ESTABLISHING AND CHARACTERIZING PATIENT-DERIVED BREAST CANCER CELL LINES

A Thesis
Submitted to the Graduate Faculty
of the
North Dakota State University
of Agriculture and Applied Science

By

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In Partial Fulfillment of the Requirements
for the Degree of
MASTER OF SCIENCE

Major Department:
Biological Sciences

August 2022

Fargo, North Dakota

North Dakota State University
Graduate School

Title

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MASTER OF SCIENCE

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ABSTRACT

Commercial cancer cell lines have long been extensively used as an important platform to study cancer. They have contributed to a plethora of discoveries in the field of cancer research. However, there are limitations with using these cell lines, such as induced mutations over the long-term in vitro culture. These mutations cause incorrect exhibition of the in vivo characteristics of the cancer cells. Here, we focused on establishing Patient-derived breast cancer cell lines and attempted to characterize them in terms of several biomarkers that are shown to be overexpressed in breast cancer cells. Patient-derived breast cancer cell lines are more reliable tools to study the molecular and cellular processes taking place in vivo, since they are freshly isolated from the tumor biopsy and do not undergo induced immortalization. We explored the CK19, Ki67, vimentin, EpCAM, E-cadherin, and N-cadherin expression in three successfully established patient-derived breast cancer cell lines.

ACKNOWLEDGMENTS

I would like to express my gratitude to my advisor, Dr. Jiha Kim, who guided me through this project. I would also like to thank my committee members, Dr. Kalpana Katti and Dr. Katie Reindl, as well as my family, friends, and lab mates for their support and help. Also, I wish to extend my special thanks to the NSF EPSCoR Track-1 grant for the financial support of this project.

DEDICATION

I would like to dedicate this work to my parents, friends especially Reza Rezaiezadeh-Roukerd, Amirhadi Alesadi, and Vikneshwari Natarajan, and colleagues for their continuous support and help throughout my career. At the same time, I want to mention the tremendous support from my siblings who have been encouraging me for years.

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LIST OF ABBREVIATIONS

CK19	Cytokeratin-19
EpCAM	Epithelial cell adhesion molecule
qPCR	Quantitative real-time polymerase chain reactions
MEFs	Mouse embryonic fibroblasts
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
FBS	Fetal bovine serum
DMEM	Dulbecco's Modified Eagle Medium
TNBC	Triple-negative breast cancer
PBS	Phosphate buffered saline
DAPI	4', 6-diamidino-2-phenylindole
EDTA	Ethylenediaminetetracetic acid
cDNA	Complementary-deoxyribonucleic acid

CHAPTER 1: INTRODUCTION

1.1. Breast cancer, an overview

Breast cancer is the most common cancer in women with a molecularly heterogeneous nature. Treatment approaches have changed over the last 10–15 years to accommodate for this heterogeneity, with a focus on more biologically focused therapies and treatment de-escalation to lessen side effects. Despite the inherent biological heterogeneity, some characteristics, such as the impact of locoregional tumor burden or metastatic patterns, are common and can affect therapy(1).

Early breast cancer refers to the stage of cancer contained to the breast or has only migrated to the axillary lymph nodes. Seventy to eighty percent of patients are now more likely to be cured as a result of advances in multimodal therapy. In contrast, advanced (metastasized) illness is not considered treatable with the current therapy choices available. However, advanced breast cancer is curable, with the primary goals of therapy being to prolong survival and control symptoms with minimal treatment-associated damage in order to maintain or improve quality of life(1). Figure 1.1 demonstrates the breast cancer statistics based on the molecular type.

1.2. Breast cancer molecular subtypes

1.2.1. Luminal breast cancer cell lines

Luminal breast cancer cell lines are characterized by positive ER and/or PR expression, notwithstanding a few unusual occurrences, such as IBEP-1 and IBEP-3 (2), where PR positivity drives their luminal phenotype. An array of genes and proteins associated with luminal features, such as luminal keratins (KRT8/18/19), transcription factors including GATA3 and FOXA1, and ESR1 (ER or ER), are highly expressed in these types of cell lines. Has-miR-501-5p, Has-miR-202, Has-miR-760, and Has-miR-626 have been found to be overexpressed in luminal cell lines in a specific manner, according to a systematic investigation on miRNA expression profiling (3). Due

to tight cell-cell connections, luminal cell lines are comparably more differentiated and less prone to migrate than those seen in tumors (4).

Although the majority of studies do not further divide luminal cell lines into luminal A and B subtypes based on their HER2 status, we embrace such a differentiation not only for the sake of achieving consistent categorization with tumor subtyping to facilitate straightforward tumor modelling but also to satisfy the requirement of drug response assays based on ER and HER2 status. For instance, a study using the breast cancer cell line BT474 (ER+HER2+) indicated the synergistic benefit of tamoxifen and Herceptin in the treatment of breast cancers (5), and MCF7 (ER-HER2) has traditionally been used to assess the tamoxifen-induced cell response (6). Since HER2 over-expression has been linked to ER down-regulation, luminal B cell lines are, in theory, more invasive and aggressive than luminal A cell lines. As a result, luminal B cells are more correctly referred to as weakly luminal and take part in the phenotypic attenuation of luminal A cells (7). Cell lines from this subtype with promise for translation in clinics have been used to successfully understand a great deal of information for luminal B malignancies (8-11). For instance, utilizing the ER+HER2+ cell line BT474, it has been revealed that the expression of quiescin-sulfhydryl oxidase 1 is related with a poor prognosis in luminal B tumors , indicating the value and significance of distinguishing luminal B cell lines from the luminal subtype (8).

1.2.2. HER2 positive breast cancer cell lines

The HER2, GRB7, PERLD1, STARD3, and C17ORF37 genes are all over-represented in the genomic profile of HER2 positive cell lines, which are characterized by ER negativity and HER2 positivity (12). These cell lines exhibit overt expression of micro RNAs such as has-let-7b, has-miR-640, has-miR-200c, has-miR-378, has-miR-141, has-miR-196a, has-miR-29c, and has-miR-18a* (3). HER2 positive cell lines are diverse and contain both luminal and basal

characteristics, bridging the gap between luminal and basal cell lines. According to the expression of luminal and basal markers in addition to ER and HER2 expression, they are classified as luminal-ERBB2+ and ER-negative-ERBB2+ (4). Since HER2 over-expression is linked to the breakdown of cell-cell junctions (7), cells of this subtype are more aggressive in terms of cell migration when compared with luminal cells (4). They are also more responsive to certain drugs, making them an excellent model for examining the response to Herceptin(4). A significant correlation between the molecular signature and biological response is revealed by Pearson's correlation test (13). Increased levels of ESR1, MAPK1/3, MEK, TYK2, FASN, and GRB7, which are primarily linked to cell proliferation, are proteins whose expression are closely correlated with such a medication response. Up-regulated expression of SFN, CAV2, GRB2, RB1, and FLNA shows a drug resistance (13). Thus, it can be inferred from research on cell lines with high levels of HER2 expression that MAPK signaling predicts the response to Herceptin, whereas Herceptin resistance is linked to the mTOR pathway, Toll-like receptor pathway, N-glycan production, and inositol-phosphate signaling (13).

1.2.3. Triple negative breast cancer cell lines

According to their name, triple negative cell lines exhibit low or no expression of all three ER-PR-HER2-related markers. It is the most diverse subtype, and basal A and basal B cell lines are common names for it in many publications. Triple negative A (basal A) lines are known as basal-like because they are enriched in basal markers such as cytokeratins (KRT4/5/6A/6B/13/14/15/16/17), integrins (ITGA6, ITGB4/6), LAMB3, LAMC2, TRIM29, S100A2, SLPI, ANXA8, COL17A1, BNC1, CD10/14/58/59, MET, LYN, CD133, GABRK, VTCN1, BST2, FABP7; exhibiting similarity with core basal tumor subtype (3, 14, 15). The mesenchymal cluster or normal-like/claudin-low triple negative B (basal B) lines overexpress

genes linked to tumor invasive and aggressive characteristics, including VIM, MSN, PLAT, TGFB1, TGFBR2, AXL, COL3A1, COL6A1/2/3, MMP2/14, TIMP1, CTSC, PLAU, PLAUR, SERPINE1/2, SPARC, FN1, FBN1, HAS2, and PRG1 (13-15), Collagens (COL3A1, COL6A1/2/3, COL8A1), proteases (MMP2/14, TIMP1, CTSC, PLAU, PLAUR, SERPINE1/2, PLAT), and proteins stabilizing cytoskeletal interactions (VIM, MSN) are significant contributors to the remodeling of the extracellular matrix necessary for cell migration, and signaling factors (TGFB1, TGFBR2, AXL) play a critical role in mediating such aggressive morphology(4).

Triple negative cell line characterization at the translational level uses a specific panel of proteins (EGFR, CAV1/2, MSN, ETS1) that, when combined with basal keratins (KRT5/6), CD10, and MET, characterize triple negative A cells and, when combined with the stemness marker CD44, identify triple negative B cells (14). Triple negative B cells have a more mesenchymal-like look and are more likely to invade. Triple negative A cells, a somewhat more developed subgroup within triple negative cell lines, can have either luminal-like or basal-like morphologies (4). Due to their abundance in epithelial mesenchymal transition (EMT) and stem-cell markers, triple negative B cells can be utilized to represent claudin-low or metaplastic breast cancer, while triple negative A lines largely match the core basal tumor subtype as shown by basal markers (16). In response to DNA damage, the protein encoded by BRCA1 forms the Rap80/Abraxas/Brca1/Brcc36 complex (17). Hereditary breast cancer, which also resembles sporadic core basal tumors, is predisposed by a mutation in this gene (18-20). The majority of the currently available BRCA1 mutant commercial breast cancer cell lines (HCC1937, MDAMB436,

SUM149PT, HCC3153) fall within the category of triple negative A lines, with the exception of SUM1315MO2 (15).

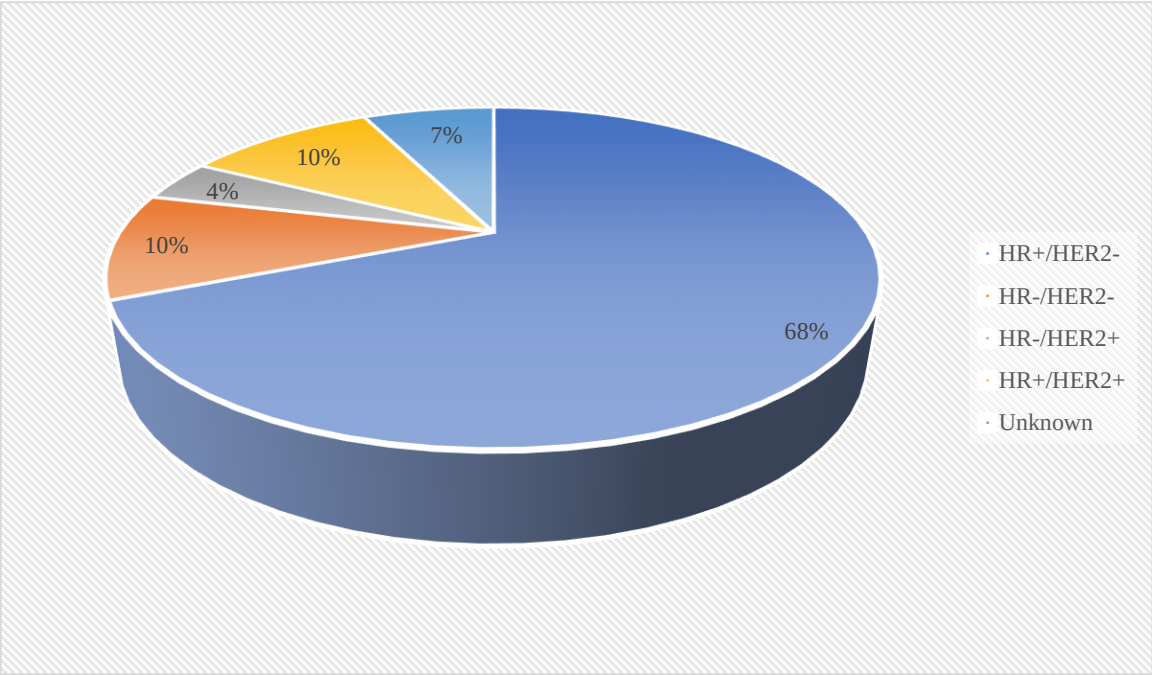

Figure 1.1. Female global prevalence of breast cancer by subtypes in 2015-2019. As shown, Luminal A (HR+/HER2-) subtype was the most common subtype of breast cancer in women (Source: National cancer institute)

1.3. Markers overexpressed in breast cancer

1.3.1. EpCAM

EpCAM is a type I transmembrane glycoprotein with a Mr of 40,000 that has two extracellular domains that resemble those of epidermal growth factor, a cysteine-poor region, a transmembrane domain, and a brief cytoplasmic tail; encoded by GA733-2 gene on the long arm of chromosome 4 (21). EpCAM has been referred to by a number of different names, including those connected to monoclonal antibodies that are specific for the cell surface antigen (MH99, AUA1, MOC31, 323/A3, KS1/4, and HEA125) and cDNA clones that were used to characterize the antigen [KS 1/4, EGP, EGP40, and GA733-2 (21)]. The name EpCAM, which more accurately

describes its function and tissue specificity, was first proposed by Litvinov et al. (22, 23). With the exception of the adult squamous epithelium and some epithelium-derived cells, including hepatocytes, epidermal keratinocytes, gastric parietal cells, myoepithelial cells, and thymic cortical epithelium, EpCAM is found at the basolateral membrane of the majority of epithelial tissues (all simple, pseudo-stratified, and transitional epithelia) (24, 25). However, during active cell proliferation, whether normal or cancerous, various cell types can also be seen to express EpCAM de novo (21). EpCAM's overexpression by the majority of human epithelial carcinomas, including colorectal, breast, prostate, head and neck, and hepatic carcinomas, is particularly intriguing. Because of this, EpCAM has received significant attention as a target for monoclonal antibody-based immunotherapy to treat a variety of cancers, most notably colorectal carcinoma. In fact, the administration of the EpCAM-specific monoclonal antibody has been effective in boosting disease-free survival in patients with minimally recurrent colon and breast cancer. These antibodies help kill tumor cells by triggering a variety of natural cytotoxic processes, including as antibody-dependent complement-mediated cytotoxicity (21).

EpCAM is a target of monoclonal antibody-based immunotherapy, and data suggests that its expression levels are correlated with proliferative activity and play a role in the development of neoplasms. In fact, poor disease-free and overall survival are linked to EpCAM overexpression in primary breast tumors. These findings imply that EpCAM is a viable target for molecular therapy and that more research is necessary (21).

1.3.2. Vimentin

The 57 KD type III intermediate filament protein known as vimentin is crucial for the mobility and deformation of tumor cells. It preserves the integrity of cells and tissues and is present during mesenchymal cell development in diverse tissues (26). The conventional MAPK signaling

system, which regulates numerous cellular functions (including proliferation, survival, differentiation, and motility), includes the MEK/Erk1/2 pathway, which is typically active in human malignancies (27).

Vimentin is extensively involved in a variety of physiological processes and is crucial for controlling how cells behave (28). Vimentin keeps cells' regular morphologies and has anchoring actions in the organelles and nucleus (29). It is involved in cell migration, differentiation, proliferation, adhesion, and invasion at the cellular level (29, 30). Vimentin is highly expressed in cells with remarkable motility, according to studies done on mouse embryos (31). Vimentin contributes to the growth of the mammary gland, the neurological system, and angiogenesis in terms of organ function. This molecule has been shown in numerous studies to control the epithelial-mesenchymal transition (EMT), which has an impact on a variety of physiological and pathological processes, including growth and wound healing (32). Vimentin is strongly linked to the occurrence and growth of tumors through modifying the EMT, which is supported by a growing body of evidence (33, 34).

The level of vimentin expression is strongly correlated with the development of cancer, according to numerous lines of evidence. Vimentin was identified by immunohistochemical detection in 145 of 295 patients with non-small cell lung cancer. It was largely expressed in highly invasive tissues, particularly in cells at the junction. Vimentin expression was highly connected with the metastasis of non-small cell lung cancer, according to the follow-up results of 193 patients (35). Vimentin expression predicts shorter overall survival, worse disease-free survival, and more severe lymph node metastases, according to a meta-analysis of 1,969 colorectal cancer patients (36). Additionally, vimentin is known to be methylated during the growth of colon cancer, and the detection of vimentin methylation in feces can help diagnose colorectal cancer (36). By interacting

with vimentin, osteopontin causes EMT and shortens the overall survival of hepatocellular cancer patients (37). Additionally, it has been discovered that the effects of osteopontin on hepatocellular cancer are completely eliminated by the knockout of vimentin residues 246 to 406 (37). Circulating tumor cells (CTCs), which circulate in the peripheral blood, have made significant advancements in the detection and monitoring of early cancers. The sensitivity and specificity of CTCs are lessened in clinical applications due to the lack of a specialized technique that may be employed to acquire circulating cells. The detection of CTCs is improved by detecting vimentin on their surface (38). According to these findings, vimentin is a potential tumor marker and therapeutic target and plays a significant regulatory role in numerous types of cancers (28).

1.3.3. Ki67

In Hodgkin lymphoma cell nuclei, ki-67 was first discovered as an antigen that is substantially expressed in cycling cells but markedly down-regulated in dormant G0 cells (39). Due to this property, Ki-67 has gained clinical significance as a proliferation marker for classifying a variety of malignancies (40), and its prognostic significance has been well-established in big investigations (41-44). Even though Ki-67 has a lengthy history of therapeutic use, its molecular functions have received far less research. An earlier description of Ki-67 localization found a nuclear protein in human cells that were proliferating (39). Additionally, research on peripheral mononuclear blood leukocytes (PBL) induced by phytohemagglutinin (PHA) revealed that G0 phase cells were negative for the Ki-67 antigen (45). We now understand that the cell cycle regulates the MKI67 gene promoter, which contains binding sites for the canonical G1-regulatory E2F family of transcription factors, and that Ki-67 mRNA levels rise during G1 phase (46, 47). Additionally, during G1, the ubiquitin proteasome complex APC/C-Cdh1 degrades the Ki-67 protein (47, 48). As a result, two competing systems regulate the levels of the protein Ki-67 in G1.

Additionally, cells in the early stages of cell cycle arrest contain modest amounts of Ki-67, which can persist after reentering the cell cycle, in contrast to severely quiescent or senescent cells (47). Thus, a small difference in the level of quiescence can lead to variations in Ki-67 levels in the initial G1 phase after cell cycle re-entry, which is probably what led to variations in the results of various experiments (45, 47, 49).

Recent significant discoveries have been made as a result of the increased interest in the functions of the Ki-67 protein. Ki-67 has numerous molecular roles that vary depending on the type of cell and are associated to various cell cycle stages. The perichromosomal layer, which makes up about one-third of the mass of mitotic chromosomes, is formed during mitosis and is coated with Ki-67, which coats the surface of chromosomes (50, 51). Because it is a component of the PCL, Ki-67's large net positive charge prevents mitotic chromosomes from adhering to one another (52). Additionally, new research suggests that the cohesin and ki-67 complexes each contribute differently to the structural integrity of mitotic chromosomes, as co-depletion of both proteins results in the formation of an amorphous "slime ball" of chromosomes (53). Therefore, there is a great deal of interest in learning the specifics of how Ki-67, as the foundation for creating the PCL, contributes to the shape of mitotic chromosomes (54).

After mitosis, perinucleolar heterochromatin is covered by Ki-67, which then relocalizes to the nucleolar periphery. The largest non-membrane-bound subnuclear structures are nucleoli (54). Research shows that the internal architecture of nucleoli depends on liquid-liquid phase separation in both Xenopus oocyte nuclei and Drosophila embryos (55). The topic of whether Ki-67 functions as a surfactant in the nucleolus' organization and whether this may help explain Ki-67's function in arranging heterochromatin surrounding the nucleolus is raised by this (46, 56, 57). Furthermore, it is unknown if there are unanticipated details about this activity because NAD

relocalization in response to Ki-67 loss has not been the subject of a thorough examination. The sensitivities of any NAD connections to the G1/S checkpoint status of the cells under study will be of special interest (54).

1.3.4. CK19

A unique epithelial cytoskeleton marker called cytokeratin-19 (CK19) is highly expressed in epithelial malignancies, and its expression is particularly tissue-specific in breast cancer. It may be a reliable diagnostic marker for finding tumor cells in cancer patients' peripheral blood (58, 59). CK19 was employed as a marker in numerous studies to find cancer cells in lymph nodes, peripheral blood, and bone marrow (59-61). Additionally, the CK19 marker is regarded as a stand-alone prognostic predictor in cancer patients (62). Reverse-transcriptase polymerase chain reaction (RT-PCR) approaches based on RNA can produce high diagnostic sensitivity that can be helpful to track the development of disease by detecting specific epithelial marker mRNA transcripts (60).

The cytoskeletal protein cytokeratin-19 (CK-19), which is found in both healthy and cancerous epithelial cells, has been widely utilized to identify breast cancer cells in mesenchymal tissues. It appears to be the most accurate tumor marker for both patients with operable and metastatic breast cancer (60).

1.3.5. E-cadherin

The type I cadherin family is made up of transmembrane glycoproteins that attach to different cell types and are essential for the normal morphogenesis and development of animal tissues (63-66). On chromosome 16q22.1, the E-cadherin gene (CDH1), which is around 100 kb long, is located (63). The gene region consists of 16 exons that range in size from 115 to 2245 bp and are separated by a total of 15 introns (63). Comparing the human CDH1 exon borders in various species and cadherin types found notable splice site conservation, suggesting gene

duplication or conversion during the coevolution of cadherin types (63). The protein called E-cadherin, which forms adherens junctions and joins epithelial cells together, was first identified by Takeichi in 1977 (67). Its related cadherin family members were subsequently identified, and they exhibit distinct temporal and geographical patterns of expression (68).

A precursor polypeptide of 135 kDa, encoded by the CDH1 gene, is translated into the E-cadherin protein. The precursor segment has the consensus protease cleavage site (Arg-Arg-Gln-Lys-Arg), which makes it easier to process the precursor segment's proteolytically into a fully developed and functioning protein with sticky characteristics (63). A 120 kDa, Ca2+-dependent transmembrane glycoprotein known as mature E-cadherin links polarized and unpolarized epithelial cells at the lateral surface via AJs (69). E-cadherin has five extracellular cadherin domains in its amino terminus, which are where its adhesive action is located and where Ca2+ ions bind (70). The extracellular domain is thus made stiffer by Ca2+ ions binding (71), which increases protease resistance (72). The three-dimensional domain swapping that is at the heart of E-homophilic cadherin's trans dimer formation across apposed cells plays a crucial role in the stiffening of the extracellular domain. Because the majority of solid tumors are carcinomas that develop from epithelial tissue, E-cadherin plays a critical role in epithelial cell adhesion and the loss of its function is a significant contribution to the advancement of cancer (73). The hallmark of a cellular process known as epithelial-mesenchymal transition (EMT), frequently linked to the development of cancer, has been observed to be diminished E-cadherin expression (63). However, some scientists assert that the loss of E-cadherin is only incidental to EMT and that its expression level may even be controlled by the EMT process itself through downstream epigenetic silencing (74). As a result, they argue that the loss of E-cadherin is insufficient to cause EMT (75). Even so,

deletion or reduction of E-cadherin expression has repeatedly been linked to poor prognosis and poor overall survival of cancers such breast cancer, colon cancer, and gastric cancer (76, 77).

1.3.6. N-cadherin

The calcium-dependent adhesion molecule family of traditional cadherins, which includes N-cadherin, directly mediates both homotypic and heterotypic cell-cell attachment . N-cadherin is a type I cadherin that has five extracellular domains connected to an intracellular domain that is functional. A tryptophan residue side-chain on an N-cadherin monomer's first extracellular domain (EC1) is reciprocally inserted into the hydrophobic pocket of the partner N-cadherin EC1 to form an engagement between the monomers on opposing cells (trans adhesion). Additionally, the His-Ala-Val (HAV) motif on EC1 and a recognition sequence on the second extracellular domain (EC2) of the lateral N-cadherin monomer (cis adhesion) are required for the clustering of adjacent monomers on the surface of the same cell in order to stabilize N-cadherin-mediated adhesion (71, 78, 79). P120 catenin, which localizes N-cadherin to cholesterol-rich microdomains, is necessary for its membrane expression and lateral clustering (80, 81). When N-cadherin extracellular domains are initially ligated, Rac, a member of the Rho GTPase family, is activated. Rac then promotes localized actin filament assembly and the development of membrane protrusions at cell-cell contacts (82, 83). By causing -catenin to be sequestered to the cadherin intracellular domain, the subsequent activation of the Rho GTPase family member RhoA, at the expense of Rac activity, promotes the maturation of N-cadherin-based cell-cell junctions (84, 85). The connection between -catenin and -catenin, which builds up at developing cell-cell junctions and inhibits actin branching, is crucial. Additionally, via actin-binding proteins like cortactin and -actinin, -catenin makes it easier for the N-cadherin-catenin complex to be anchored to the actin cytoskeleton, encouraging the maturation of cell-cell interactions (86, 87). Notably, post-translational changes

of the N-cadherin-catenin complex control the adhesive activity of N-cadherin. For instance, the phosphorylation status of N-cadherin and the accompanying catenins, which is regulated by tyrosine kinases, such as Fer and Src, and the tyrosine phosphatase PTP1B, is strongly dependent on the stability of the N-cadherin-catenin complex (88, 89). Additionally, N-cadherin EC2 and the third extracellular domain's branching N-glycosylation regulate N-cadherin-dependent cell adhesion, at least in part, by regulating the lateral clustering of N-cadherin monomers (90).

1.4. Epithelial-mesenchymal transition (EMT)

The phenotypic change known as epithelial-mesenchymal transition (EMT) is connected to metastasis(91, 92). Developmental biologists initially characterized EMT as a morphological change that took place at particular locations in embryonic epithelia to give rise to distinct migratory cells, but similar findings have been made between developmental EMT and cancer metastasis. Epithelial cells undergo EMT processes in which they acquire mesenchymal characteristics, exhibit decreased intercellular adhesion and increased motility, and are also capable of penetrating the basal membrane and migrating over great distances as a result of significant changes to their cytoskeleton architecture (91, 93, 94).

EMT has been divided into three categories based on the biological context (91, 94, 95). Type 1 EMT includes the development of the embryo. Organ fibrosis, tissue regeneration, and type 2 EMT are all connected. When epithelial cancer cells undergo type 3 EMT, the malignancy progresses and metastasizes. Although the three basic forms of EMT represent very distinct biological processes, several genetic components and regulatory systems have remained relatively untouched. It implies that the embryonic EMT mechanism is hijacked by tumor cells for metastasis. A deeper comprehension of EMT regulation in the metastatic cascade will result in the development of novel targeted therapy methods because metastatic breast cancer is generally

regarded as an incurable illness. Numerous in vitro and in vivo investigations have shown the importance of EMT in breast cancer (96, 97).

Loss of expression of the important epithelial cell-cell adhesion protein E-cadherin is one of the characteristics of EMT. E-cadherin functions as a guardian of the epithelial phenotype by assisting in the formation of epithelial cell sheets and preserving the quiescence of the cells inside them (98). Numerous signaling pathways have been connected to the control of EMT. The Snail/Slug family, Twist, EF1/ZEB1, SIP1/ZEB2, and E12/E47 are a few transcription factors that respond to various microenvironmental stimuli and serve as master molecular switches for the EMT program (99). These transcriptional factors have the ability to connect to the so-called E-Box at the E-cadherin promoter, which will then attract transcriptional corepressors and histone deacetylases for the silencing of E-cadherin (100). The most extensively researched effector of E-cadherin suppression and EMT is the snail. It was initially discovered to play a crucial role during EMT in mammalian cells after being initially identified in Drosophila as a repressor of the transcription of shotgun (an E-cadherin homologue) to govern embryogenesis (101-103). Snail increases the expression of genes linked to a mesenchymal and invasive phenotype16 in addition to repressing the expression of E-cadherin and other epithelial components like as claudins, occludins, and mucin-1. Invasive breast cancer cells with high levels of Snail expression were seen in both epithelial and endothelial cells (99, 104). In individuals with breast cancer, it has been connected to tumor grade, metastasis, recurrence, and a poor prognosis(104-106). Additionally, to coordinate the regulation, Snail family proteins work with other transcription factors, such as Twist and ZEB1 (99).

1.5. Breast cancer research tools: Cell lines

Cell lines serve as models for the study of cancer biology and linking genetic variations to drug response can aid in comprehending the response of cancer patients to treatment (110). To

correlate the genomic and pharmacologic characteristics of cell lines, various massive databases have been produced. The first of these databases was the NCI-60, a pharmacological screening of sixty cancer cell lines (111). Later, genetic characteristics of these cell lines were defined, and CellMiner was populated with all NCI-60-related data (112). Targeted analysis of a panel of breast cancer cell lines has shown the pathways and processes directly influenced by anticancer drugs (113, 114). Additional pharmacogenomics datasets, including the Connectivity Map (115), Genomics of Drug Sensitivity in Cancer (116), the Cancer Cell Line Encyclopedia (117), and the Cancer Therapeutics Response Portal (118) have increased the number of cell lines, drugs, and cancer types. These works have resulted in advancements in our understanding of the cellular response to medications and have provided the data essential to construct prediction algorithms that try to correlate the response with genomic characteristics (110).

Despite the widespread use of cancer cell line models, the same question has persisted since the isolation of the first cell lines in the 1950s: to what extent do in vitro cell line models replicate the biological mechanisms underlying in vivo illness and therapeutic response? Are tumor-derived cell lines representative genomic models of in vivo disease progression and treatment response? In vitro research based on established tumor cell lines continue to serve as the foundation for designing effective in vivo and translational clinical studies (114). Several known breast cancer cell lines, representative of the various mammary tumor subtypes (115), are available and are frequently used to study breast cancer heterogeneity as well as breast cancer susceptibility to various medications or to uncover novel possible pharmacological targets (116). Thus, the availability of additional appropriate cellular models may significantly improve our understanding of the molecular heterogeneity of breast cancer, the discovery of new biomarkers useful for

predicting drug response or for use as drug targets, and our overall understanding of breast cancer (110).

Collections of tumor-derived cell lines are widely employed as models in cancer research because they contain hundreds to thousands of mutations that originated in the tumor from which they were produced. Numerous biological processes are studied using cancer cell lines, which have also been extensively utilized in pharmacogenomics research (110). Recent research by Sharma and colleagues examined the benefits and drawbacks of cell lines as a platform for drug screening (119). Since the publication of this study, genomic measures for hundreds of cancer cell lines have become accessible, and these data present fresh opportunity to correlate genomic profiles to therapy response (110).

1.6. Primary cell cultures for personalized therapy

Massive improvements in the understanding of cancer biology have resulted in a significant investment in drug research in the era of cutting-edge technologies. However, the clinical success of these new target medications has been restricted due to their high toxicity or ineffectiveness. For these reasons, it is essential to develop fresh high-throughput screening models for testing new medications in preclinical research in order to convey the results from the laboratory to the clinic. Since the 1970s, the approach for anticancer drug screening has been refined, and both in vitro and in vivo models have been utilized. For many years, tumor cell lines were the primary drug screening models; nevertheless, they have significant limitations and low clinical predictive ability. Other approaches that more closely match a patient's tumor, such as patient-derived xenografts (PDXs), circulating tumor cells (CTCs), and primary cancer cell cultures, have grown more appealing and effective. PDX is an in vivo model that preserves the majority of the complexity and heterogeneity of the original tumor (120, 121).

However, engraftment efficiency is dependent on the aggressiveness of the tumor and might be affected by the mice model in question. This model's limitations for drug screening and tailored therapy include the lengthy period required for engraftment and cost-effectiveness problems (122-124). CTCs represent a metastatic stage and are appropriate for studying molecular alterations arising from the initial tumor to the metastasis. Poor yield and purity are the primary limitations of using CTCs for individualized therapy at now (125).

A number of primary ovarian cancer cell cultures were created by Kar et al. using the ascitic fluid of the corresponding patients. Ovarian cancer cells that had undergone four to six passages were devoid of fibroblasts and used to test how paclitaxel, carboplatin, and curcumin affected their behavior. Upon treatment with the individual medicines, each primary culture displayed a variable percentage of apoptotic cells; however, combination trials revealed greater efficiency. For instance, the inclusion of curcumin boosted apoptosis to 45% while the combination of carboplatin and paclitaxel improved the average percent from 14% (individual therapies) to 22%. Primary cultures revealed the vast range of responses to particular medications, which likely resulted from various tumors' molecular profiles. This demonstrates how primary ovarian cell cultures have the potential to be used for customized therapy (126).

Kodack et al., attempted to establish primary cancer cell lines for 568 patient tissues representing a range of tumor forms and sources. With a 26% success rate on average, most of the established cell lines belonged to lung cancer. The rate of pleural effusions was higher (42%) than the rate of core biopsies (23%). A low concentration of cancer cells in the patient specimens, followed by stromal fibroblast expansion, was the primary factor in the failure to initiate cell culture. Since fibroblasts often react more quickly to cell detachment, it was proposed that these challenges could be overcome by microscopic monitoring and differential trypsinization.

Additionally, they found out that a culture system containing irradiated fibroblast feeder cells and medium with a specific composition (Dulbecco's Modified Eagle's Medium, F-12, FBS, endothelial growth factor (EGF), insulin, adenine, hydrocortisone, cholera toxin, and Rho-associated protein kinase inhibitor) was more efficient than conventional growth media for producing primary cell lines from needle biopsies (77% Further research revealed that cancer cells of epithelial origin can be recognized by their expression of cytokeratin 8 (CK8) and cytokeratin 18 (CK18), including cells with epithelial to mesenchymal transition. They went on to develop an immunofluorescence assay for cell viability applicable to biopsy cultures in the presence of stromal cells. Breast, bladder, colorectal, and pancreatic cancer cells could all be recognized by CK8/CK18, as well as NSCLC and squamous cell lung carcinoma. The antibody combination could replicate the dose-response curves of pure cancer cells created using an MTS-metabolic viability assay but did not stain human fibroblasts. The same outcomes were observed when irradiated feeder fibroblasts were cocultured with cancer cells. The development of the primary cultures can be slowed down by the requirement to remove EGF and insulin from the medium composition in order to preserve the response to EGFR and ALK tyrosine kinase inhibitors. Tyrosine kinase inhibitor sensitivity of the NSCLC biopsy cultures was discovered to be compatible with the patients' response (127).

1.7. Long-established breast cancer cell lines

In addition to MCF-7, which was generated in 1973 from a pleural effusion at the Michigan Cancer Foundation (128) and is the most extensively used breast cancer cell line in the world, a variety of other cell lines are routinely employed as breast cancer models. The majority of the long-established breast cancer cell lines in use today are not generated from primary breast tumors, but rather from tumor metastases, such as aspirates or pleural effusions (128). This indicates that the vast majority of these cell lines are produced from more aggressive and frequently metastatic

tumors, as opposed to the primary lesion. This is manifestly unrepresentative of the many forms of tumor, which are reflected by the unique types, grades or stages, and progression indicators identified in primary breast cancer. Thus, research based on such lines will be biased toward more rapidly progressing kinds of breast carcinoma and late-stage disease, as opposed to low-grade and early-stage breast malignancies. For these reasons, it would be more clinically meaningful to employ cells that are directly produced from a primary tumor, especially since the majority of pharmacological regimens target these cells (128).

1.8. Recently established breast cancer cell lines

A number of unique breast cancer cell lines have been developed and characterized over the past few years (129-132). Several of these have the benefit of being established from the primary lesion rather than a distant metastasis. The establishment of these new lines has been a time-consuming and patient endeavor, with the majority requiring several months to achieve the accepted standards for a continuous cell line. This includes changed cytomorphology, enhanced growth, decreased serum dependence, increased clonogenicity, a trend toward anchorage-independent growth, ploidy alterations, tumorigenicity in nude mice, and an unlimited lifespan (128).

Two studies have compared the characteristics of newly created breast cancer cell lines to those of the tumor from whence the cell lines were extracted (131, 132). Considerable consistency was obtained with regard to morphology (87%), immunohistochemical examination of estrogen and progesterone receptors (87% and 73%, respectively), HER2/neu (93%), p53 (100%) and allelic loss (82–100%). A related study (133) utilized comparative genomic hybridization to identify recurrent genetic changes in 38 breast cancer cell lines and the degree to which these cell lines resembled uncultured tumors. In addition to more recently created cell lines, this investigation includes some of the cell lines listed in Table 1 that have a lengthy history of use. The most frequent

chromosomal gains were found in 1p, 1q, 3q, 5p, 7p, 7q, 8q, 17q, 20p, and 20p, whereas the most frequent chromosomal losses were observed in 1p, 4p, 8p, 10q, 11q, 18p, 18q, 19p, Xp, and Xq. There was an average of 19 genetic changes, with nine losses and ten gains per cell line, which is 2.5 more per cell line than in tumors; however, the most prominent modifications remained the same.

Several high-level amplifications were also identified in breast tumor cell lines that had previously been described. Although multiple recurring and high-level amplifications were detected in these cell lines, some of which have also been found in uncultured breast malignancies (such as 1q32, 8p11, 8q23, 11q13, 17q23, 17q24, and 20q13), the majority of amplification sites were novel (e.g. 1p13, 7q21, 7q31, 9p23 and 11p13). These may be alterations linked with in vitro culture. Although there were no direct comparisons between chromosomal modifications in traditional and newly developed cell lines, the 10 most strongly elevated genes were detected in three of the former (MCF-7, SKBr3, and ZR75.1wt) and just one of the latter (SUM52). Notably, SUM52 was isolated from pleural effusion rather than a primary breast tumor (134). Another study (135) employed comparative genomic hybridization to discover chromosomal alterations in a panel of eleven novel and well-characterized breast cancer cell lines derived from original tumors. This demonstrated repeated chromosomal gains at 1q, 3q, and 8q. These results align with those found in fresh tumor tissue (136). Thus, these more recently developed cell lines may be more typical of breast cancer as a whole, as they can represent a variety of tumor kinds, grades, and stages. Clearly, these new cell lines are intriguing models, and because some of them have been deposited in reputable cell banks (such as the American Type Culture Collection; http://www.atcc.org), they are now widely accessible to breast cancer researchers (128).

1.9. Primary cell culture

Developing primary cultures obtained directly from tumors is a feasible alternative to employing cell lines, whether they are classic or newly developed. This provides a variety of benefits. Not only are cells isolated directly from the tumor location, but complete pathology is also provided to compare the properties of the culture to those of the original tumor. In general, such cultures can be created either as explants, in which mixed cell populations develop from small tissue fragments, or as enriched populations of specific cell types, the latter of which is preferable (128).

1.10. Explant culture

Early attempts at primary culture of breast tumors were unsuccessful due to the expansion of epithelial cells and stromal fibroblasts, which have been aptly described as "weeds in the tissue culture garden". Rapidly multiplying and outgrowing their slower epithelial neighbors, fibroblasts quickly adapt to in vitro settings. This is a particular issue with explants, thus scientists have resorted to isolating enriched populations of specific cell types to address the issue (128).

1.11. Culture of individual cells

The simplest of these techniques is the spillage approach, which was first described more than four decades ago (128). This entails slicing the tumor and collecting the cells that fall off the sliced surface. McCallum and Lowther (130) have utilized this technique successfully in recent years to produce 10 novel cell lines from 135 unselected primary breast tumors. The majority of other documented procedures rely on enzymatic dissemination of tumor pieces after mechanical disaggregation. A partial enzymatic breakdown of tumor stroma for up to six hours has been described (137), which allows for the enrichment and proliferation of breast epithelial cells in vitro. Approximately 66% of samples had tumor epithelial cells with the ability to proliferate. The same group described a sandwich culture (138) in which dissociated breast tumor cells are sandwiched

between two microscope slides made of glass. The slides are submerged in culture media, which fills the space between them to generate a diffusion gradient for oxygen, nutrients, and metabolic wastes. Under these conditions, only malignant cells can survive, exhibiting the cytokeratin (CK) profile of lumenal epithelia, with CK7, 8, 18, and 19 predominating and CK4 and 5 being absent (138).

Other strategies for cell separation hinge on the varying sedimentation rates of cells of different sizes and involve differential centrifugation. This is performed after enzymatic dispersal of tissue fragments, with collagenase III being the most effective (139). This technique, initially described by Emerman and Wilkinson (140) and updated and enhanced by Burdall et al. (128), is a robust technique for short-term culture of epithelial-enriched cells. This technique permits the multiplication of significant amounts of cells with a specified phenotype for further cell and molecular biology research (141-143). The tissue digest yields three distinct fractions, named organoid, epithelial, and stromal. The organoid fraction is comprised of minute fragments of partially digested tissue, from which cell expansion is detected. Due to the heterogeneous nature of this population, which contains both epithelial and fibroblast cells, the fibroblasts may overrun the epithelial cells if no precautions are taken. This is accomplished by using a well-defined, serum-free baseline medium. As with explant cultures, this percentage has the potential to generate a population of rapidly reproducing genetically normal cells (141, 144). The epithelial fraction is composed primarily of single cells with the characteristic cobblestone appearance of epithelial cells. These cells can be grown selectively with increasing time on selective media and have been described by immunohistochemical, biochemical, and molecular biology techniques, as well as flow cytometry (141, 142). When cultured in conditions supplemented with heat-inactivated fetal bovine serum, the fibroblast fraction produces cells with the bipolar spindle form characteristic of

fibroblasts. These cells have also been characterized using the procedures described above (141-143).

1.12. Pros and cons of breast cancer primary culture

Utilizing enhanced primary cultures offers numerous advantages. In other instances, cells are maintained in culture for a finite period of time, and they have little opportunity to undergo the alterations observed in immortalized cell lines cultured for extended periods. Because the cell–cell interactions that exist in tissue are lost in vitro, it is possible that cells isolated from a breast tumor may react differently in culture compared to their response when they are part of a tissue/organ (128).

Primary culture has constraints. These include delayed population doubling rates and the short lifespan prior to senescence; frequently, only two or three cell passes will survive. For certain experimental approaches, a large number of cells is required, which is sometimes a limiting constraint because this is frequently not possible until after multiple passes. As measured by a rise in initial cell production and the capacity to undergo a greater number of passes without detectable phenotypic change, cells maintained in the later medium appear to have a higher growth rate and longer lifespan (128). Moreover, for certain tumors, such as infiltrating grade I ductal carcinomas and tubular carcinomas, in which mitotic activity is never or rarely observed in whole sections, it is likely that even the use of an enrichment technique will not provide an appropriate in vitro model due to the extended doubling times (128).

Because tumors are heterogeneous, scientists attempting to generate cultures of breast tumor epithelial cells face the possibility of contamination by normal epithelial cells. There is no phenotypic difference between cultivated tumor and normal epithelium, with the former being simple to culture, even from tumors (130). Since tumors are formed from lumenal epithelium, immunopositivity for cytokeratin CK7, 8, 18 and 19 (145) is frequently used to differentiate tumor

from normal. In this regard, CK19 has shown to be very popular (138, 141, 143). However, culture circumstances can selectively impact cell phenotypic, and even normal mammary epithelial cells can express CK19 in vitro (130, 146). Consequently, for tests conducted on low-passage CK19+ cells thought to be tumor cells, other, more strong markers (such as telomerase) are required (147).

1.12.1. Ethical approval and tissue availability

Prior to considering the use of human clinical material for research, it is necessary to address the problem of patient consent, which is now rigorously regulated in the United Kingdom as a result of the Redfern report on organ retention (128). For therapeutic purposes, surgery always removes more tissue than is required for diagnosis; frequently, excess tumor tissue is left over after diagnosis and staging. In the past, it was reasonably easy to collect this material from a cooperative breast surgeon and/or pathologist under the assumption of implicit agreement, but today, explicit patient approval must always be obtained. To ensure the success of this project, the most macroscopically representative portion of the tumor must be obtained with the assistance of an enthusiastic pathologist who is willing to collaborate. Priority must be given to pathology diagnosis, and as a result, scientists are frequently left with decreasing amounts of tumor for investigation. This is not a concern, however, because viable tumor cells may be successfully identified and amplified from a fine needle aspirate (148).

1.13. Novel applications of primary culture for translational research

There is a massive pressure on scientists to limit or eliminate the use of animals in laboratory research, and primary cell culture may be a viable alternative, particularly in preclinical drug testing. Predicting how patients will respond to chemotherapy treatments is another hot topic. In the latest extreme drug resistance experiment, live tumor specimens are cultured in agarose for five days in the presence of chemotherapeutic medicines (149). In the final 48 hours, tritiated thymidine is added, and liquid scintillation counting determines the amount of radioactive label

that has been integrated (indicative of cell proliferation). This technique was recently tested on breast tumors. The approach was most effective when tumors were greater than 1 g, of high grade, from younger patients, and lacking progesterone receptor expression (150). Overall, 70% of analyzed samples offered information on chemotherapy resistance, suggesting that in vitro results could be used to guide the selection of the most appropriate adjuvant therapy (128).

A comparable method is the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium (MTT) assay, in which dissected tumor samples of approximately 1mm are cultured with chemotherapeutic medicines at concentrations intended to replicate the plasma levels attained in vivo (151). On the basis of the MTT-to-formazan product conversion, the 50% inhibitory concentration is then estimated. With the inevitable development of tumor-specific medications that are tailored to patient requirements, appropriateness, and gene profile, breast cancer will be viewed in the future as a patient-specific entity rather than a single disease. Conventionally, tumor-derived cell lines are obtained using immortalization methods, which may result in the selection of subpopulations with changed phenotypes that are no longer indicative of the original cancer tissue (152).

Ex vivo culture of fresh patient tumor tissue or establishment of patient-derived xenografts (PDX) in mice yields models that closely reflect the heterogeneity and medication response of patient tumors (153). Previously, a group of scientists have developed several breast cancer PDX models using primary breast cancer patient tumors obtained through the BEAUTY (154, 155). Using these PDX models has allowed for coming up with novel pharmacogenomic biomarkers associated with a variety of treatments (155, 156). However, the maintenance costs and lengthy turnaround time required to manufacture PDX restrict their widespread application in laboratory settings. In contrast, the generation of immortalized primary breast cancer cell lines from PDX

tumors could provide relatively inexpensive tools for mechanistic investigations and the elucidation of alternative therapeutics (157).

The procedure of acquiring immortalized cancer cell lines is intricate and unpredictable (158, 159). The capacity of cancer cells to proliferate on plastic tissue culture plates depends on cancer cell histology, tumor grade, the presence of particular genetic abnormalities, and the provision of adequate nutrition (153). The extensively researched human breast cancer cell lines (MCF-7, T47D, MDA-MB-231, and SKBR-3) were derived from metastatic lesions, making it less probable that they precisely reflect the genetic makeup or biological behavior of original breast tumors (4). While others have successfully produced cell lines from tumors (127, 160), BEAUTY patients only provided biopsies, restricting the number of tumors for in vitro subculture. Cavotelli et al. and Matossian et al. have previously established the benefit of using in vivo methods to create immortalized cell lines (161-163). They hypothesized that sub-culturing tumor biopsies in vivo not only increases the number of tumor cells, but also allows tumors to adapt to their surrounding milieu, ensuring proliferative proliferation and simple passage—traits important to the immortalization of tumor cells (163). When PDX tumors were therefore cultured in a single-cell solution, immortalized cell lines were produced. These instances give the basis for investigating if immortalized cell lines can be derived from primary breast cancer PDX tumors.

Scientists have spent decades attempting to create techniques for growing and analyzing primary cancers and normal cells outside the human body (164, 165). Traditional established cell lines have formed the cornerstone of cell, molecular, and cancer biology until this point. However, the poor success rate (1–10 percent, depending on the tissue of origin and stage of disease progression) impedes the formation of tumor cell lines (166, 167). In addition, the complex heterogeneity of primary tumors is frequently absent from these cell lines, and normal cell lines

from many organs do not exist, restricting the use of these cultures for predicting tumor cell responses (168). Tumor heterogeneity is detectable at both the microscopic (immunohistochemistry) and genetic (DNA mutations and/or RNA expression alterations) levels. Genetic study of original tumor samples has uncovered additional differences within tumor groupings and even the relationship between cancers originating from other tissues (169). Thus, genetic analysis is the foundation of predicting tumor biology and therapy response. Despite the immense promise for using genomic analysis as the basis for patient treatment, it is evident that in vitro and/or quick ex vivo models are the best way to analyze the interplay of genetics, epigenetics, signaling changes, and cell–cell interactions (170).

1.14. Development of the protocol for establishing patient-derived breast cancer cell lines

In contrast to other model systems, this procedure has been shown to facilitate the establishment of patient-derived CR cell cultures from both normal and cancerous tissues that are capable of growing indefinitely without genetic manipulation (171-173). Figure 1.2 summerizes the procedure through which we propagated the patient-derived breast cancer cell lines, emphasizing the tumor dissociation and CR culture approaches. To proliferate epithelial cells, this method employs irradiation mouse fibroblast cells and the Rho-associated kinase (ROCK) inhibitor (Y-27632). Y-27632 was initially found in a caspase/kinase inhibitor library as having the potential to increase the cloning efficiency of human embryonic stem (ES) cells (174) and, in subsequent research, as having the ability to increase the viability of human keratinocyte stem cells (175). When introduced to Green11 keratinocyte/feeder cocultures, Y-27632 stimulated indefinite cell proliferation (171). Unexpectedly, it was found that feeder cells and Y-27632 can be employed to produce normal and tumor cell cultures in non-keratinocyte tissues (170). It has also been observed that the capacity of feeders and Y-27632 to cause infinite cell proliferation is comparable

to the immortalizing capacity of the HPV-16 E6 and E7 oncogenes. E6 and feeder cells both activate telomerase (172), whereas E7 and Y-27632 both damage the actin cytoskeleton (176) and inactivate Rho (177). The effects of Y-27632 are totally reversible (173), in that CR cultures cease to proliferate or differentiate terminally upon its removal, depending on the culture conditions.

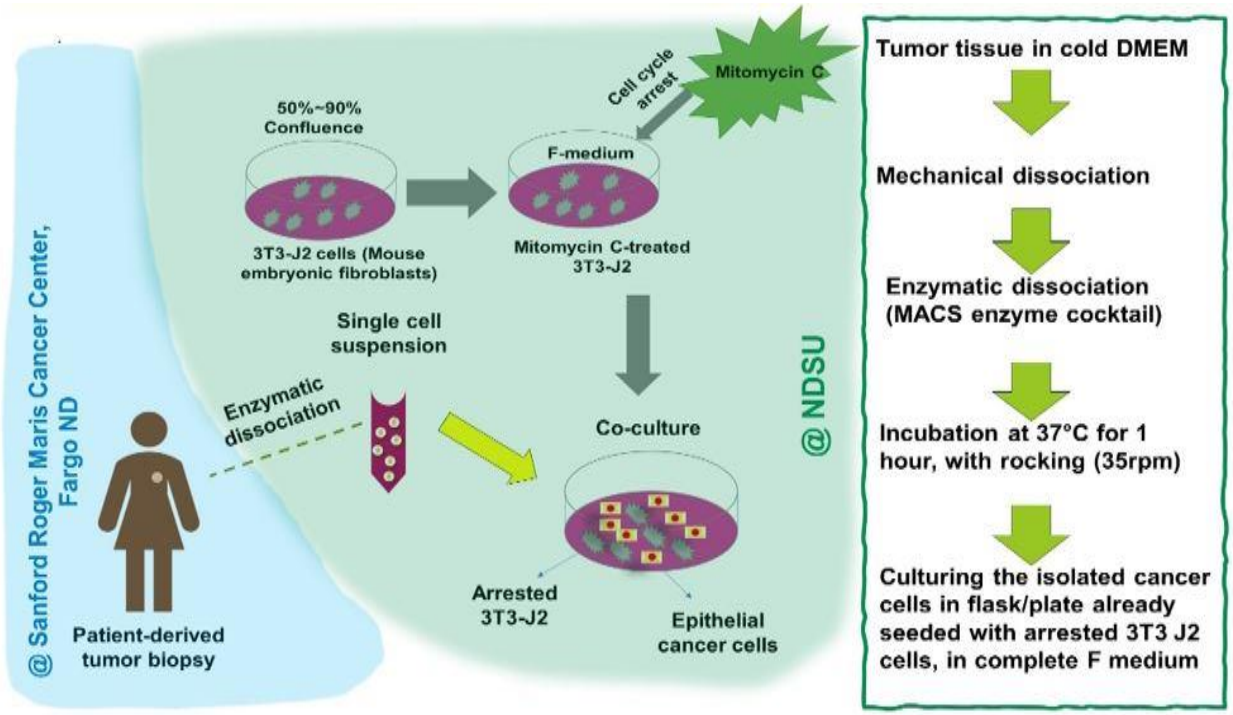

Figure 1.2. Workflow of patient-derived breast cancer cell line establishment. This workflow is based on the Conditional reprogramming technology approach for cell line establishment, in which feeder layer cells are used in co-culture with isolated breast cancer cells in F-media

The rapid induction of CR (within 2 days) is due to reprogramming of the cell population rather than clonal selection (173), as is the case with normal cell lines. In contrast to embryonic stem (ES) and induced pluripotent stem (iPS) cells, CR cells from normal tissue do not exhibit large quantities of Sox2, Oct4, Nanog, or Klf4 (173) and do not generate teratomas in mice (172). In addition, CR cells retain their developmental potential and do not require intricate manipulation to differentiate into their originating tissue (171-173). In tumor CR cell cultures, the phenotypic and genotypic characteristics of the source tumor are preserved (172), and the approach was

recently employed to discover an acceptable therapy for pulmonary papillomatosis (178). As stated by independent laboratories, the CR approach can be used for live biobanking (172), fundamental research (179-184), diagnostic (185, 186), therapeutic (184, 187, 188), and regenerative medicine (184, 189, 190) applications.

CHAPTER 2: ESTABLISHMENT AND CHARACTERIZATION OF PATIENT-DERIVED BREAST CANCER CELL LINES IN 2D CULTURE SYSTEM

2.1. Abstract

Commercial cancer cell lines have long been extensively used as a an important platform to study cancer. They have contributed to a plethora of discoveries in the field of cancer research. However, there are limitations with using these cell lines, such as induced mutations over the long-term in vitro culture. These mutations cause incorrect exhibition of the in vivo characteristics of the cancer cells. Here, we focused on establishing Patient-derived breast cancer cell lines and attempted to characterize them in terms of several biomarkers that are shown to be overexpressed in breast cancer cells. Patient-derived breast cancer cell lines are more reliable tools to study the molecular and cellular processes taking place in vivo, since they are freshly isolated from the tumor biopsy and do not undergo induced immortalization. We explored the CK19, Ki67, vimentin, EpCAM, E-cadherin, and N-cadherin expression in three successfully established patient-derived breast cancer cell lines.

2.2. Introduction

Commercial cancer cell lines have been utilized for a long time in cancer research. One of the key advantages of using cultured cell lines in cancer research is that they provide an infinite supply of a relatively homogeneous, self-replicating cell population in standard cell culture medium (191). However, the long-term culture of these cell lines has led to the acquisition of genetic aberrations which makes them unable to mimic the actual condition that happens in vivo. Many efforts have been made to develop efficiently in vitro culture for a wide variety of cancers, including breast cancer (BC).

The use of 2D cell lines derived from a patient's own tumor has a positive economic outlook and reduces the excessive use of animal models (192). Given that the transcriptomic and

proteomic profiles correspond to the behavior of patient tumors, the use of well-characterized cell lines could facilitate a faster and more efficient screening of drugs (192). The primary limitations, however, include the fact that these 2D cell cultures do not depict or adhere to a number of important cancer biology factors, such as the stromal and matrix components of cancer, among other microenvironment factors (193). Specifically, in the case of generic cell lines, a number of studies have demonstrated that established cancer cell lines contain a greater number of genetic mutations and abnormalities than patient-derived tumors. Developing an efficient and stable 2D culture of patient-derived cancer cells is challenging and needs a lot of effort and time. In addition, in some cases, cancer cells undergo genetic changes due to long-term culture in 2D setting.

The use of patient-derived cell lines is the core component in cancer research, from the biological understanding of cancer cells to the development of new treatments for this disease. Patient-derived cancer models, like cell lines, organoids, and xenografts, are the main elements in cancer research. They allow the screening of the cancer-related gene function and different pathways and validation effectiveness of drugs in cancer treatments. These cells were derived from cancer patients and have been adapted to grow in vitro. The patient-derived biopsied tumor tissues are applied in monolayer (2D culture) for the identification and characterization of cell lines.

Historically, culturing cancer cells from solid tumors has neither been swift nor straightforward. In addition to this difficulty, patients presenting with metastatic disease frequently undergo diagnostic needle biopsies as opposed to surgical resection, and the biopsied material may be sparse. Recent work by Dr. Richard Schlegel and colleagues (170, 172, 173) has established conditions that enable robust and, at times, otherwise unattainable efficiency in culturing cancer cells from surgical or biopsy specimens. Schlegel and colleagues reported "conditional reprogramming" as a method to generate chemo-sensitive cell cultures from normal and malignant

recurrent respiratory papillomatosis cells (178). The chemotherapy that was determined to be the most effective in vitro had a long-lasting cytostatic effect on the patient.

In this recent study, we focused on the establishment and characterization of three BC patient-derived cell lines in monolayer culture and attempted to evaluate the efficacy of monolayer culture system in developing efficient, stable patient-derived breast cancer cell lines.

2.3. Methods

2.3.1. Tumor dissociation

Fresh surgical biopsy samples were collected from breast cancer patients admitted in Sanford hospital. Tumor tissues were transported to the laboratory in cooled-DMEM and immediately dissociated into single cell suspension using both mechanical and enzymatic dissociation. Briefly, mechanical dissociation was done by mincing the tissue via scalpel, followed by mechanical digestion using tumor dissociation kit, human (miltenyi biotec, cat no. 130-095-929), with an incubation time of 1 hour at 37°C with gentle rocking (35rpm). The tube containing single cells and tissue debris was then centrifuged at 200rcf for 5min, aiming to remove the digestion media including enzymes. Single cells were then cultured on 3T3 J2 cells (Mouse embryonic fibroblasts, Kerafast, cat no. EF3003) which were already mitotically arrested by 10µg/ml Mitomycin C (Sigma-Aldrich, cat no. M4287) and seeded in the flask one day before tumor dissociation. F-media, consisted of complete DMEM+ F-12 nutrient mix supplemented by 5 µg/ml insulin; 250 ng/ml amphotericin B, 10 µg/ml gentamicin, 0.1 nM cholera toxin, 0.125 ng/ml EGF, 25 ng/ml hydrocortisone, and 10µM ROCK inhibitor, was used to coculture the isolated tumor cells and feeder layer cells. Cells were incubated at 37°C until confluence, with media change every other day.

2.3.2. Arresting Swiss 3T3 J2 cells (MEFs)

Mitomycin C (Sigma-Aldrich, Cat no. M5353) was used in a concentration of 10µg/ml for 2h at 37°C to arrest MEFs that were grown in a flask and had reached 70% confluency. After treatment with mitomycin C, cells were rinsed 4-5 times with PBS to eliminate the residual Mitomycin C. MEFs were then trypsinized and used for the co-culture with cancer cells in a seeding density of $10^4/cm_2$ surface area.

2.3.3. Trypsinization and maintenance of the culture

Once the flask of cancer cells co-cultured with MEFs reached 70-90% confluency, the MEFs were partially removed by differential trypsinization. Cells were first trypsinized by adding 0.025% trypsin-EDTA solution with incubation at room temperature for 3 minutes and observing the MEF cells detachment under the microscope. Once a significant number of MEFs were detached, the trypsin solution was removed and the flask (which mostly contained the cancer cells) was rinsed with PBS. The cancer cells in early passages were detached from the flask by using 0.25% trypsin-EDTA. After the centrifugation and resuspension of cancer cells, they were seeded in a new flask with arrested MEFs already cultured. The split ratio for maintaining cultures was 1:3 dilution and the seeding density for MEFs in the co-culture was 10^4 cells/cm^2 surface area.

2.3.4. Immunocytochemistry

The expression of cytokeratin 19, EpCAM, E cadherin (as epithelial cell markers), Ki-67, vimentin and N cadherin (as EMT markers) were explored in established breast cancer through immunocytochemistry. Table 2.1 lists all the studied markers with their function as well as their location in the cells. Cells were seeded on gelatin-coated round coverslips in 24-well plate and incubated at 37°C for 24 hours for cell attachment. Fixation was done using 4% PFA (15 min at 4°C, followed by permeabilization by 0.1% Triton X-100 (10 min at room temperature). Two-hour

incubation in 5% BSA was done to block the unspecific regions. Primary antibodies were Anti-cytokeratin 19 antibody ((A53-B/A2): sc-6278, Santa Cruz biotechnology), CD326 (EpCAM) mouse anti-human (Clone: 1B7, eBiosience), Ki-67 Recombinant Rabbit Monoclonal Antibody (Invitrogen, SP6), and Vimentin (D21H3) XP Rabbit mAb. Following overnight incubation with primary antibody, Alexa fluor 546 goat anti-mouse IgG (A11003, Invitrogen) and Cy5 goat anti-rabbit (A10523, invitrogen) were used as secondary antibodies depending on the host species of primary antibodies. After 1 hour incubation, coverslips were transferred onto slides and mounted using vectashield antifade mounting media with DAPI (H-1200, Vector laboratories) and imaged using fluorescent microscopy.

Table 2.1. The list of different breast cancer biomarkers tested in this study. The function of each marker as well as their location in breast cancer cells.

Marker	Function	Location
CK19	Associated with aggressiveness in cancer cells	Cytoskeleton marker, cytoplasm
Vimentin	Associated with increased migration/invasion of cancer cells	Cytoplasm
EpCAM	Cell-cell adhesion, stimulating or inhibiting diverse cancer signaling pathways	Cell surface
Ki67	Involved in cell proliferation	Nucleus
N-cadherin	Promotes tumor cell survival, migration and invasion	Cell surface
E-cadherin	Cell adhesion, affect tumor invasion by inhibiting cell migration away from the epithelial layer	Cell surface

2.3.5. Quantitative Real-time polymerase chain reactions analysis

Quantitative Real-time PCR was done to explore the relative gene expression of two breast cancer cells markes, CDH1 (E-cadherin) and CDH2 (N-cadherin). The RNA extraction was performed using the Direct-zol RNA MicroPrep Kits (Zymo Research, Irvine, California) according to the manufacturer's protocol. Complementary DNA was produced using the cDNA Synthesis Kit (Thermo Fisher Scientific) following manufacturer's instructions. qRT-PCR was performed using the PowerUp™ SYBR™ Green Master Mix (Applied Biosystems, Darmstadt, Germany) and the QuantStudio 3 (Applied Biosystems) to determine the expression levels of target genes, shown in Table 2.2.

Table 2.2. Primers used for qPCR

Gene of interest	Primer name	Sequence 5′ - 3′
E-Cadherin	huCDH1 for	CCAAGTGCCTGCTTTTGATGA
	huCDH1 rev	CCCCTACCCCTCAACTAAC
N-Cadherin	huCDH2 for	GACAATGCCCCTCAAGTGTT
	huCDH2 rev	CCATTAAGCCGAGTGATGGT

2.3.6. Estrogen treatment

During the establishment of patient-derived breast cancer cell lines, we noticed that the NT013 cells stopped growing in passage 5. We decided to treat these cells with 1nM estrogen. The rationale behind this is that the NT013 cell line is hormone receptor positive/HER-2 negative, and treatment with estrogen could result in the increased cancer cell's proliferation in this molecular type. Treatment begun at passage 5 and continued for 2 passages.

2.3.7. Statistical analysis

In order to analyze the immunostaining data, the number of cancer cells that were positive for the marker of target were counted for 5 images and devided by the total cell number to obtain

the percentage of positive cells. Data were then statistically analyzed via One-way Anova test using GraphPad Prism v.9. For qPCR, we used triplicate and data were analyzed via One-way Anova test using GraphPad Prism v.9. P-value$\leq$0.05 was considered as statistically significant.

2.4. Results

In this study, we attempted to establish and characterize the patient-derived breast cancer cell lines using conditional reprogramming technology. List of patient samples received from Sanford hospital is provided in Table 2.3. Participants of the study were assigned a unique code maintained by Sanford research team. No identification information was shared outside of Sanford. Our research team at NDSU just received the coded tussue along with the unique code. We optimized the tumor dissociation protocol using tumor dissociation kit, human (Miltenyi biotec, Cat no. 130-095-929) which included various enzymes required for obtaining single cell suspension. After dissociation, F-media and Swiss 3T3 J2 cells (as feeder layer cells) were utilized to support the growth and survival of breast cancer cells. After expanding the cultures, these cultures were tested for the presence of key breast cancer biomarkers such as CK19, EpCAM, vimentin, Ki67, E-cadherin, and N-cadherin, to verify the successful establishment of the cancer cell lines.

Table 2.3. List of patient tumor biopsies. Fresh tumor tissues were collected from Sanford hospital after careful histological evaluation and were transferred to the lab in transport media (DMEM 10% FBS, 1X P/S)

Sample ID	Molecular type	Success/failure
NT005	Hormone receptors positive, HER-2 negative	Ongoing expansion of the cells
NT 008	Hormone receptors positive, HER-2 negative	Failure in establishing the cell line. Cells underwent senescence
NT 013	Hormone receptors positive, HER-2 negative	Successfully established
NT 015	Hormone receptors positive, HER-2 negative	Successfully established
Sample ID	Molecular type	Success/failure
NT 017	Hormone receptors positive, HER-2 negative	Ongoing culture
NT 021	Hormone receptors negative, HER-2 positive	Failure in establishment of a cell line, small sample size
NT 023	Hormone receptors negative, HER2 negative (TNBC)	Successfully established
NT028	Triple-negative breast cancer	Failure in establishment of a cell line
NT032	Hormone receptors negative, HER-2 positive	failure in establishment of a cell line
NT040	Her2 positive, hormone receptor negative	Very small sample size, failure in establishment of a cell line

2.4.1. Immunocytochemistry for CK19 and vimentin detection in established cell lines

Cells were seeded on coverslips in 24-well cell culture plates using the F-meda (for NT013, NT015, and NT023) and complete DMEM(for MDA-MB 231, MCF-7, and BJ fibroblasts) and incubated for 24h in 37℃. Cells were then washed with PBS, fixed with 4% PFA in PBS, and permeabilized using 0.1% Triton X-100 in PBS. There were three-times washing steps between these treatments. Cells were then blocked using 5% BSA in PBS for 1h and then double-immunostained with anti-cytokeratin 19 and anti-vimentin IgG for 1h, followed by washing with PBS and incubation with secondary antibodies for 1h. Finally, the cells were mounted on microscope slides and DAPI solution was used to stain the nuclei. Imaging was done using

fluorescent microscopy. This experiment was done in triplicate and 5 images were taken for each coverslip (i.e. there were 15 images to check the target molecule in each cell line). Data quantification was done as follow (example: Ck19):

$$\%\ \text{of CK19 expressing cells} = \frac{\text{the average of total CK19 expressing cells for all images}}{\text{the average of total number of cells for all images}}$$

To count the number of cells, we counted the number of nuclei stained with DAPI, which represented the presence of cells.

Figure 2.1 shows the CK19 and vimentin staining results for the established cell lines. Data showed that NT013 cells expressed CK19. However, this expression was significantly high in a percentage of cells, while the rest of cells expressed a lower level of CK19. Interestingly, cells with high CK19 expression showed a low vimentin expression, while cells with low CK19 expression had a high level of vimentin expression. A similar pattern of CK19 and vimentin was observed for NT015 cell line. For NT023 cell line, all the cells weakly expressed CK19, while there was a higher expression of vimentin. MDA-MB 231 (triple-negative breast cancer cell line) and MCF-7 (HR+ breast cancer cell line) cell lines were used as CK19-positive controls, and Bj fibroblasts (normal human fibroblasts from foreskin) was used as CK19-negative control. For vimentin, MDA-MB 231 and Bj fibroblasts were considered as positive control, while MCF-7 cells were considered as vimentin-negative control.

A

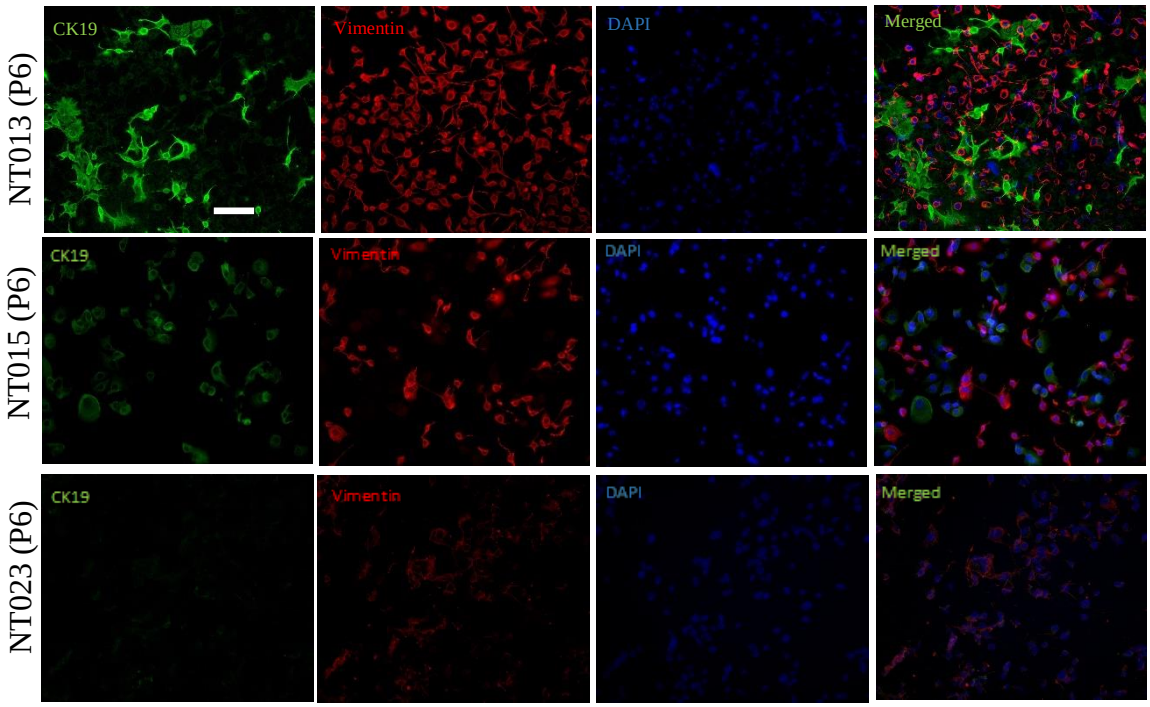

Figure 2.1. A) The expression status of CK19 and vimentin in the established patient-derived breast cancer cell lines using double-immunocytochemistry (Scale bar: 100µm). B) quantitative analysis of CK19 and vimentin expression in established breast cancer cell lines. As shown, all three cell lines expressed CK19. However, the NT023 weakly expressed CK19. Regarding vimentin, NT023 cells significantly expressed high levels of vimentin, and NT013 cells expressed higher level of vimentin than NT015 cells. For CK19, MDA-MB 231 and MCF-7 cell lines were considered as positive control, and Bj fibroblasts considered as negative control. For vimentin, MDA-MB 231 and Bj fibroblasts were considered as positive control and MCF-7 cells were considered as negative control. (One-way Anova test, P-value≤0.05 considered as statistically significant).

A (Continued)

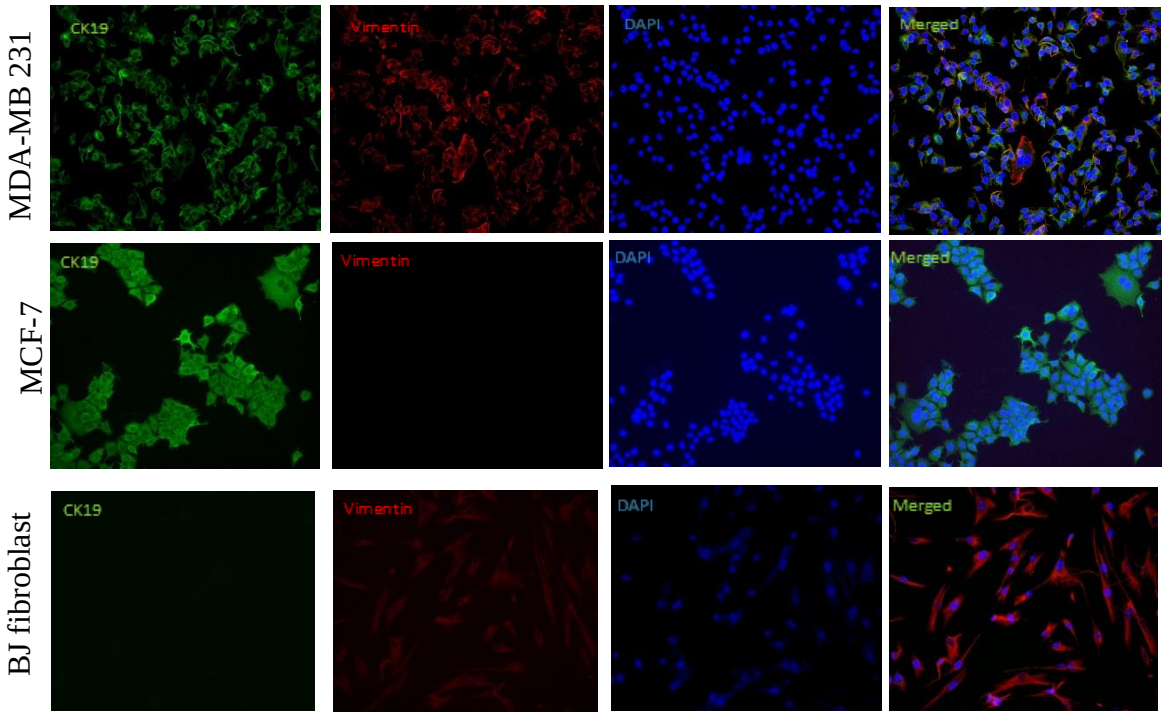

Figure 2.1. A) The expression status of CK19 and vimentin in the established patient-derived breast cancer cell lines using double-immunocytochemistry (Scale bar: 100µm). B) quantitative analysis of CK19 and vimentin expression in established breast cancer cell lines (Continued). As shown, all three cell lines expressed CK19. However, the NT023 weakly expressed CK19. Regarding vimentin, NT023 cells significantly expressed high levels of vimentin, and NT013 cells expressed higher level of vimentin than NT015 cells. For CK19, MDA-MB 231 and MCF-7 cell lines were considered as positive control, and Bj fibroblasts considered as negative control. For vimentin, MDA-MB 231 and Bj fibroblasts were considered as positive control and MCF-7 cells were considered as negative control. (One-way Anova test, P-value≤0.05 considered as statistically significant).

B

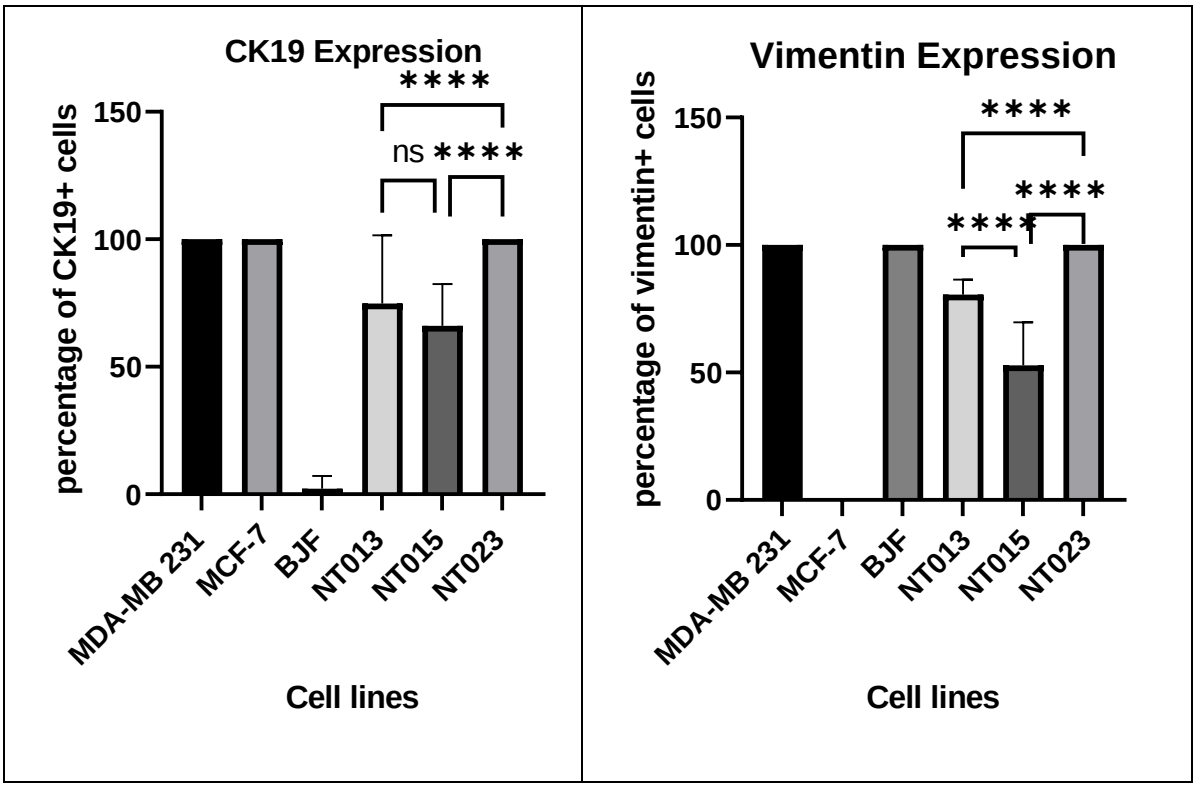

Figure 2.1. A) The expression status of CK19 and vimentin in the established patient-derived breast cancer cell lines using double-immunocytochemistry (Scale bar: 100µm) (continued). B) quantitative analysis of CK19 and vimentin expression in established breast cancer cell lines. As shown, all three cell lines expressed CK19. However, the NT023 weakly expressed CK19. Regarding vimentin, NT023 cells significantly expressed high levels of vimentin, and NT013 cells expressed higher level of vimentin than NT015 cells. For CK19, MDA-MB 231 and MCF-7 cell lines were considered as positive control, and Bj fibroblasts considered as negative control. For vimentin, MDA-MB 231 and Bj fibroblasts were considered as positive control and MCF-7 cells were considered as negative control. (One-way Anova test, P-value≤0.05 considered as statistically significant).

2.4.2. Weak CK19 and vimentin expression in a portion of NT013 and NT015 cell lines

Our data revealed that the status of CK19 and vimentin expression, in NT013 and to some extent in NT015 cell line, was as follows: High CK19/low vimentin and low CK19/high vimentin; which was interesting. Results also demonstrated that there was a significantly higher weak expression of CK19 in NT013 cells compared to strong CK19 expression (p-value≤0.05). On the other hand, there were significantly more cells expressing high level of vimentin than those

expressing low vimentin (p-value≤0.05). The figure 2.2 shows the quantification data for CK19 vs. vimentin expression level in NT013 cell line.

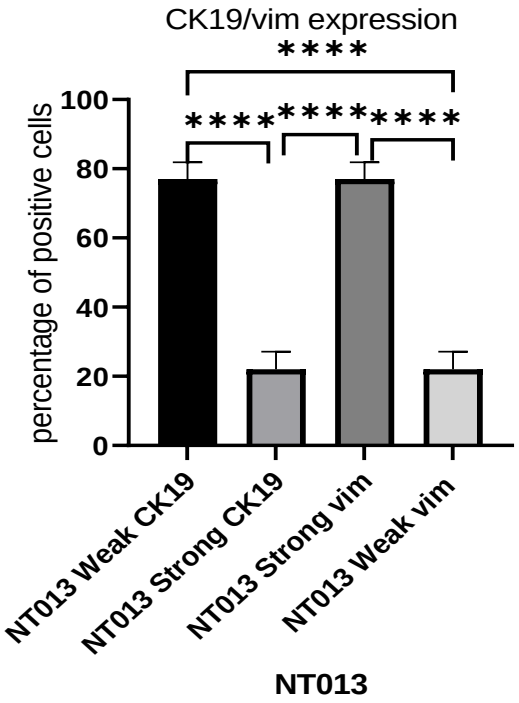

Figure 2.2. quantitative analysis of CK19&vimentin expression in two levels (strong vs. weak) based on fluorescence intensity in NT013 cell line. As shown, there were significant number of NT013 cells with weak CK19 expression than those with strong CK19 expression. Also, there were significantly higher number of NT013 cells with strong vimentin expression than those with weak vimentin expression (One-way Anova, p-value≤0.05 was considered as statistically significant

2.4.3. EpCAM and Ki67 expression status of the established cell lines

Cells were seeded on round coverslips in a 12-well cell culture plate in the corresponding media mentioned above. Similar staining protocol was used, with anti-human EpCAM mouse antibody and anti-human Ki67 rabbit antibody used to detect EpCAM and Ki67 molecules in the cell lines, respectively. Figure 2.3 shows the staining images and quantification data for EpCAM and Ki67 expression in the established cell lines. Based on the staining results, EpCAM was not expressed in NT023 cell line, which was consistent with our finding about the corresponding control cell line, MDA-MB 231 cell line. However, there was a low EpCAM expression especially

in NT013 cell line, and NT015 cells EpCAM expression was significanly higher than that in NT013 cells (p-value<0.05). Since these two cell lines were hormone receptors-positive, HER2 negative; their EpCAM expression was compared with that of MCF-7 cell line, and we found that unlike MCF-7 cells, not all NT013 and NT015 cells expressed EpCAM on their surface. For Ki67 expression, data showed that all the NT023 cells expressed this marker, which was consistent with the expression in MDA-MB 231 cells. This reflects the high proliferative feature of triple-negative cell lines. NT023 cells showed a significantly higher Ki67 expression compared to NT013 and NT015 cells (p-value<0.05). Also, NT013 cells expressed a significanly higher Ki67 than NT015 cell line (p-value<0.05).

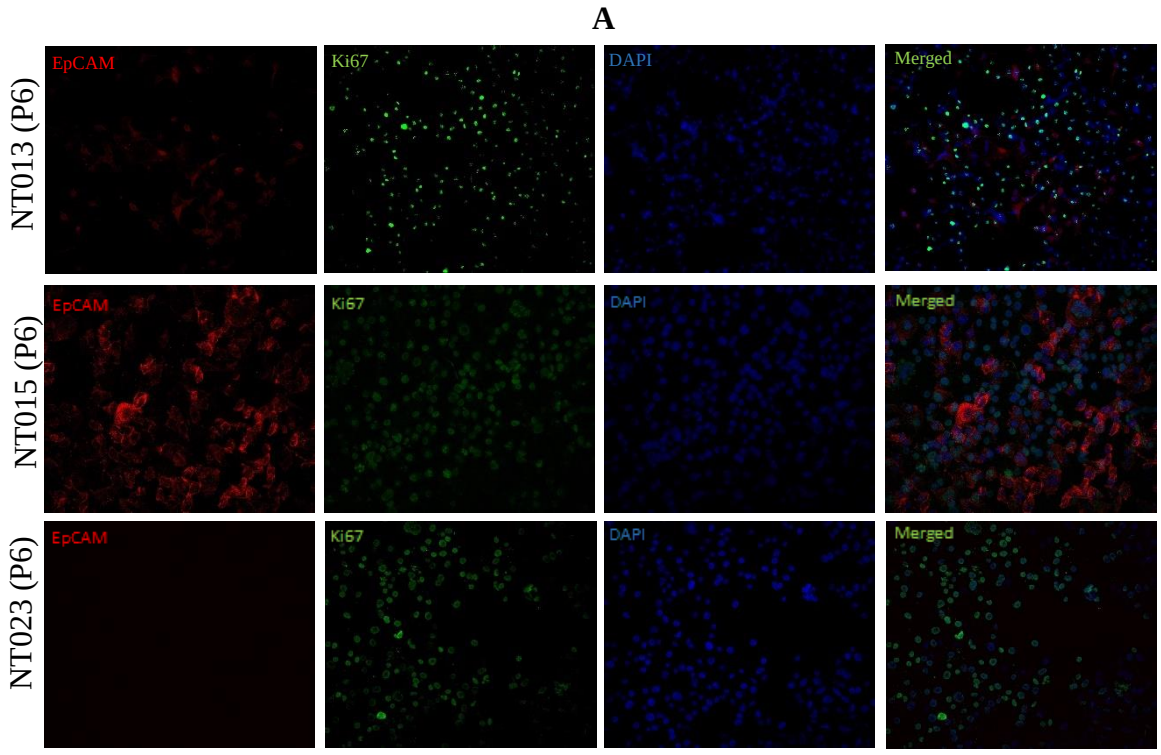

Figure 2.3. The expression status of two breast cancer biomarkers in the established patient-derived breast cancer cell lines using immunocytochemistry. A&B) EpCAM/Ki67 double staining. NT023 cells showed no EpCAM expression, while a population of NT013 and NT015 cell lines significantly expressed EpCAM. All the cell lines showed Ki67 expression (Scale bar: 100µm). B) quantitative analysis of the EpCAM and Ki67 epression in established breast cancer cell lines. There was a significantly higher EpCAM expression in NT015 compared to NT013 cell line. As expected, NT023 cell line showed no EpCAM expression. NT023 cells highly expressed Ki67, and NT013 cells expressed significantly higher Ki67 than NT015 cell line (One-way Anova test, P-value≤0.05 considered as statistically significant).

B

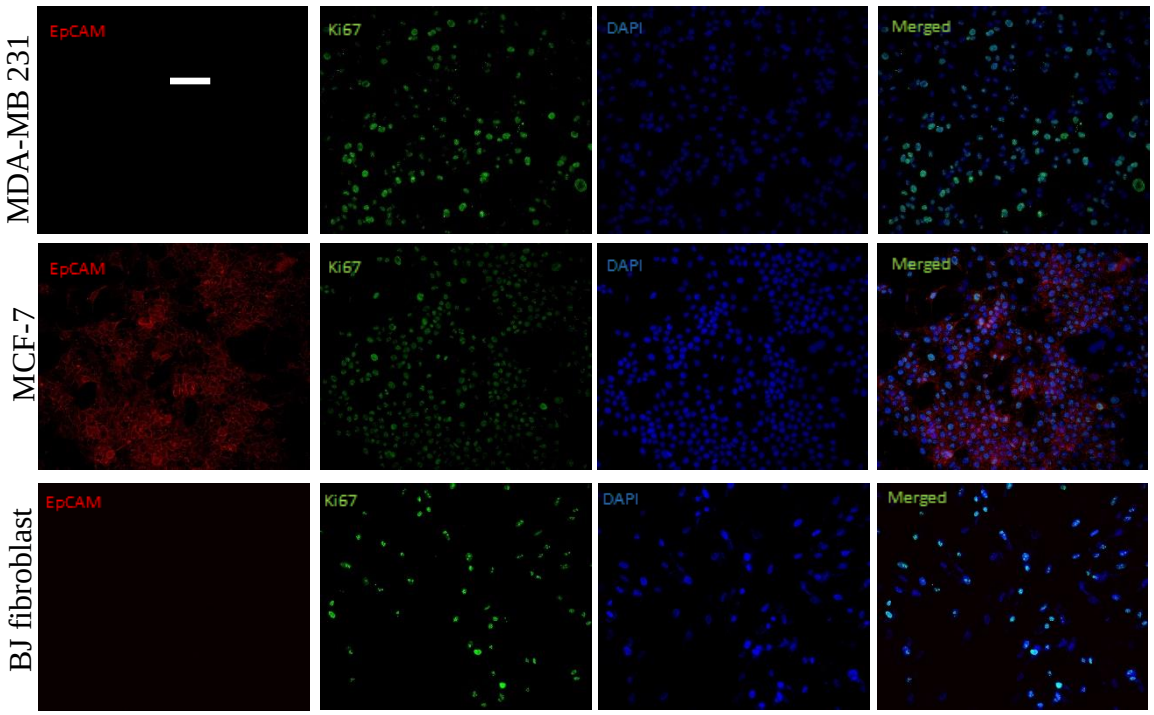

Figure 2.3. The expression status of two breast cancer biomarkers in the established patient-derived breast cancer cell lines using immunocytochemistry (continued). A&B) EpCAM/Ki67 double staining. NT023 cells showed no EpCAM expression, while a population of NT013 and NT015 cell lines significantly expressed EpCAM. All the cell lines showed Ki67 expression (Scale bar: 100µm). B) quantitative analysis of the EpCAM and Ki67 epression in established breast cancer cell lines. There was a significantly higher EpCAM expression in NT015 compared to NT013 cell line. As expected, NT023 cell line showed no EpCAM expression. NT023 cells highly expressed Ki67, and NT013 cells expressed significantly higher Ki67 than NT015 cell line (One-way Anova test, P-value≤0.05 considered as statistically significant).

C

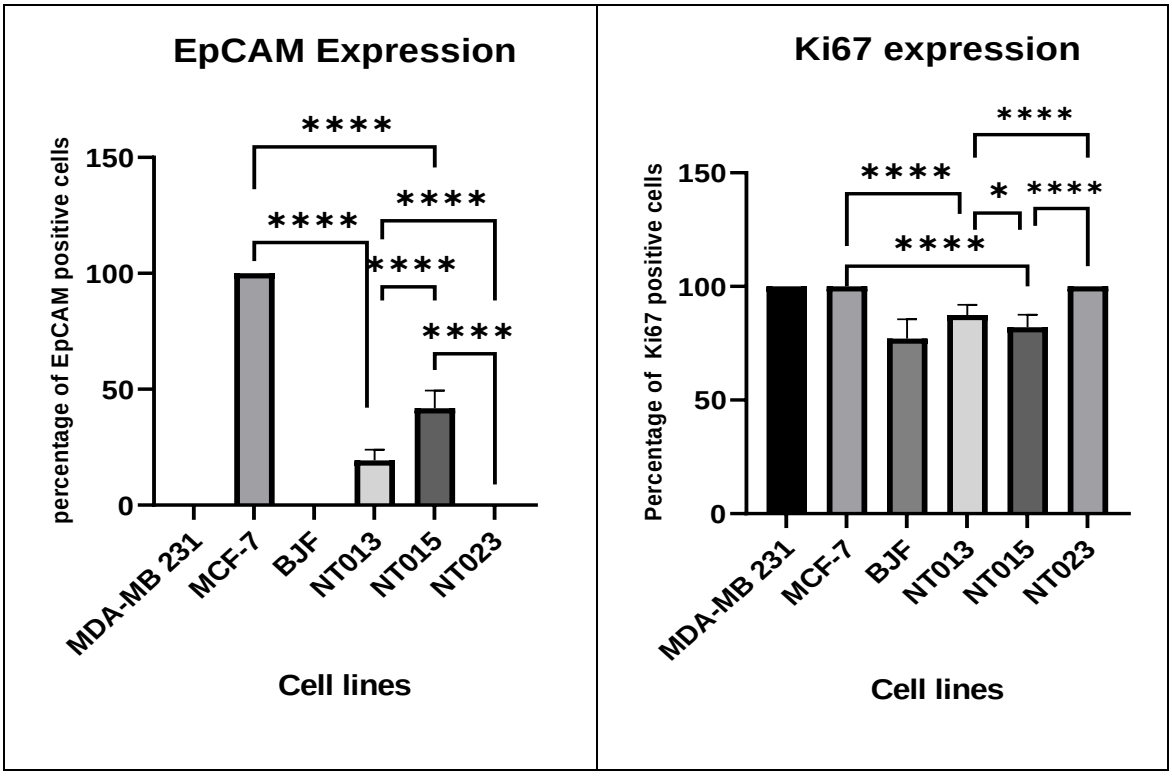

Figure 2.3. The expression status of two breast cancer biomarkers in the established patient-derived breast cancer cell lines using immunocytochemistry (continued). A&B) EpCAM/Ki67 double staining. NT023 cells showed no EpCAM expression, while a population of NT013 and NT015 cell lines significantly expressed EpCAM. All the cell lines showed Ki67 expression (Scale bar: 100µm). B) quantitative analysis of the EpCAM and Ki67 epression in established breast cancer cell lines. There was a significantly higher EpCAM expression in NT015 compared to NT013 cell line. As expected, NT023 cell line showed no EpCAM expression. NT023 cells highly expressed Ki67, and NT013 cells expressed significantly higher Ki67 than NT015 cell line (One-way Anova test, P-value≤0.05 considered as statistically significant).

2.4.4. E-cadherin and N-cadherin expression status of the established cell lines

In order to check the E-cadherin and N-cadherin expression levels, q-PCR was conducted. Cells were seeded in 6-well plates and after reaching 90% confluency, were trizoled to lyse the cells and release RNA. RNA extraction was done and the concentration and quality of the extracted RNA was checked using nanodrop. After C-DNA synthesis, the concentration and quality of C-DNA was also checked by nanodrop. Q-PCR was done using 200ng concentration of the C-DNA

for each sample. This experiment was done in both biological and technical triplicates. Our q-PCR results showed that the NT013 and NT015 cells significantly expressed E-cadherin, while NT023 cells did not show any E-cadherin expression. Also, none of the three established cell lines expressed N-cadherin (Figure 2.4).

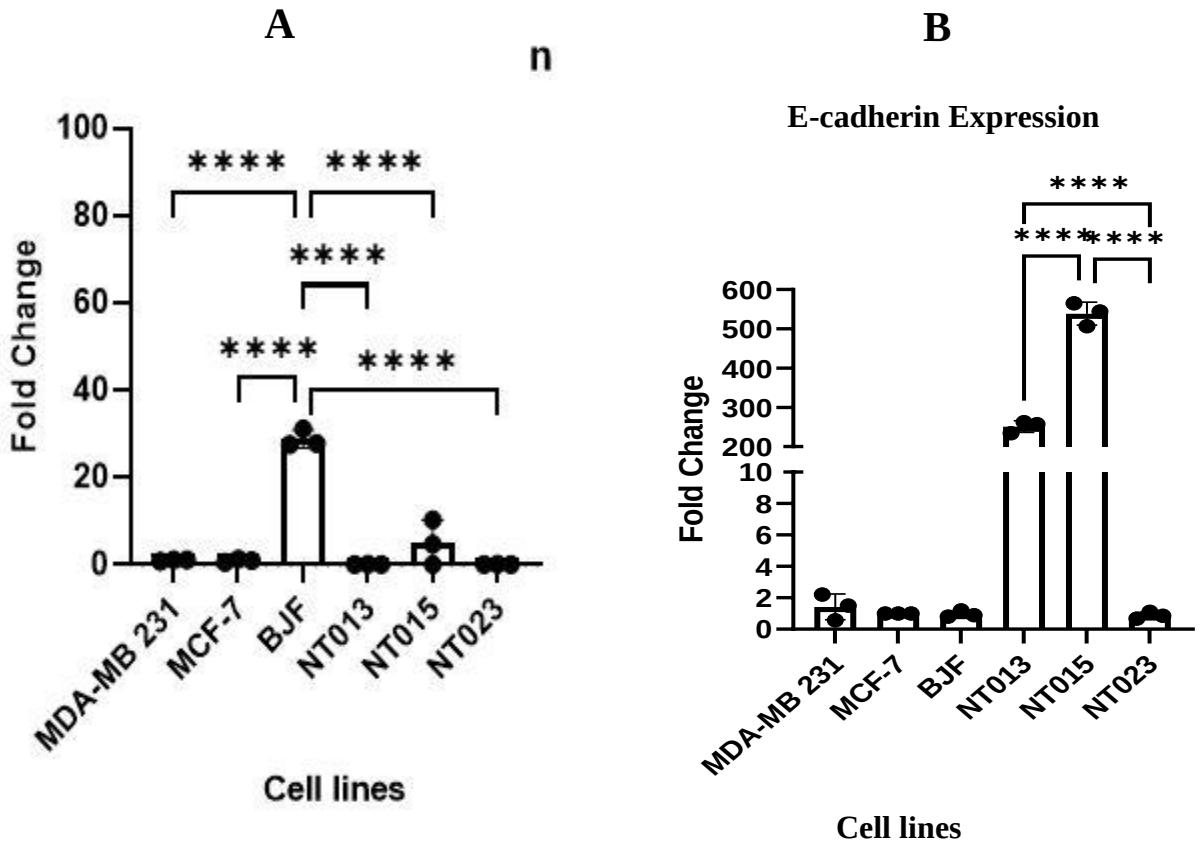

Figure 2.4. The expression status of two breast cancer biomarkers in the established breast cancer cell lines by q-PCR. A) N-cadherin expression. As shown, none of the established patient-derived breast cancer cell lines significantly expressed N-cadherin. BJ fibroblasts were considered as the positive control. B) E-cadherin expression. NT015 and NT013 cell lines significantly expressed E-cadherin, while there was no E-cadherin expression for NT023. Experiment was done in biological and technical triplicates. (One-way Anova test, P-value≤0.05 considered as statistically significant).

2.4.5. Changes in proliferation rate following estrogen treatment

As the passage number increased, cells showed slow growth rate in passage 5. We started treating the NT013 cells with 1nM estrogen (Sigma-Aldrich, catalog number: E8875) in P5 in order to facilitate cancer cells proliferation. Following estrogen treatment, we noticed a faster proliferation rate starting from passage 6, exhibited by increased Ki67 expression in estrogen-treated NT013 cells (Figure 2.5). However, we found out changed morphology and decreased CK19 expression (compared to NT013 cells in P6, shown in figure 2.1A) in these cells, while estrogen-treated MCF-7 cells maintained CK19 expression (figure 2.6). The sustained CK19 expression in MCF-7 cells might be due to the long-term adaptation to the 2D culture. Another scenario might be that the changes found in NT013 cells were spontaneous and were not due to estrogen treatment. Also, NT015 cells were treated with estrogen in the same way and we could not see any improved proliferation after estrogen treatment in this cell line (data not shown).

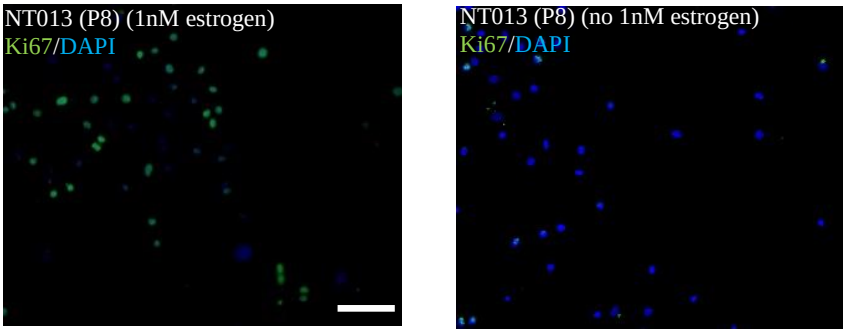

Figure 2.5. Higher Ki67 expression in NT013 cells treated with 1nM estrogen (left), compared to untreated NT013 cells (right). This reflects a higher proliferation rate following estrogen treatment in NT013 cell line (Scale bar: 100µm).

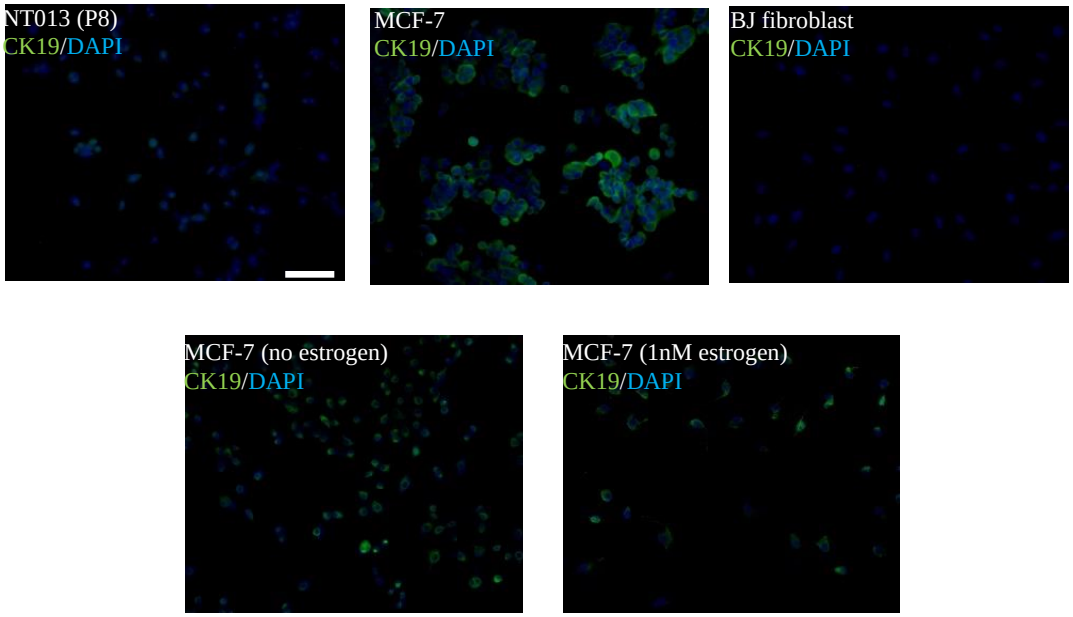

Figure 2.6. Loss of CK19 expression in NT013 cells treated with 1nM estrogen. Following treatment with estrogen, NT013 cells lost their CK19 expression, while MCF-7 cells could retain their CK19 expression even following estrogen treatment.

2.5. Discussion

Commercial breast cancer cell lines, despite their advantages, have shown limitations in representing the actual in vivo condition of the cancer cells. This is mainly due to the existence of key mutations over the long-term in vitro culture of cancer cells. Patient-derived cancer cell lines are valuable tools to understand the biology of tumor microenvironment as well as to demonstrate a relatively close resemblance to the in vivo condition of the cancer cells, since they are just isolated from the patient tumor and have not undergone genetic alterations. However, based on our findings, long-term culture of patient-derived breast cancer cell lines in monolayer condition was to some extent challenging and time-consuming. This was demonstrated by lack of efficient growth and survival in several breast cancer tumor samples that we obtained. However, the strength of our study was successful establishment of three patient-derived breast cancer cell lines. In our way to establish these cell lines, we overcame challenges such as cell scenesence, inefficient

cancer cell growth, etc. The success rate of breast cancer cell line establishment has been 25% so far. We had several samples in which the cancer cells could not survive and died due to the stress of transition from in vivo to in vitro. However, we successfully established three cell lines, two of which being hormone receptors (Estrogen and/or progesterone) positive, HER2 negative breast cancer; and one being Triple-negative (Estrogen/progesterone negative, HER-2 negative) breast cancer cell line.

Cells in passages 6 and 7 exhibited some changes in the CK19, vimentin, and EpCAM expressions. As shown in the result section, NT013 and NT015 cell lines began to express vimentin, accompanied by reduced CK19 expression. For NT023 cell line, the CK19 expression level significantly reduced. These findings demonstrated some degrees of inconsistency compared to their corresponding commercial cell lines, MDA-MB 231 and MCF-7. There are different potential reasons for these changes. One possible reason for established HR+ HER2- cell lines, NT013 and NT015, might be the occurrence of EMT in vitro. Epidermal growth factor (EGF) is known as a trigger of EMT via the phospho-Smad2/3-Snail signaling pathway (194). High glucose levels have also been demonstrated to enhance EMT in breast cancer cells, mediated by ROS formation (195). Furthermore, TGF-β is proven to induce EMT in cancer cells (196) via the induction of the H3K27me3 demethylation in Snail1 promoter, resulting in Snail1 overexpression and thus EMT induction (197). TGF-β is a component found in serum. Since we used F-media containing TGF-β, EGF, and high glucose DMEM, these factors might have induced EMT in our HR-positive HER-2-negative established breast cancer cell lines. Loss of CK19 expression is explained as one of the phenomena associated with EMT (198).

We did not find loss of E-cadherin and gain of N-cadherin at the same passage number as we noticed CK19 and vimentin expression changes, which is one of the reasons that challenges

the EMT induction hypothesis, which makes us take into account another scenario: genetic alterations induced due to 2D culture of the established breast cancer cell lines. Liu et al. showed that the 2D cell lines carried significant genomic alterations due to the culturing effects. They emphasized that the culturing media could not guarantee mimicking tumor microenvironment, thereby making the cancer cells susceptible to genetic changes (199).

Kuo et al. aimed to establish two basal-like breast cancer cell lines and found out that there was a strong CK19 expression in both MDA-MB 231 and MCF-7 cell lines, while there was an extremely weak CK19 expression in the two patient-derived basal-like breast cancer cell lines (200), similar to the NT023 cell line in passage 6 Furthermore, Fujisue et al found out tha in patients with luminal A subtype, there was 45.3% CK19 positivity and 40.7% CK19 negativity among the studied patient samples, while these percentages were 30.2% and 14.8% in luminal B subtype (201).

Fu et al. reported that vimentin was not expressed by MCF-7 cells (202), while Wei et al. showed that MDA-MB 231 cells highly expressed vimentin (203). The high vimentin expression in NT023 correlated with the results by Wei et al. Also, we did not detect vimentin in MCF-7 cells, while a population of NT013 and NT015 cells highly expressed vimentin.

Martowicz et al. aimed to checked the EpCAM expression in MCF-7 and MDA-MB 231 cell lines and western blotting results showed that MCF-7 cells, but not MDA-MB 231 cells, highly expressed EpCAM, which was consistent with what we observed in these two cell lines. We also did not observe EpCAM expression in NT023 cells, while in some population of NT013 and NT015 cells, there was EpCAM expression (204).

Chao et al. demonstrated that MCF-7 cells could express E-cadherin, while there was no E-cadherin expression in MDA-MB 231 cells (205). In our research, we also did not find E-

cadherin expression in MDA-MB 231 cells, while MCF-7 cells showed E-cadherin expression. NT013 and NT015 cell lines highly expressed E-cadherin, while NT023 cells did not. These data suggest that the E-cadherin expression status of the established cell lines correlated with what has been reported by others.

In 2016, Schlegel et.al (170)attempted to propagate both normal and tumor cells isolated from the individuals in 2D culture system using conditional reprogramming technology, and successfully established patient-derived breast cancer cell lines. They also showed that conditional reprogramming demonstrated an efficacy higher than that of cell transformation by SV40 virus large T-antigen or by overexpression of hTERT. Zhuang et al. (157) established two patient-derived breast cancer cell lines using the following culture media: mouse embryonic fibroblast (MEF) media (DMEM with 10% fetal bovine serum (FBS) supplemented with glutamax, MEM NEAA, sodium pyruvate, and 5 μM Y-27632-inhibitor. The presence of ROCK inhibitor and Swiss 3T3 J2 feeder cells in our culture system was to contribute to a more efficient survival and proliferation of the cancer cells. Feeder cells cooperate with ROCK inhibitor and induce the immortalization of the cancer cells through a complex interaction between various genes, including hTERT, Myc, E6, pRB, etc (172). Our established breast cancer cell lines initially showed high proliferation rate in co-culture with MEFs, while later in passage 5, the presence of MEFs did not support the proliferation of cancer cells, and the cells stopped growing. Only switching from 2D culture to 3D culture, and then 3D to 2D culture, led to the enhanced proliferation in the established breast cancer cell lines.

In estrogen treatment experiment, although we found enhanced proliferation and reduced CK19 expression when the NT013 cell were treated with 1nM estrogen, NT015 and MCF-7 cell did not show such changes. This may be due to an spontaneous change in CK19 and Ki67

expression in NT013 cell line. On the other hand, we could speculate that only NT013 cell line showed these responses after treatment with estrogen. However, further research needs to be conducted to evaluate the estrogen effect on breast cancer subtypes regarding their biomarker expression and proliferation status.

In conclusion, we found out that the established breast cancer cell lines could express the key breast cancer biomarkers, with tendency towards expressing higher levels of vimentin. our results indicated the need for an alternative option for maintaining long-term and stable patient-derived breast cancer cell lines which can mimic the in vivo properties of the tumor. These options could include 3D spheroids and Patient-derived organoids.

CHAPTER 3. SUMMARY AND FUTURE DIRECTION

Commercial cell lines have long been used in both basic and translational cancer research and have been considered as a valuable tool to understand the biology of cancer as well as developing innovative therapeutic strategies for cancer patients. However, there are limitations that necessitates the development of alternative modalities in order to have a better understanding of biological events occurring in Tumor microenvironment (TME). Due to long-term in vitro culture, commercially available cell lines have undergone key mutations that make them ineffective in recapitulating the actual TME in vivo.

Many efforts have been made to come up with more efficient in vitro approaches that could sustain the genetic characteristics of breast cancer. Patient-derived breast cancer cell lines are promising tools that have shown to be effective alternatives for commercially available cell lines. However, establishment of long-term, stable breast cancer cell lines is a daunting task, and many efforts have failed due to lack of efficient growth, genetic alteration, and senescence. Characterization of the established patient-derived breast cancer cell lines is a key to verify the successful establishment of cell lines as well as to keep a track of their genetic characteristics overtime, thereby enabling us to draw conclusion about the efficacy of 2D culture system for maintaining the patient-derived breast cancer cell lines.

We successfully established three patient-derived breast cancer cell lines, overcoming the challenges such as cell scenescence and lack of cancer cell growth and propagations. We could verify that the cancer cells expressed well-known breast cancer biomarkers such as CK19, vimentin, EpCAM, and E-cadherin. These cell lines could be valuable tools for further research on testing drug sensitivity as well as being considered as platforms for utilization in 3D models to

understand the biological characteristics of breast cancer as well as testing drug sensitivity and gene expression profiling in these models compared to 2D model.

We checked the status of several biomarkers that are shown to be overexpressed in breast cancer cells, CK19, Ki67, Vimentin, E-cadherin, N-cadherin, and EpCAM. Our findings demonstrated that our established breast cancer cell lines underwent morphological and marker expression alterations overtime, reflecting the need for alternative approaches to develop the effective culture of patient-derived cancer cells with appropriate growth rate and sustained genetic characteristics of the original tumor in vivo.

We successfully established these cell lines, overcoming cell scenescence and inefficient cancer cell growth. Also, our cell lines, to some extent, maintained key breast cancer biomarkers. These cell lines are being used in 3D culture systems such as hanging drop method and biomimetic scaffolds in order to recapitulate breast cancer bone metastasis and shed lights on for better understanding of the breast cancer biology. Using these cell lines in biomimetic scaffold approach, a wide variety of therapeutic drugs could be tested and screened for further precise administration in clinical trials and develop efficient and promising treatments in personalized medicine area, where treatment is specifically developed for each patient based on the samples obtained from their own body. This is of particular importance due to the tumor heterogeneity among different individuals. Furthermore, it would be helpful to develop patient-derived breast cancer cell lines from the bone metastasis samples and use both breast-derived and bone-derived cell lines for personalized medicine, and compare the effectiveness of treatment in these two types of cell lines.

Therefore, future research requires to focus on comparing the 2D versus 3D culture systems and investigate the efficacy of 3D and other novel ex-vivo systems in recapitulating the biological processes happening in vivo, so that they could be offered as promising platforms to test the

effectiveness of various therapeutic modalities for cancer. This can especially be useful to be implemented in personalized medicine.

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