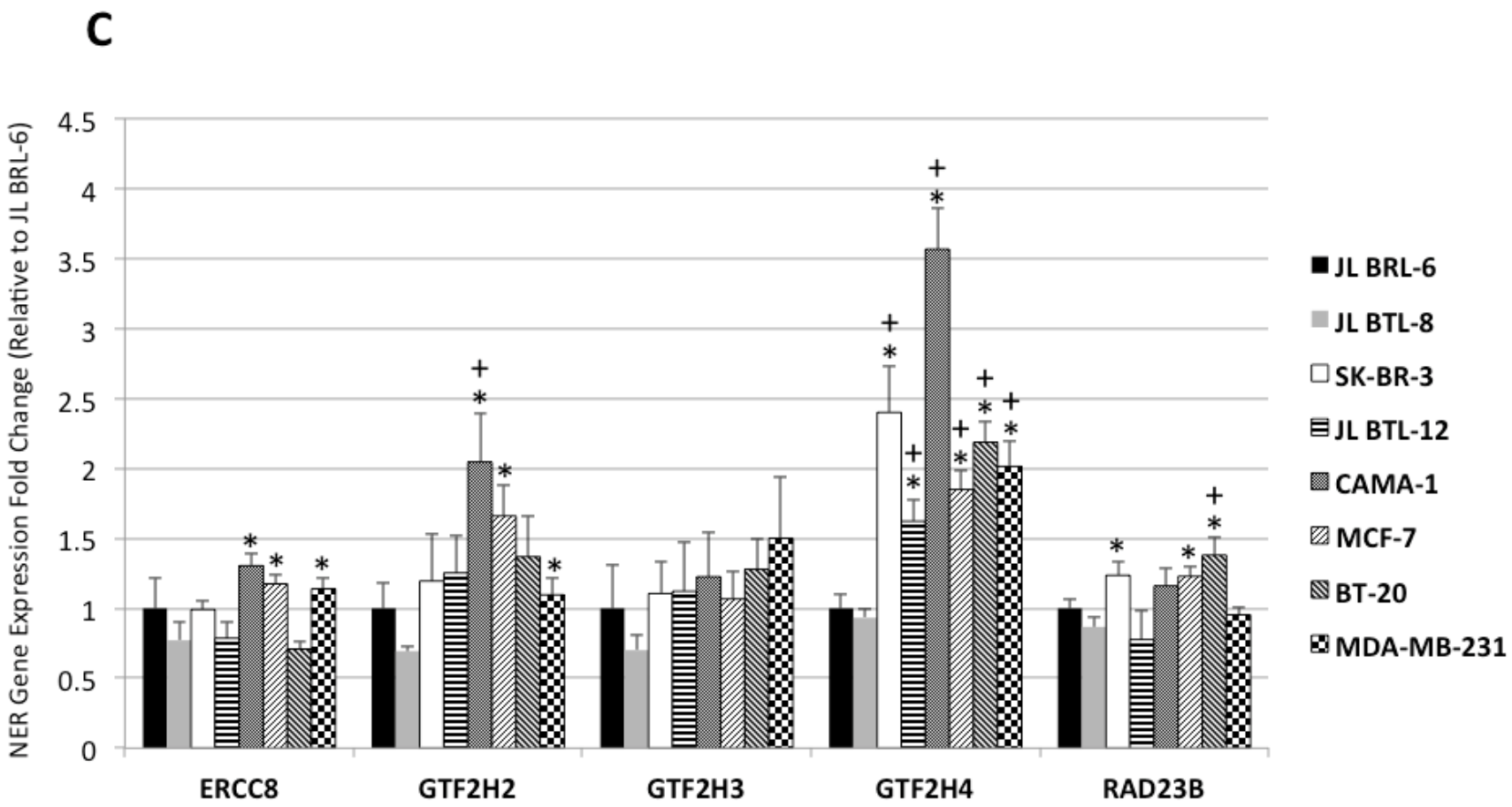
4
3.5
3
2.5
2
1.5
1
0.5
0
CCNH
CDK7
DDB1
DDB2
ERCC1
JL BRL-6
JL BTL-8
SK-BR-3
JL BTL-12
CAMA-1
MCF-7
BT-20
MDA-MB-231


B
NER Gene Expression Fold Change (Relative to JL BRL-6)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
ERCC2
ERCC3
ERCC4
ERCC5
ERCC6
JL BRL-6
JL BTL-8
SK-BR-3
JL BTL-12
CAMA-1
MCF-7
BT-20
MDA-MB-231


C
NER Gene Expression Fold Change (Relative to JL BRL-6)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
ERCC8
GTF2H2
GTF2H3
GTF2H4
RAD23B
JL BRL-6
JL BTL-8
SK-BR-3
JL BTL-12
CAMA-1
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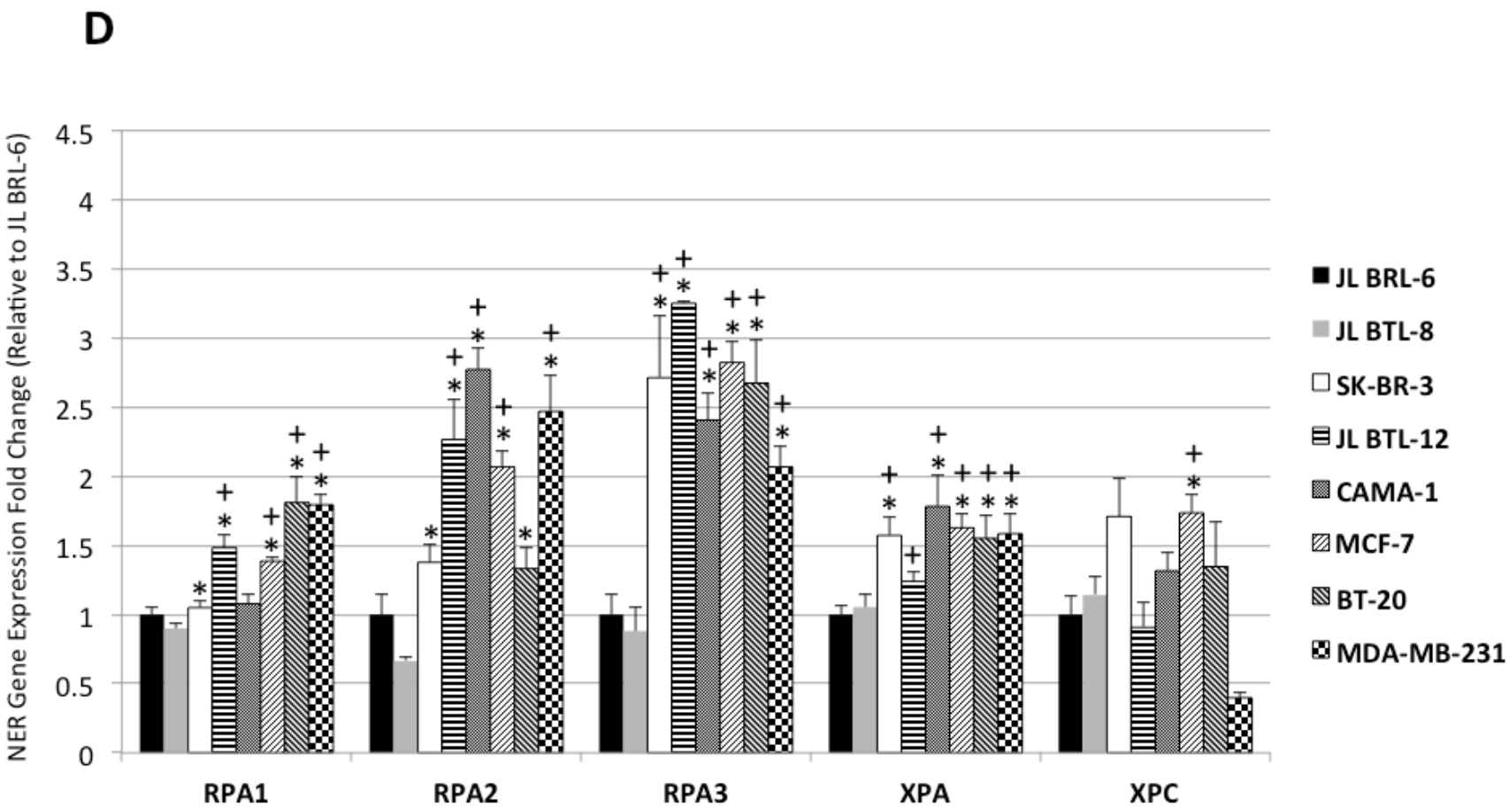
D
NER Gene Expression Fold Change (Relative to JL BRL-6)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
RPA1
RPA2
RPA3
XPA
XPC
JL BRL-6
JL BTL-8
SK-BR-3
JL BTL-12
CAMA-1
MCF-7
BT-20
MDA-MB-231

Figure A1.3 **Comparative expression of the 20 NER canonical genes in the established breast cancer cell lines and the stage III explant JL BTL-12 relative to the non-diseased breast epithelial tissue explant JL BRL-6 and the stage I breast tumor explant JL BTL-8.** The microarray molecular analyses of NER genes are shown in four subfigures. (A) *CCNH*, *CDK7*, *DDB1*, *DDB2*, and *ERCC1*. *CDK7* was significantly upregulated in JL BTL-12 ($P = .033$) ($P = .018$) compared to JL BRL-6 and JL BTL-8, respectively, and in MDA-MB-231 ($P = .003$) compared to only JL BTL-8. *DDB1* was significantly overexpressed in SK-BR-3 (P = .021) compared to JL BRL-6. The upregulation in *DDB2* reached a statistical significant in JL BTL-12 ($P = .009$) ($P = .022$) and MCF-7 ($P < .001$) ($P < .001$) compared to JL BRL-6 and JL BTL-8, respectively. (B) *ERCC2*, *ERCC3*, *ERCC4*, *ERCC5*, and *ERCC6*. *ERCC3* was significantly upregulated in SK-BR-3 ($P = .004$) ($P = .002$), CAMA-1 ($P = .009$) ($P = .004$), MCF-7 ($P < .001$) ($P < .001$), BT-20 ($P = .008$) ($P = .006$), and MDA-MB-231 ($P = .001$) ($P < .001$) compared to JL BRL-6 and JL BTL-8, respectively, while in JL BTL-12 (P = .045) compared to only JL BTL-8. *ERCC4* was significantly overexpressed in CAMA-1 ($P = .001$) compared to JL BTL-8. (C) *ERCC8*, *GTF2H2*, *GTF2H3*, *GTF2H4*, and *RAD23B*. *ERCC8* was significantly unregulated in CAMA-1 ($P = .005$), MCF-7 ($P = .008$), and MDA-MB-231 ($P = .021$) compared to JL BTL-8. *GTF2H2* was significantly overexpressed in CAMA-1 ($P = .036$) ($P = .013$) compared to JL BRL-6 and JL BTL-8, respectively, while in MCF-7 ($P = .023$) and MDA-MB-231 ($P = .043$) compared to only JL BTL-8. *GTF2H4* was significantly upregulated in SK-BR-3 ($P = .012$) ($P = .010$), JL BTL-12 ($P = .013$) ($P = .006$), CAMA-1 ($P < .001$) ($P < .001$), MCF-7 ($P = .003$) ($P = .002$), BT-20 ($P < .001$) (P < .001), and MDA-MB-231 ($P = .005$) ($P = .004$) compared to JL BRL-6 and JL BTL-8, respectively. *RAD23B* was significantly overexpressed in BT-20 ($P = .043$) ($P = .015$) compared to JL BRL-6 and JL BTL-8, respectively, while in SK-BR-3 ($P = .021$) and MCF-7 ($P = .014$) compared to only JL BTL-8. (D) *RPA1*, *RPA2*, *RPA3*, *XPA*, and *XPC*. *RPA1* was significantly upregulated in JL BTL-12 ($P = .006$) ($P = .002$), MCF-7 ($P < .001$) ($P < .001$), BT-20 ($P = .011$) ($P = .006$), and MDA-MB-231 7 ($P < .001$) ($P < .001$) compared to JL BRL-6 and JL BTL-8, respectively, while in SK-BR-3 ($P = .049$) compared to only JL BTL-8. *RPA2* was significantly overexpressed in JL BTL-12 ($P = .009$) ($P = .003$), CAMA-1 ($P < .001$) ($P < .001$), MCF-7 ($P < .001$) ($P < .001$), and MDA-MB-231 ($P = .003$) ($P = .002$) compared to JL BRL-6 and JL BTL-8, respectively, while in SK-BR-3 ($P = .003$) and BT-20 ($P = .008$) compared to only JL BTL-8. *RPA3* was significantly upregulated in SK-BR-3 ($P = .019$) ($P = .014$), JL BTL-12 ($P < .001$) ($P < .001$), CAMA-1 ($P = .001$) ($P < .001$), MCF-7 ($P < .001$) ($P < .001$), BT-20 ($P = .004$) ($P = .003$), and MDA-MB-231 ($P = .001$) ($P < .001$) compared to JL BRL-6 and JL BTL-8, respectively. *XPA* was significantly overexpressed in SK-BR-3 ($P= .011$) ($P = .017$), CAMA-1 ($P = .020$) $P = .027$), MCF-7 ($P = .004$) ($P = .007$), BT-20 ($P = .027$) ($P = .040$), and MDA-MB-231 ($P = .027$) ($P = .039$) compared to JL BRL-6 and JL BTL-8, respectively, while in JL BTL-12 ($P = .031$) compared to only JL BRL-6. *XPC* was significantly upregulated in MCF-7 ($P = .008$) ($P = .022$) compared to JL BRL-6 and JL BTL-8. *GTF2H4* and *RPA3* were the only two genes that showed a significant increase in the cell lines and JL BTL-12 when compared to both JL BRL-6 and JL BTL-8. NER gene expression values were expressed as relative to JL BRL-6. + and * indicate a significant increase in gene expression compared to JL BRL-6 and JL BTL-8, respectively. $P < .05$ considered statistically significant using one-tailed unpaired student's t test.

Gene expression clustering analysis of the established breast cancer cell lines

Overall NER gene expression patterns were examined in JL BRL-6, JL BTL-8, JL BTL-12, and the five established cell lines using a hierarchical supervised clustering analysis. This analysis revealed two main clusters; the first cluster contained the non-diseased breast tissue explant JL BRL-6 and the stage I tumor explant JL BTL-8, while the other cluster included the five late stage breast tumor derived cell lines and the late stage explant JL BTL-12 (Figure A1.4B). This clustering demonstrates that the established cell lines and JL BTL-12 have a widely different NER gene expression pattern, influencing the NER function, compared to the early stage breast tumors and questions the use of these cell lines to study early stage-related molecular or biological features. Interestingly, the late stage, luminal type JL BTL-12 clustered closely with the most common luminal type cell line MCF-7, suggesting that MCF-7 molecular identity as a luminal tumor has been maintained despite that it has been cultured in laboratories for a long time.

These results were consistent with previous supervised clustering analyses based on 521 probe sets covering replication and DNA repair genes using a larger set of cell lines that have been created by Nancy Lalanne, a former graduate student in our laboratory, including non-diseased breast reduction, DICS with matching contralateral and ipsilateral non-tumor adjacent explants, stage I, stage II and JL BTL-12 as well as the late stage commercial breast cancer cell lines (Figure 3.4A). In this analysis, JL BTL-12 clustered with the commercial cell lines, away from the other two clusters containing the

non-diseased reduction cell lines, DCIS, stage I, and stage II cell lines. For replication/repair genes and many other gene sets this is the continuum we see based upon stage. The clustering of our JL-BTL12 with the stage IV commercial cell lines demonstrates that the discrimination of our early stage tumors lines from these established cell lines is not an artifact of our culture system, and it is due to intrinsic molecular differences between late stage and early stage breast cancer.

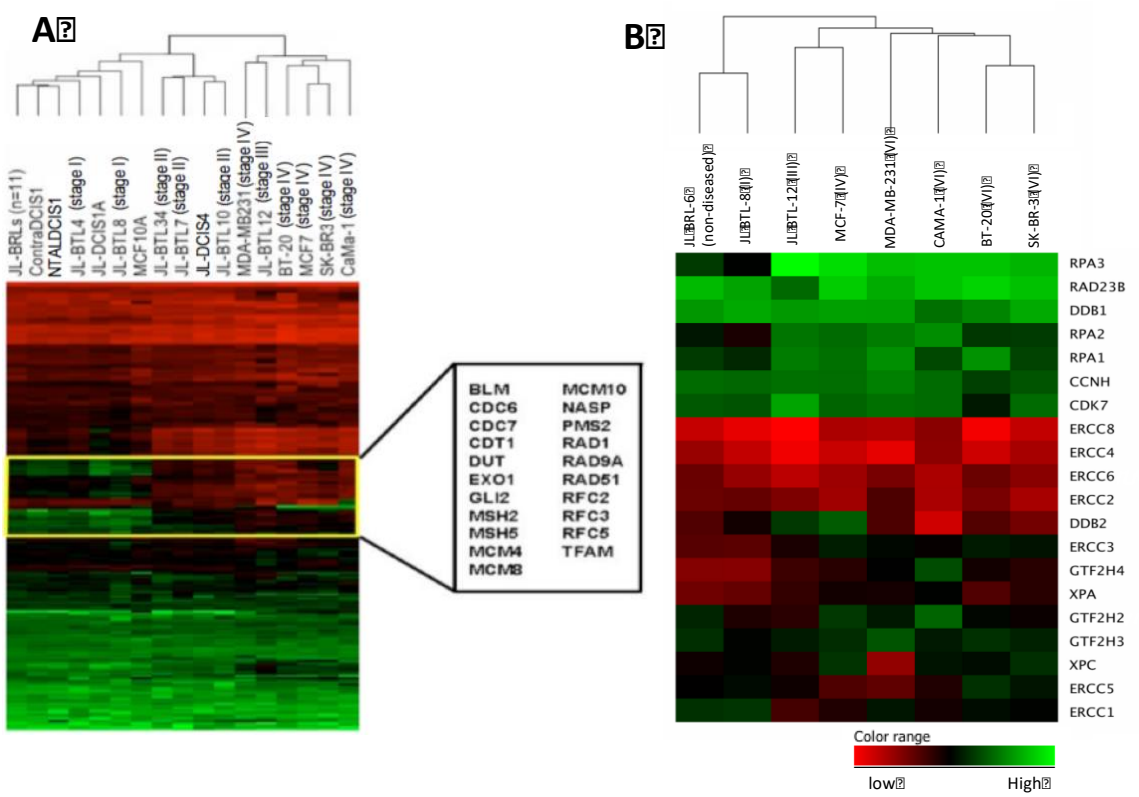

Figure A1.4 **Hierarchical supervised clustering dendrograms.** (A) Based on supervised analysis of 521 probe sets covering replication and DNA repair genes. This figure shows three major clusters: On the far right, the commercial stage IV pleural effusion cell lines and our late-stage lone JL-BTL12; 2. In the middle, stage II cell lines created in our laboratory; 3. On the left, stage I cell lines, DCIS cell lines with matching contralateral and ipsilateral non tumor adjacent explants as well as Breast Reduction Lines (BRLs) on the far left. (B) Based on the 20 NER canonical genes. This clustering analysis yields two main clusters, the first cluster is comprised of the non-diseased breast tissue explants JL BRL-6 and the stage I tumor explants JL BTL-8 while the other cluster included the five established cell lines and the late stage explants JL BTL-12. The <5 percentile of expression is the brightest color green and the > 95 percentile of expression is the brightest color red. The Affymetrix HGU133 Plus 2.0 Array was utilized.

A1.3.4 Silencing RPA3 gene expression

RPA3, as mentioned earlier, was the most overexpressed NER gene in all the late stage established cell lines and JL BTL-12 relative to JL BRL-6 and JL BTL-8 that might have a significant impact influencing NER function in late stage breast cancer. We decided to examine such impact experimentally by silencing RPA3 gene expression in three late stage cell lines.

After evaluating the transfection efficiency in MDA-MB-231, MCF-7, and JL BTL-12 (Figure A1.5; Figure A1.6), we assessed the efficacy of RPA3 siRNA in silencing RPA3 gene expression in these three cell lines using RT-PCR. RPA3 expression of the RPA3 siRNA transfected samples were compared to two control groups, mock and negative control (Figure A1.7).

In the most transfectable cell line MDA-MB-231, 97% of RPA3 gene expression was successfully silenced in the RPA3 siRNA treated samples compared to both the mock ($P < .001$) and the negative control samples ($P < .001$). In the second most transfectable cell line MCF-7, 85% and 83% of the total RPA3 gene expression was suppressed in RPA3 siRNA transfected samples compared to the mock ($P < .001$) and the negative control samples ($P < .001$), respectively. JL BTL-12 RPA3 siRNA transfected cells had 69% reduction in RPA3 gene expression relative in mock cells ($P = .006$), and 68% when compared to negative control treated cells ($P = .003$). These results showed that we were able to efficiently suppress RPA3 gene expression in all three cell lines to

an extent sufficient to allow us to examine the effect of such reduction on NER function, especially in MDA-MB-231, where the RPA3 gene expression was almost completely silenced.

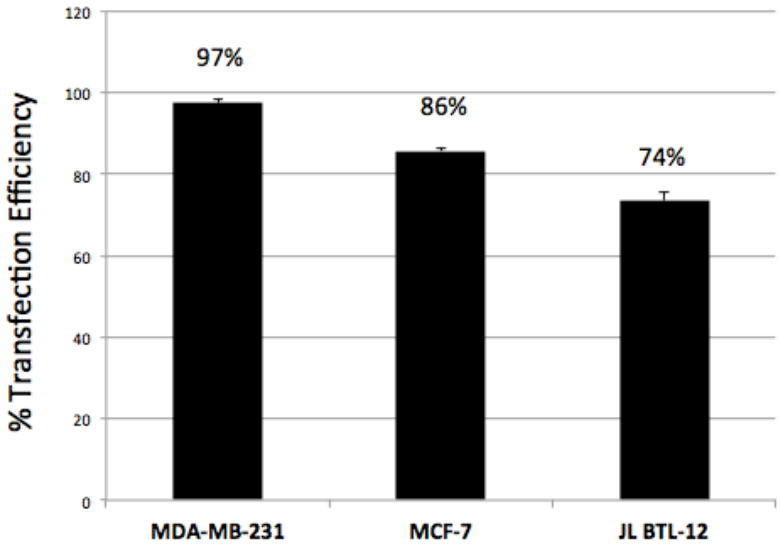

Figure A1.5 **Lipofectamine transfection efficiency of MDA-MB-231, MCF-7, and JL-BTL12.** MDA-MB-231 had the highest transfection efficiency followed by MCF-7 then JL BTL12. The transfection efficiency percentage for each cell line is shown. Error bars represent the standard error of three independent experiments for each cell line.

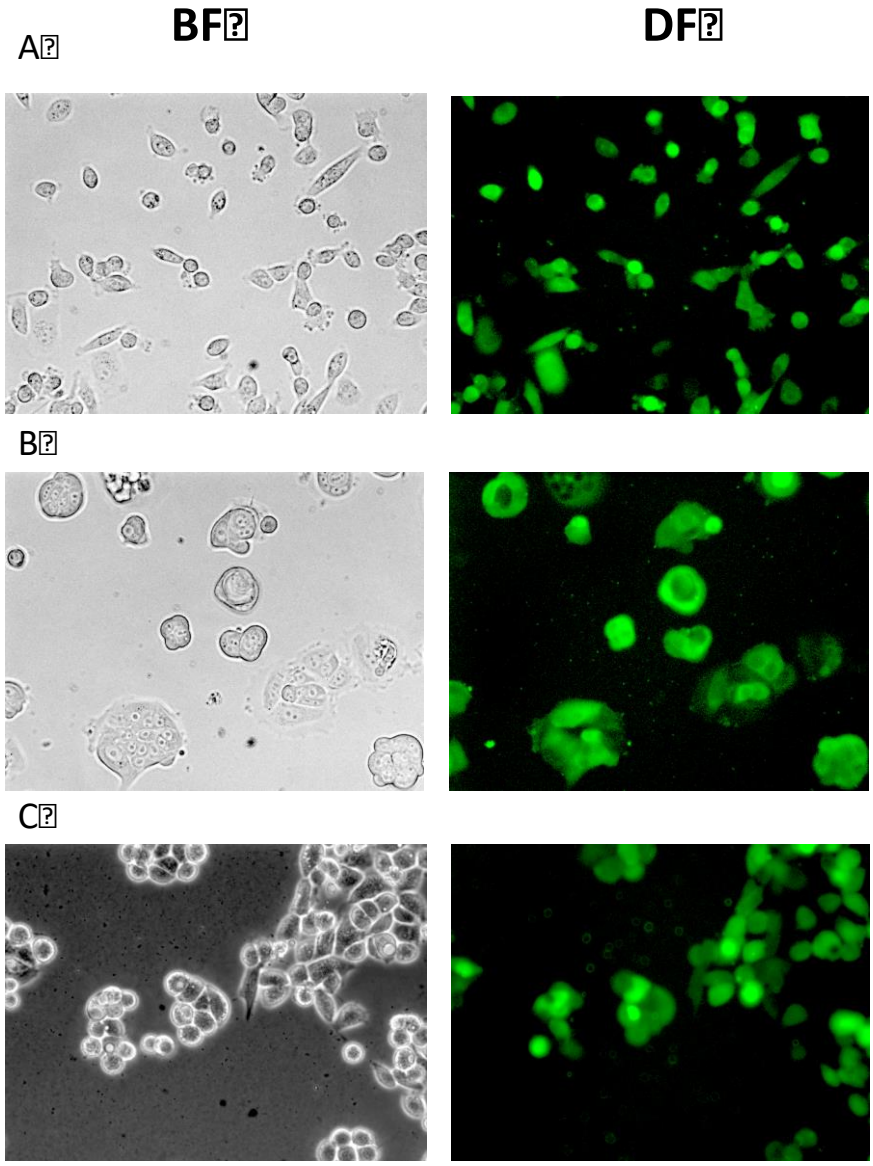

Figure A1.6 **Representative microscopic fields of fluorescent-tagged negative control transfected cells of (A) MDA-MB-231, (B) MCF-7, and (C) JL BTL-12.** Transfection efficiency was assessed 24 hours after transfection using an Olympus® IX51 microscope. Five 20x microscopic fields were captured for each negative control sample using a Hamamatsu® digital camera (model# C848-03G02) and MetaMorph® software 7.7.4.0v then evaluated for transfection efficiency using ImageJ software. Representative microscopic bright field (left) and dark field (right) images are shown for each cell line. BF; bright field DF; dark field (fluorescence).

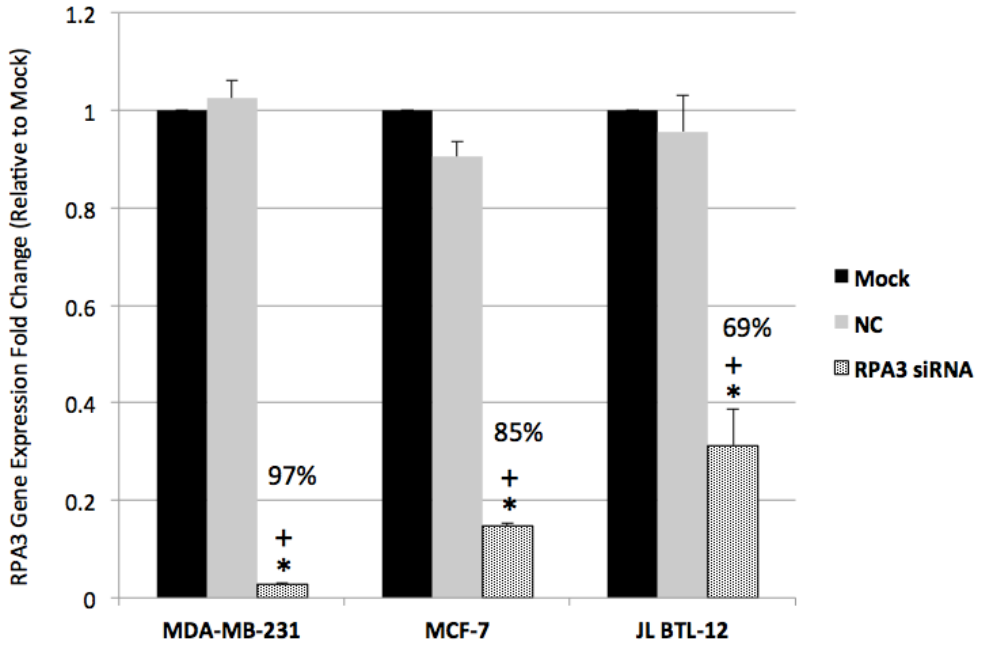

Figure A1.7 **RPA3 gene expression of the RPA3 siRNA transfected samples compared to mock and negative control samples in MDA-MB-231, MCF-7, and JL BTL-12.** JL BTL-12 was plated on matrigel, which was later shown to reduce transfection efficiency. RPA3 gene expression was significantly reduced by RPA3 siRNA transfection in all three cell lines. The percentage of reduction is given in the figure relative to the concurrent mock treated controls. RPA3 gene expression was expressed relative to the mock samples. Error bars represent standard errors over three independent experiments for each cell line. + and * indicate a significant decrease in RPA3 gene expression when compared with mock and negative control samples, respectively. $P < .05$ was considered statistically significant using one-tailed paired student's t test. NC; Negative control samples.

A1.3.6 Impact of silencing RPA3 gene expression on NER function and replication

After we validated the RPA3 siRNA efficacy in three different advanced stage breast cancer cell lines, we selected the most transfectable cell line, MDA-MB-231, in which RPA3 gene expression was almost completely silenced, to assess the impact of

such silencing on NER function using the functional unscheduled DNA synthesis assay. Processed grain counts derived from application of the unscheduled DNA synthesis assay on three slides transfected with RPA3 siRNA were compared to three slides that were treated with negative control scrambled RNA duplex.

RPA3 siRNA transfected cells (n = 300) had a 16% decrease in NER capacity relative to negative control treated cells (n = 300, Figure A1.8). This reduction was found to be statistically significant ($P < .001$). These data suggest that RPA3 is significantly involved in the increased NER function that late stage breast cancer acquired.

Since RPA3 has a role in replication, we evaluated the impact of RPA3 siRNA transfection on proliferation by calculating S-phase indices for both negative control and RPA3 siRNA transfected slides. RPA3 siRNA transfected slides had a significantly lower S-phase index by 25% ($P < .001$) (Figure A1.9). These results indicate that silencing RPA3 gene expression significantly reduced MDA-MB-231 cell proliferation.

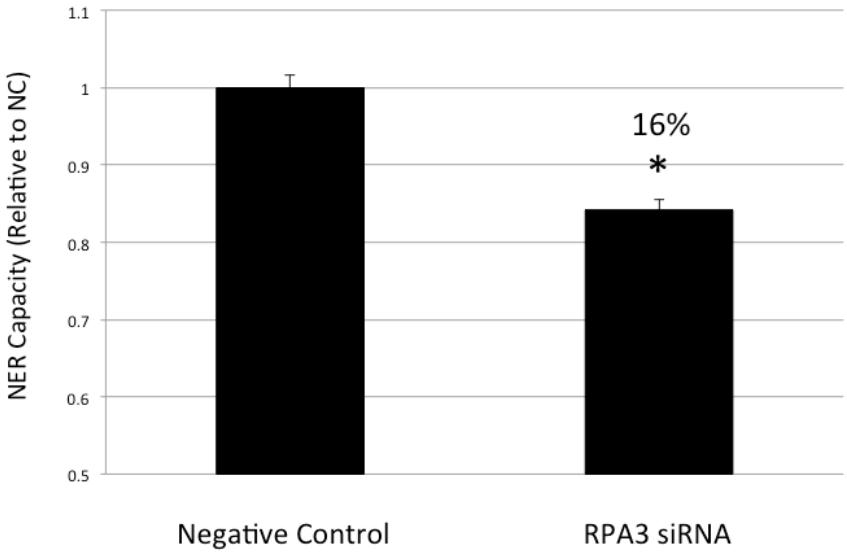

Figure A1.8 **The impact of silencing RPA3 gene expression on NER function in MDA-MB-231.** NER function was significantly reduced in RPA3 siRNA treated cells (n = 300) relative to negative control cells (n = 300, P < .001). The percentage of NER function reduction is given in the figure. The NER function was expressed as relative to negative control cells. Error bars represent standard errors of the pooled grain counts over the counted nuclei in three slides of each treatment group. *P* < .05 considered statistically significant using one-tailed paired student's t test. NC; Negative control cell

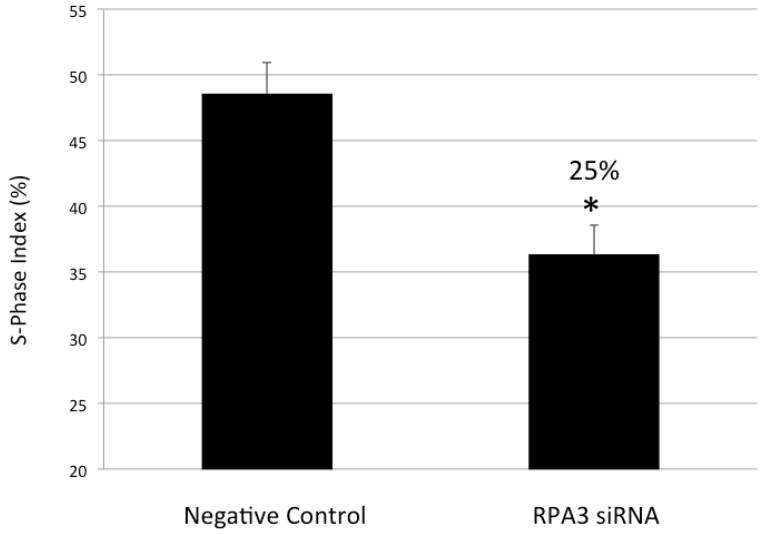

Figure A1.9 **The impact of silencing RPA3 gene expression on cell proliferation in MDA-MB-231.** The S-phase index was significantly reduced in the in RPA3 siRNA slides relative to negative control slides (P < .001). The percentage of S-phase index reduction is given in the figure. Error bars represent the standard error of the pooled S-phase indices of total counted fields in three slides in each treatment group. *P* < .05 was considered statistically significant using one-tailed paired student's t test. NC; Negative control samples

A1.4 Discussion

The established, late stage, commercially available breast cancer cell lines have been the predominant models in breast cancer research. In order to improve breast cancer patient outcomes using these cell lines, especially with downstaging of the most commonly diagnosed breast cancers, it is essential to ensure that these cell lines are representative breast cancer models.

Our laboratory has previously shown that sporadic stage I breast tumors had an intrinsic loss in NER function and gene and protein expression compared to non-diseased breast epithelial tissues (Latimer et al., 2010). However, this low repairing phenotype was not shown to be present in the most commonly used late stage established available breast cancer-derived cell lines (Figure A1.1). In fact, the established cell lines instead showed significantly higher NER function by a factor 2.35 and 5.15 times, when compared to primary cultures of non-diseased breast epithelial tissues and stage I breast tumors, respectively (Table A1.1). These results indicate that these widely used breast cancer cell lines are not representative of stage I breast tumors when it comes to NER capacity, which is a fundamental molecular characteristic associated with genomic instability and carcinogenesis.

There are several factors that might contribute to the increase in NER function that was observed in the established cell lines compared the primary stage I tumor cultures. The first factor is the tumor stage from which these cell lines and primary

cultures have been isolated. The majority of the established breast cancer cell lines that were examined in this study have been derived from pleural effusion of women with a stage IV metastatic breast cancer (Cailleau et al., 1978; Keydar et al., 1979; Lippman & Bolan, 1975; H D Soule et al., 1973; Trempe, 1976). Therefore, the observed increase in NER function is likely associated with cancer progression and/or drug resistance. Late stage tumors from the era that produced these commonly used cell lines may represent the most abnormal subpopulation that has survived in culture. These cells have gone through a serial of cancer cell selection rounds due to the natural tumor selection process or as a result of chemotherapy treatment. This aggressive subpopulation of tumor cells survived chemotherapy treatment because they have an efficient high NER function.

Another progression-related possibility for increasing NER function in late stage cancer is that the tumor itself has evolved and restored NER capacity either by altering NER gene or protein regulation or amplifying some of NER genes since cancer cells are genomically unstable. This would assume that most stage I tumors, if they progress, naturally become higher in NER capacity than they were as stage I entities. In fact, JL BTL-12 that has been established from chemotherapy native stage III tumor had already high NER function (Figure A1.1). This finding supports this possibility and indicates that the increased NER function is an intrinsic phenotype in late stage breast cancer.

The second factor that might play a role in driving the significant difference in NER function between the established cell lines and the primary cultures of stage I

tumors is the culture conditions in which these cultures are maintained. Established cell lines grow as a monolayer on plastic culture flask, while the stage I tumor primary cultures our laboratory created are maintained on a reconstituted basement membrane that allows the cancer cells to grow and communicate with each other in a three-dimensional environment, which is closer to the tumor *in vivo* microenvironment as compared to the classical *in vitro* cell culture methods (Lee, Kenny, Lee, & Bissell, 2007). For instance, the HER2 overexpressing cell line SK-BR-3 has been shown to respond significantly better to trastuzumab grown in a three-dimensional model compared to on plastic (Pickl & Ries, 2009). A number of MCF-7 cell lines that were obtained from different laboratories have been shown to have significant biological and genomic parameters such as growth rate, hormonal response, and karyotype, despite the fact that they all had the same origin (Bahia et al., 2002; Wenger et al., 2004). The fact that JL BTL-12 manifested high NER capacity consistent with what we observed in the established cell lines, when compared to both non-diseased breast epithelial tissues and stage I tumors that were maintained under the same cell culture conditions indicates that the increased NER function in the cell lines was not due to culture technical artifacts; it is most likely an intrinsic progression-related characteristic in late stage breast cancer. JL BTL-12 explants that were derived from a patient with a luminal type breast tumor clustered closely with the most common luminal type cell line MCF-7 in the hierarchical NER supervised clustering analysis (Figure A1.4B), demonstrating that MCF-7 still

exhibits the luminal molecular characteristics despite it has been maintained in culture for a long time.

The established cell lines that were selected for this study represent different molecular subtypes of breast cancer that allowed us to examine whether the molecular subtypes have different NER function profiles. Triple negative breast cancer patients tend to have more aggressive tumors that are less responsive to chemotherapy, and subsequently have worse clinical outcomes compared to patients with other molecular subtypes (Bauer et al., 2007). One possible mechanism by which triple negative breast tumors are less responsive to chemotherapy is by acquiring an increased NER capacity. The triple negative breast cancer cell lines BT-20 and MDA-MB-231 had significantly higher NER capacity compared to luminal type cell lines CAMA-1, MCF-7, and MCF-7/LY2 and the luminal type breast tumor primary culture JL BTL-12 (Table A1.5). These data indicate that NER might be a molecular factor contributing to tumor aggressiveness and chemotherapy resistance that is seen in triple negative breast cancer patients.

However, these results contradict a recent report that has showed that NER function was low in triple negative breast cancer compared other molecular subtypes. Matta, Ortiz, Encarnación, Dutil, & Suárez (2017) examined NER function in peripheral blood lymphocytes as a surrogate for the tumor cells, which might not reflect the actual repair in the primary tumor. In fact, the unaffected distant tissue would not show a

progression-related event such as acquiring an increased NER capacity that occurs only in the primary tumor. In addition, Matta's group used the host cell reactivation assay that specifically measures the transcriptional-coupled repair to assess NER function in triple negative patients, which might not be indicative of the total repair function. Therefore, we believe we addressed the repair in triple negative late stage breast cancer represented by MDA-MB-231 and BT-20 in a more specific approach compared to the study that was discussed.

We also investigated the possibility of an association between antiestrogen resistance and NER capacity. Advanced breast cancer tumors manifest acquired antiestrogenic therapy resistance (Broom et al., 2009; Kuukasjärvi, Kononen, Helin, Holli, & Isola, 1996). MCF-7/LY is a stable variant of MCF-7 that is known for its tamoxifen resistant phenotype. Although MCF-7/LY2 had a higher NER function by 23%, compared to its parent antiestrogen sensitive MCF-7, this increase did not reach significance (Figure A1.1). Therefore, NER is as likely as not be involved in developing resistance to tamoxifen. Such resistance might be explained by other tumor progression-related phenomena such as altering tamoxifen pharmacological target or loss of estrogen receptor expression (Viedma-RodríGuez et al., 2014).

After we established that the established breast cancer-derived cell lines had a higher NER function relative to non-diseased breast tissue and stage I tumor primary cultures, we evaluated individual NER gene expression to identify the genes that might

play an important role in increased NER function in late stage. Supervised analysis using NER genes showed that the stage I tumor cell line JL BTL-8 grouped together with non-diseased breast epithelial tissue explants JL BRL-6, whereas the established stage IV cell lines and JL BTL-12 formed another cluster, indicating that even though JL BTL-8 is a tumor, it is still more related to non-diseased JL BRL-6 than to late stage cancer with respect to NER gene expression patterns (Figure A1.4B). This supports that early stage breast cancer has widely different NER molecular characteristics compared to late stage breast cancer. An extended clustering analysis utilizing more than 500 probes representing genes involved in DNA repair mechanisms and proliferation revealed similar results (Figure A1.4A). Thus, the established cell lines that are derived from late stage patients are inappropriate models for studying NER-related features in early stage breast cancer.

NER genes, overall, were increased in expression in the cell lines and JL BTL-12 relative to JL BRL-6 and JL BTL-8, as we hypothesized. However, the number and identity of upregulated genes that individually reached statistical significance varied from one cell line to another, even amongst cell lines with the same molecular subtypes. *GTF2H4* and *RPA3* were the only NER genes consistently significantly overexpressed in all the established cell lines and JL BTL-12 compared to both JL BRL-6 and JL BTL-8 (Figure A1.3), indicating that these two genes might play an important role in the increased NER function phenomenon seen in the late stage breast cancer.

GTF2H4 is a member of the TFIIH complex that is essential for opening the site of DNA damage, stabilizing the repair bubble, and initiating the repair process. *GTF2H4* is a non-enzymatic protein that has a structural function maintaining the stability of TFIIH complex (Compe & Egly, 2016). TFIIH also plays an important role in transcription initiation of protein encoding genes (Compe & Egly, 2012). Therefore, the increase in *GTF2H4* gene expression could be also impacted by its role in transcription.

RPA3 is a subunit in replication protein A (RPA) complex that binds to the opposite (i.e. undamaged) strand, preventing premature re-annealing of the DNA helix and protecting the undamaged strand from degradation by nucleases. The RPA complex is also involved in replication, playing essentially the same role as it does in repair . The RPA complex has been an appealing therapeutic target in cancer drug development due to its essential role in replication. Several compounds have been developed to target the RPA complex to inhibit cancer cell proliferation, such as NSC15520, HAMNO, MC113E, TDRL-551, TDRL-505 and fumaropimaric acid (Glanzer et al., 2013, 2014; Glanzer, Liu, & Oakley, 2011; Neher, Bodenmiller, Fitch, Jalal, & Turchi, 2011). MC113E, TDRL-551, and TDRL-505 have sensitized cancer cells to chemotherapy agents that are associated with NER pathway, such as cisplatin, illustrating the important role that RPA complex plays in NER function (A. K. Mishra, Dormi, Turchi, Woods, & Turchi, 2015; Neher et al., 2011; Shuck & Turchi, 2010).

Here in our study, we showed that silencing a subunit in this complex, RPA3, in the high repairing MDA-MB-231 significantly reduced its NER capacity, although by no means abrogating the ability to perform NER. Silencing *RPA3* also significantly inhibited MDA-MB-231 proliferation. Although these two processes have been shown to be independent, the dual impact on proliferation and DNA repair may make *RPA3* a potential therapeutic target in breast cancer treatment. Silencing *RPA3* might synergize the genotoxic effect of chemotherapy agents by directly interfere with the ability of cancer cell to replicate and suppress NER repair-induced drug resistance mechanism. However, such statistically significant reduction in NER function and proliferation needs to be evaluated in the context of the biological significance. Future experiments should involve *RPA3* siRNA transfection accompanied by DNA damaged-induced chemotherapy agents such as cisplatin to see whether this reduction in NER function caused by silencing *RPA3* is significant enough to sensitize cancer cells to this classical NER remediating genotoxic agent.

In summary, we demonstrated that five commonly used established breast cancer cell lines and one similar cell line our laboratory established from a primary culture had significantly higher NER function relative to primary cultures of non-diseased breast epithelial cultures and stage I breast tumor primary cultures. In addition, the established cell lines had widely different NER gene expression profiles compared to stage I breast cancer. It is a clinical reality that cancers of increasing stage are harder to treat

effectively. This study suggests that with increasing stage, breast cancer may become more resistant to chemotherapy because of increasing DNA repair capacity.

These findings question the use of the established cell lines as models to study genomic instability-related phenotypes associated cancer etiology and emphasizes the need to develop better and more representative *in vitro* models, especially stage I breast tumor-derived models, since stage I is by far the most diagnosed stage, 62% of the total breast cancer cases. Our laboratory has successfully established novel cell lines from early stage breast tumor primary cultures. Hopefully, the availability of such models would be the start to shift the focus from the traditional use of the late-stage established cell lines to utilize our cell lines or similar early stage models in the field of breast cancer research. In fact, some early stage commercially available breast cancer cell lines have emerged (Gazdar et al., 1998).

Finally, this study also provides early evidence for the development of a new NER-targeted therapeutic approach via precisely inhibiting RPA3 gene expression in concert with genotoxic agents to suppress cell proliferation and reverse chemotherapy resistance, after recurrence.

Appendix B

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