

Characterization of UMD227 cells:
A novel murine mammary cancer cell line

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Dedication

This thesis/project is dedicated to my husband and my dad. The two men in my life. One that pushes me into reaching my dreams and one that loved me no matter what I choose to do.

Abstract

Breast cancer is a heterogeneous disease, which makes it a challenging disease to treat. Breast cancer can be classified by histology as well as by molecular markers. In order to develop a relevant *in vitro* model, our lab has cloned and isolated a cell line, UMD227, from a mammary tumor of our mouse model which expresses transforming growth factor alpha under control of promoter, neu-related lipocalin (NRL-TGF α). When UMD227's are transplanted back into our mouse model the resulting UMD227 tumors exhibit a higher grade than the primary tumor from which the cell line was derived and tumors following transplant have a distinct spindle cell like morphology, suggesting mesenchymal like characteristics. These characteristics suggest that this cell line represents basal-like tumor characteristics. My hypothesis was that the UMD227 cell line is a model of basal-like breast cancer. I began by determining the biological response of UMD227 cells to estrogens and antiestrogens *in vitro* and evaluation of marker proteins that distinguish mature differentiated epithelial cells from cells undergoing epithelial mesenchymal transition from progenitor/stem cells. My research concludes that the UMD227 cells are basal-like mammary gland cancer cells.

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Hypothesis

The goal of this project is to characterize the novel murine mammary cancer cell line UMD227R2 (UMD227), to determine what type of breast cancer it best represents. We know that breast cancer make-up is heterogeneous. This makes it difficult to study breast cancer as well as apply simple diagnoses and treatment regimens. Cancer, in general, exists at different levels and stages ranging from benign to metastatic. Breast cancer alone exists as at least 15 different types (American Cancer Society, 2008). Models are needed that represent all types of cancer and so that the heterogeneous behaviors are better understood and relevant treatments for each type are identified.

The UMD227 cell line has some initial qualities that suggest it may be a novel model of breast cancer that warrants further scrutiny. Characterizing the UMD227 cells is the key to their utility for studies in breast cancer tumorigenesis, prevention and therapy. The UMD227 cell line originated from a mammary tumor from a genetically engineered mouse which expressed transforming growth factor alpha under control of promoter, neu-related lipocalin (NRL-TGF α). NRL-TGF α tumors express estrogen receptors but not progesterone receptors. The UMD227 cell line was one of 12 cell lines derived from 12 independent tumors of NRL-TGF α transgenic mice and the only cell line that formed tumors following transplantation (A. Skildum, unpublished data). UMD227 tumors exhibited a higher grade than the primary tumor from which the cell line was derived and tumors following transplant have a distinct spindle cell like morphology, suggesting mesenchymal like characteristics. Which suggest that this cell line represents basal-like tumors. The hypothesis is, therefore, that the UMD227 cell line is a model of basal like breast cancer.

Introduction

Mammary Gland Development

In almost all cases, the origin of breast cancer is undoubtedly mammary epithelial cells. The mammary gland separates the Mammalian class from the rest of the animal kingdom with a unique gift of providing nourishment and immunological protection to their young (Lanigan, O'Connor et al. 2007). The development of human mammary

glands occurs in stages that are influenced by hormones and growth factors, and cellular interactions between epithelial and stromal cells (Lanigan, O'Connor et al. 2007). The human mammary gland initially starts to develop 35 days after conception and continues to develop through adulthood (Parmar and Cunha 2004). There are 10 stages in mammary gland development which range from the formation of the mammary placode in the developing fetus to lobuloalveolar formation with pregnancy of the adult female (Russo and Russo 2004). However, the mammary gland does not fully develop in utero (Lanigan, O'Connor et al. 2007). Further development is initiated by the secretion of hormones and growth factors, which occurs at puberty. Between the time the maternal hormone subsides in a new born and puberty, the development of the mammary gland is silent (Russo and Russo 2004).

Puberty is the start of dramatic changes in the female mammary gland, caused by the establishment of the hypothalamic-pituitary-ovarian axis. Ovarian hormones have specific roles in the development in the mammary gland. For instance, estrogen is responsible for ductal formation and progesterone is responsible for ductal side branching and alveologensis during pregnancy (Lanigan, O'Connor et al. 2007).

Lobules with 4 different configurations are found in the human breast and are described as lobule type 1 to 4 (Russo and Russo 2004). Lobule type 1 is first to develop after the first menstruation. Lobule types 2 and 3 occur as the breast further develops in the nulliparous individual and continues to develop until about 50 years of age (Russo and Russo 2004). The hormones of pregnancy promote further development of lobules in preparation for lactation, which are referred to as type 4 lobules (Russo and Russo 2004). The breast ultimately becomes a network of epithelial ducts and lobes that in humans are collectively known as terminal ductal lobulo-alveolar units (TDLU) (Morrison, Schmidt et al. 2008). TDLUs consist of two layers; inner luminal cells and outer myoepithelial cells with the basement membrane outside of the myoepithelial layer (Flannigan, O'Connor et al. 2007).

The human breast eventually undergoes a process that is referred to as involution. There are two types of involution, postlactational and lobular (Radisky and Hartmann 2009). Postlactational involution occurs at the time of weaning and results in lactational

lobules returning to lobules type 2 and/or 3 (Russo and Russo 2004). Most of the epithelial cells that differentiated during pregnancy and lactation undergo cell death, leaving undifferentiated epithelial cells to remodel into type 2 or 3 lobules (Radisky and Hartmann 2009). Lobular involution occurs with age and is distinguished by the loss of TDLUs, and ductal tree size and density (Radisky and Hartmann 2009).

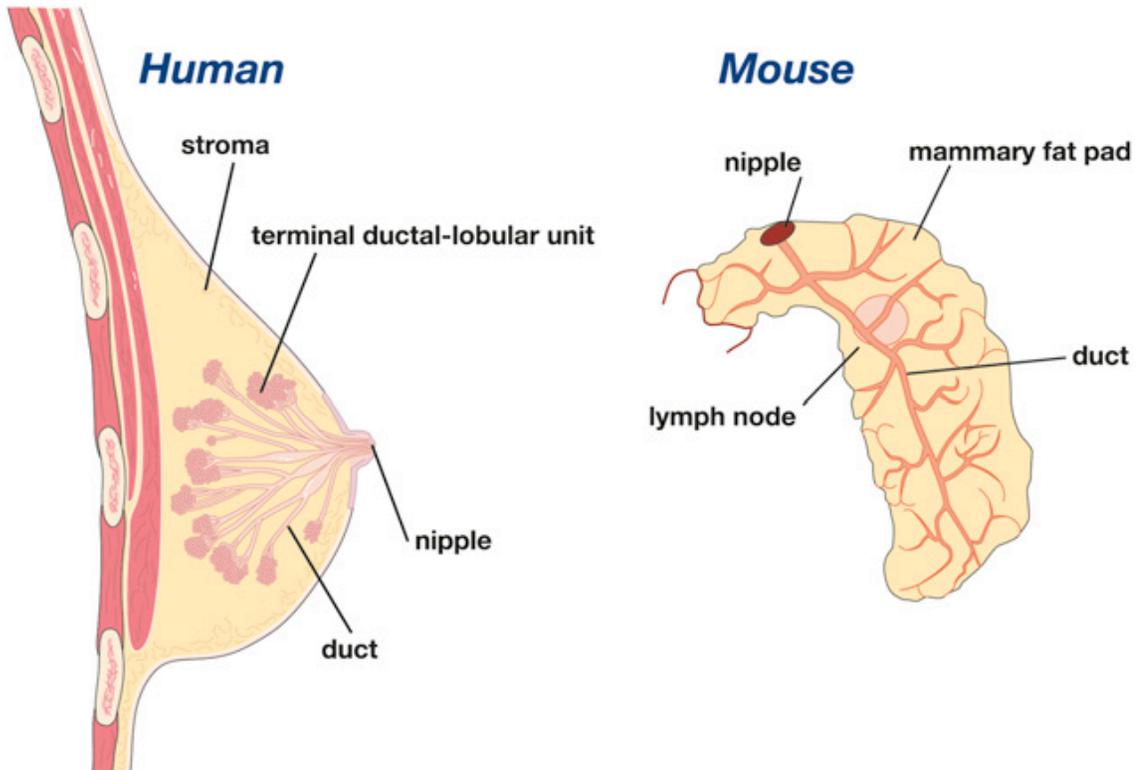


Figure 1. The structural differences between human and mouse breast Visvader, J. E. (2009).

The developmental and anatomical structure between mouse and human mammary gland are highly similar (Fig. 1). The most significant difference is that a nulliparous mice have less developed lobules and fewer fiberblasts compared to a nulliparous woman. However, development cues which drive puberty and pregnancy are the same. Ductal elongation occurs at the terminal ends of the ducts at sites called terminal end buds or TEB (Visvader 2009) in both species. Cap cells are group of cells at the end of the TEB that contain stem cells which are responsible for cell division. Cap cells therefore are instrumental to establishing a ductal tree that extends through the stroma (Hinck and Silberstein 2005). Lobulo-alveolar growth during pregnancy is

similar between mice and women and is largely driven by estrogen, progesterone, prolactin and glucocorticoid. Importantly, both mouse and humans ducts and lobules consist of two layers; the inner luminal cells and the outer myoepithelial cells. The similarity in the architecture and endocrinology between mouse and human mammary glands are important because the UMD227 cells allow us able to compare and extrapolate these findings from mouse to human.

Breast Cancer

In United States, breast cancer is the second leading cause of cancer death among women with lung cancer being the first. Smoking causes the majority of lung cancer. However, there is no clear cause of breast cancer, and further research must be done to bring an end to breast cancer. According to the American Cancer Society (ACS, 2009), there are millions of people who have or had cancer. Roughly about 192,000 American women will develop breast cancer in 2009 and 40,000 will die from it. (ACS, 2009) A woman has a 1 in 8 chance of developing breast cancer in her life time and a 1 in 35 chance of dying from it (ACS, 2009). In the 1950's, the number was a 1 in 6 chance of dying from breast cancer (Sugarbaker and Wilfley 1950). The decrease in deaths is due to improved methods of early detection and treatments. In addition, with improvements in detection, the number of breast cancer cases identified has increased.

It is believed that breast cancer is the result of a complex interaction of internal (genetic, epigenetic) and external (environmental) elements plus time (Pollner 1993). There are several risk factors associated with breast cancer, including factors as simple as gender all the way to factors as complex as genetic make-up. The risk factors can be divided into two categories, one category of which the contributing factors cannot be changed and one category of which the contributing factors that can be changed. All of the risk factors discussed in this paper are summarized by the American Cancer Society and pertain to American women. Risk factors are factors which increase the probability of developing breast cancer.

Some risk factors cannot be altered by the individual, such as age and genetic make-up. For example, older white women with a first generation family member

diagnosed with breast cancer or a personal history of breast cancer, have an increased risk for breast cancer; and women born into a family with a history of women developing breast cancer are likely to have inherited a gene mutation which increases susceptibility to breast cancer. Five to 10% of breast cancers are linked to gene mutations, of which the most commonly known ones are BRCA1 and BRCA2. Finally, breast cancer risk increases in women with early menses and/or late menopause after the age of 55.

Risk factors that can be altered by an individual include lifestyle choices. For example, women who drink more than 5 alcoholic drinks per day, eat a high fat diet AND do not exercise are at higher risk for breast cancer. Birth control pills and the combined replacement hormone therapy of estrogen and progesterone are also thought to increase one's risk for breast cancer. On the other hand, a decrease in risk is seen with women who bear children before the age of 30 and breastfeed them.

Although several breast cancer risk factors have been identified, it should be pointed out, that "some women who have one or more risk factors never get breast cancer. And most women who get breast cancer don't have any risk factors" (ACS, 2008). This observation points to the fact that there is much to learn about breast carcinogenesis and the steps required to prevent and treat it. Characterization of the UMD227 may provide another tool for studying breast cancer.

Types of Breast Cancers

One of the difficulties of studying breast cancers is that they are heterogeneous. Breast cancer can be classified into groups according to histological characteristics as well as molecular features. Breast cancers typically are evaluated for the presence of estrogen receptor (ER) and progesterone receptor (PR) status. Estrogen is a breast mitogen in normal and cancerous breast epithelial cells. Treatment of breast cancer frequently includes endocrine therapy directed at modulating ER signaling by either: (a) reducing levels of estrogen; (b) antagonizing ER function with antiestrogens such as tamoxifen or using the pure antiestrogen, such as ICI 182,870, or (c) altering the amount of estrogen available by use the use of aromatase inhibitor (Schiff, Massarweh et al. 2004). Only ER+ breast cancers are sensitive to antiestrogen, which makes up 75% of

cases of breast cancer (Clarke, Liu et al. 2003). However, not all ER⁺ breast cancers are responsive to antiestrogen therapies; some ER⁺ patients are *de novo* resistant and most ER⁺ cancers, which initially are sensitive to antiestrogen, become resistant with time (Clarke, Liu et al. 2003).

PR is an emerging marker for breast cancer therapy as well. Transcription of the PR gene normally is up regulated by estrogen in breast and reproductive tissues. However, ER⁺ breast cancers may or may not display PR. Recently it was demonstrated that ER⁺ breast cancers that lack PR expression are less responsive to endocrine therapy than those that express PR (Arpino, Weiss et al. 2005), suggesting that ER⁺/PR⁻ tumors are more likely to be estrogen resistant. In addition, ER⁺ breast cancers with up-regulated epidermal growth factor receptor/ human EGFR receptor 1 (EGFR /HER1) and/or human EGFR receptor 2 (HER2) receptors are correlated with an increased risk of antiestrogen resistance and found more often in ER⁺/PR⁻ cancers than in ER⁺/PR⁺ cancers (Schiff, Massarweh et al. 2005). This indicates that breast cancers can be classified in ways other than the occurrence of ER or PR.

HER2 (or ErbB2, the equivalent found in mouse, but will be referred to as HER2) has been shown to be over expressed in ER⁻ cancers and some ER⁺ cancers, most frequently in ER⁺ cancers when tamoxifen sensitivity is lost (Chu, Blackwell et al. 2005). HER2 is a member of the HER family, which are transmembrane protein receptor tyrosine kinases (RTK). There are four members of the HER family including HER1, HER2, HER3 and HER4. There are over 25 ligands (Moasser, Basso et al. 2001) known to bind to these receptors. Ligands responsible for activating the HER receptor members are called epithelial growth factor (EGF)-related growth factors (Holbro and Hynes 2004), and include ligands such as transforming growth factor alpha (TGF- α), which binds specifically to HER1. Transduction of HER receptors depends on homo- or heterodimerization with one of the four family members with subsequent autophosphorylation of tyrosine, which initiates a series of signal transduction cascade. Ligands are specific, but dimerization partners are not. HER2 does not have a ligand-binding domain so it must dimerize with one of the other members of the HER family for induction of activity. HER3 has a defunct catalytic domain with impaired kinase activity

hence it is thought to act in a dominant negative fashion (Warren and Landgraf 2006). HER4 is the least defined receptor of all of the HER family. The biological outcome of the signal in the mammary gland depends on the level of homo- and/or heterodimerization of the respected receptors. Ultimately, the activation of the HER receptors are responsible for cell proliferation, differentiation, apoptosis, adhesion, and cell mobility (Holbro and Hynes 2004).

In breast cancer, the activities of HER1 and HER2 are most well known. For example, it has been shown that HER1/HER2 dimerization activates the mitogen-activated protein kinase pathway (MAPK), which is responsible for cell proliferation (Knowlden, Hutcheson et al. 2003). It has been found that an increased activity of MAPK in ER+ breast cancer patients is linked to tamoxifen insensitivity, resulting in a poor survival rate (Knowlden, Hutcheson et al. 2003). HER2 is found to be over expressed in 30% of breast cancer cases, which is linked to poor prognosis for the patient (Chu, Blackwell et al. 2005). HER2 over expression predominantly results from amplification of the HER2 gene (Nahta, Yu et al. 2006). In addition, there is an inverse relationship between ER activity and HER1 and HER2 expression and the over expression of these RTKs is associated with a decrease in sensitivity to endocrine therapy as well as a poor prognosis (Knowlden, Hutcheson et al. 2003).

Recently, a class of breast cancer has been identified as triple negative. Triple negative breast cancer has been histologically characterized as having low to no ER and PR and low or no over-expression of HER2 (Viale and Bottiglieri 2009). Patients diagnosed with triple negative breast cancer do not respond to antiestrogens, HER2 therapies or traditional chemotherapy such as Taxol, 5-fluoruracil and mitomycin (ACS, 2008), which makes for a poor prognosis (Irvin and Carey 2008). Between 10-20% of all breast cancers are of triple negative type (Reis-Filho and Tutt 2008). There are several subclasses of triple-negative breast cancer, which include basal-like cancers, which are described below.

Breast cancers have also been categorized into different groups based on molecular characteristics, including gene expression or responses to treatment (Reis-Filho and Tutt 2008). These characteristics are summarized in Table 1. These groups include;

luminal A and B, which generally express ER and PR proteins (ER+, PR+), HER2 + and basal-like tumors (Viale and Bottiglieri 2009). Luminal A and B type are generally classified as slow growing luminal cells, low grade and can be treated with hormonal therapy (ACS, 2008). HER2+ cancers can be treated with specific inhibitors but with mixed outcomes and are classified as high grade and fast growing (ACS, 2008). Basal type cancer is distinguished by expression of cytokeratins 5/6 and 14 and lack of ER expression (Jumppanen, Gruvberger-Saal et al. 2007). Basal cancers are considered to be a high grade tumor with poor prognosis as it is highly resistant to therapy (ACS, 2008). The point is that breast cancer classification is carried out using multiple approaches and these approaches can be used to classify novel models of breast cancer. Appropriate classification of models is paramount for their utility. Once accurately characterized, the UMD227 cell line could be another tool to study breast cancer behavior.

Table1. Summary of the types of breast cancer in humans and the common characteristics used to classify them.

Characteristics	Luminal A/B	Basal	Triple Negative
ER/PR	+/+	-/- or -/+	-/-
HER2 amplified	yes	no	no
Grade *	low	high	high
Response to treatment	hormonal therapy	no response	No response
Luminal/Basal	luminal	basal	not well defined

* Tumor grade refers to the histological assessment of nuclear and cytoplasmic morphology

Theories Addressing the Origin of Breast Cancer

There are several overlapping theories exist that address the question: Where do breast cancer cells originate? One theory is the stochastic theory (Molyneux, Regan et al. 2007). This theory implies that all the cells within a tumor mass have equal chances to continue tumor growth, but the odds are low (Molyneux, Regan et al. 2007). Another theory is the clonal evolution theory; which basically states that cancer is caused by a few mutated cells which expand to form a tumor (Molyneux, Regan et al. 2007). This could

be supported by the fact that there is a delayed tumor development after exposure to a carcinogen.

Then there is the hierarchical or cancer stem cell theory. The idea behind this theory is that cells in the mammary gland develop in a hierarchical manner similar to the hematopoietic system (Molyneux, Regan et al. 2007). Stem cells are defined as a small population of cells that are able to self-renew, able to remodel and maintain tissue health (Molyneux, Regan et al. 2007). Stem cells would maintain their population of cells by asymmetric division; which means stem cells are able to divide into one identical cell and one common progenitor cell (Cariati and Purushotham 2008). Stem cells are considered to be at the top of the hierarchy with the progenitor cells giving rise to committed progenitor cells at the next level and then differentiated cells (Cariati and Purushotham 2008).

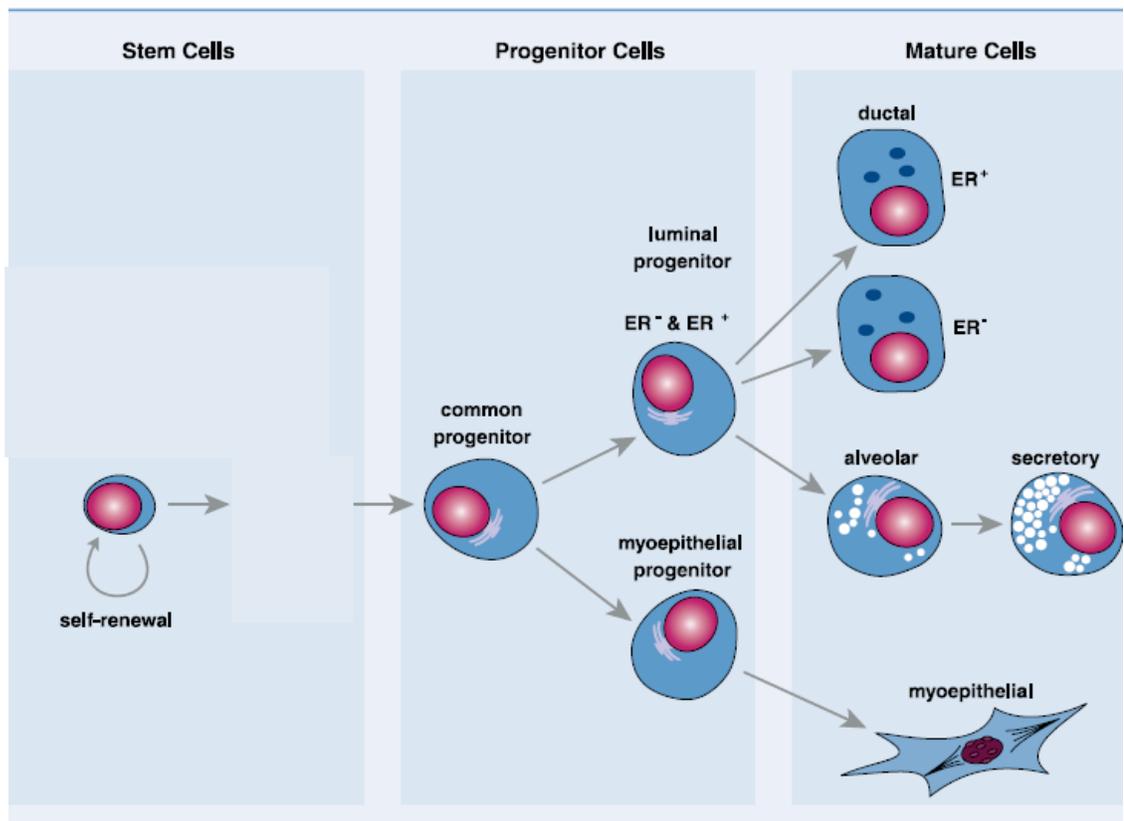


Figure 2. Cell hierarchy as found in a mouse (Figure adapted from J. Visvader & G. Lindeman, 2006)

Figure 2 depicts one proposed hierarchy for mammary epithelial cells in mice. The thought is the mammary gland is similar to other organs that have stem cells. Unique to the mammary gland, however is that stem cells not only respond to the initial development of the organ at puberty, but also respond to the physiological demand of pregnancy and involution, which can occur numerous times in a life time. Therefore, mammary stem cells must organize in a way to rapidly and efficiently respond to the physiological demand.

Common progenitors are the population of cells that give rise to progenitors that are committed to further differentiate into adult cells (Visvader and Lindeman 2006). In mammary glands, the common progenitor creates committed progenitor cells that are either luminal or myoepithelial. The luminal progenitor differentiates to ductal or alveolar cells whereas the myoepithelial progenitor differentiates into myoepithelial cells (Figure 2; Visvader and Lindeman 2006). These factors and accessible location of mammary glands has made them a valuable organ system in which to study stem cell activity.

Cancer stem cells are thought to have some of the same properties as normal stem cells; a small population of cells that are able to self renew and appear to maintain the integrity of a tumor, but cancer stem cells follow different rules in terms of proliferation and apoptosis (Cobaleda, Cruz et al. 2008). As part of my investigation of the UMD227 cells, I will use the same markers that identify cancer stem cells to see if the UMD227 cells have stemness.

Cell Markers that Identify Mammary Epithelial Cell Subtype

It is important to mention that most breast cancers arise from the epithelial cells within the mammary glands (ACS, 2009). The mammary epithelial cell types can be categorized by their location including the inner luminal cells and the outer myoepithelial cells. Because these cells have structural differences, epithelial markers are used to distinguish between the two. An example is cytokeratin 18, an intermediate filament that is found in luminal cells whereas cytokeratin 6, another intermediate filament, is found mainly in myoepithelial cells (Stingl, Eirew et al. 2006). Additionally, epithelial cells are

thought to be able to undergo alterations so that they appear to be more mesenchymal like. Mesenchymal cells are all cells that are not epithelial cells such as fibroblasts, adipocytes, and endothelial cells. Epithelial-mesenchymal transition (EMT) is thought to convey the ability to migrate and invade (Thompson, Newgreen et al. 2005), and an important process in embryogenesis (Thiery 2002).

There are several commonly used antibodies used to define epithelial and mesenchymal cell types. The choice to a large extent depends on whether the characterization is geared for a cell line or cells characterized directly after isolation from a tissue such as the mammary gland. For this study, which had a goal to determine the biological characteristics of a novel murine mammary cancer cell line UMD227, I evaluated five proteins that are commonly used for breast cancer classification into one of the breast types (i.e., luminal, HER2, triple negative); vimentin, smooth muscle actin (SMA), cytokeratin 6 (CK6), cytokeratin 18 (CK18) and mucin 1 (MUC1). Vimentin and SMA are found in EMT cells as well.

Vimentin is an intermediate filament that is one of three filament that makes up the cytoskeleton of different cell types such as fibroblasts, leukocytes and other mesenchymal cells as well as some epithelial cells (Evans 1998) and (Kokkinos, Wafai et al. 2007). This makes vimentin a good marker for mesenchymal cells as well as for myoepithelial cells. SMA identifies cells that are differentiated myoepithelial cells (Gusterson, Ross et al. 2005), and provides the cells with the ability to contract, thereby moving milk within the mammary gland. MUC1 is a secretory mucinous protein that is produced by luminal epithelial cells and over-expressed in many breast tumor types (Barratt-Boyes 1996). Recently, MUC1 has been suggested as a possible marker for mammary stem and luminal progenitor cells (Engelmann, Shen et al. 2008).

Surface protein markers are used to distinguish epithelial cell types in the stem cell hierarchy. Some of the commonly used surface markers are CD24 and CD49f. They are transmembrane proteins that have a cell to cell and/or cell to surface relationship with the extracellular matrix (Pontier and Muller 2009). Integrins maintain communication between extracellular matrix and cell, allowing for control of the cell's fate including proliferation, migration, and cell death (Giancotti and Tarone 2003). CD24 and CD49f

are integrins that have important functions in the immunological processes. As adhesion molecules, integrins help wound healing by adhering lymphocytes and leukocytes to blood vessel walls or into tissues for repair (Janeway's Immunobiology, 432-434). CD24 or heat-stable antigen is an adhesion molecule which promotes cell to cell interaction (Al-Hajj, Wicha et al. 2003). Studies have shown that only a low number of cells with the phenotype CD24^{-/low} appear to cause tumor growth, as opposed to a large number of cells with the phenotype CD24^{+hi} (Al-Hajj, Wicha et al. 2003). CD49f (also referred to as $\alpha 6$) is another adhesion molecule found in epidermal stem cells and in mammary glands (Li, Simmons et al. 1998) and (Stingl, Eaves et al. 2001). CD49f is a marker for progenitors that generate both luminal and myoepithelial lineages (Stingl, Eaves et al. 2001).

Table 2. Cell markers used in this thesis and what type of cells they indicate in human normal and cancerous mammary glands.

Cell Markers	Luminal	Myoepithelial	EMT	Progenitor/Stem	References
Cytokeratin 6	no	yes	yes	yes	(Stingl, Eirew et al. 2006)
Cytokeratin 18	yes	no	no	no	(Gusterson, Ross et al. 2005)
Mucin 1	yes	no	no	yes	(Engelmann, Shen et al. 2008)
Vimentin	no	yes	yes	no	(Evans 1998), (Kokkinos, Wafai et al. 2007)
Smooth muscle actin	no	yes	yes	no	(Gusterson, Ross et al. 2005)
CD24 (expression level)	high	low/med	????	med	(Al-Hajj, Wicha et al. 2003)
CD49f	low	low	low	high	(Stingl, Eaves et al. 2001)

Experimental Goal and Design

The purpose of my thesis was to characterize the UMD227 cell line. Our laboratory is interested in the development of *in vivo* murine models. There are a limited number of murine cell lines that represent the diversity of human breast cancers and cell lines available, few form tumors when grafted into syngeneic immune competent mice. UMD227 cells developed into tumors upon transplantation, which led us to further characterize the cell line. We know that the UMD227s are ER^{low} /PR⁻, do not have amplified levels of HER2 and express EGFR. This suggests that the tumors are triple negative breast cancer but the initial characterizations are not conclusive and triple negative breast cancers have a diverse phenotype. In order to better define the tumorigenesis process of UMD227, my goal was to determine (1) the biological response of UMD227 cells to estrogens and antiestrogens *in vitro* and (2) evaluation of marker proteins that distinguish mature differentiated epithelial cells from cells undergoing epithelial mesenchymal transition from progenitor/stem cells. Methods utilized to carry out these characterizations included Sulforhodamine B colorimetric assay, western blot analysis, immunocytochemistry and flow cytometry. Sulforhodamine B colorimetric assays were done to determine the effect of estrogen and antiestrogen on the UMD227 proliferation rate. Western blot analysis was done to determine if protein was expressed in the entire cell population. The purpose of immunocytochemistry was to confirm the location of the protein within expressing cells. The purpose of flow cytometry was used to determine the fraction of the cells that expressed each marker. UMD227 cells were compared to the well-characterized human breast cancer cell lines, MCF-7 and MDA-MB-231. MCF-7 cells form low grade, ER+, luminal type cancers and are feebly invasive, while MDA-MB-231 cells forms higher grade, ER-, mesenchymal like cancers that metastasize at a high frequency (Lacroix and Leclercq 2004).

Methods

Cell culture

MDA-MB-231 and MCF-7 cell lines were purchased from American Type Tissue Collection (ATCC) and maintained in Dulbecco's modified Eagle's medium (Mediatech, Inc Manassas, VA) supplemented with 10% fetal clone serum (HyClone, Logan, Utah) and 5% penicillin and streptomycin antibiotics (Mediatech, Inc. Herndon, VA). The UMD227 cell line was derived from a mammary tumor in a NRL-TGF- α transgenic mouse (A. Skildum, unpublished data). The UMD227 cell line was maintained in Improved modified Eagle's medium and F12 (Mediatech, Inc, Manassas, VA) supplemented with 10% fetal clone serum and 5% penicillin and streptomycin antibiotics. All cells were incubated at 37°C with 5% CO₂. The UMD227 cells utilized in these studies were of passages less than 10. Cells were allowed to reach 70% - 90% confluency prior to passage into a new culture flask.

Sulforhodamine B Colorimetric assay (SRB)

The effect on cell viability and growth of estradiol 17 β and the mixed antagonist/agonist, 4-hydroxytamoxifen and the pure antagonist, ICI 182,780 were evaluated using SRB assay. Cell proliferation was determined and averaged over three experiments. Each experiment was carried out in triplicate for both control and treatment samples. Phenol-red free Improved modified Eagle's medium (Mediatech, Inc Manassas, VA) was supplemented with charcoal stripped serum (Biotechnics Research, Lake Forrest, CA) and 5% antibiotics were used for this assay. Cells were seeded in a 24 well plate at 1×10^4 cells per well. Treatment with estradiol 17 β , 4-hydroxytamoxifen and ICI 182,870 (10^{-8} M to 10^{-12} M) began when cells reached 20-30% confluence. All hormones and inhibitors were purchased from Sigma (St. Louis, MO). Stock solutions were diluted in 100% ethanol, aliquoted and stored in -20° C. There were two different control groups; wells that were treated with ethanol at 1:100 ratio and wells with no treatment and no ethanol added. Sulforhodamine B colorimetric assay (SRB) (Sigma, St. Louis, MO) was used to determine the number of cells in a dish after 72h. Media was aspirated and cells washed once with phosphate buffered saline (PBS) (Lonza, Walkersville, MD).

Washed cells were fixed with methanol with 1% acetic acid and incubated for at least 1hr at -20° C. The fixative was then removed and the cells were oven dried. A solution of 0.5% sulforhodamine B in 1% acetic acid was added to the wells for 45 minutes at 37° C. Excess sulforhodamine B was washed away with 1% acetic acid and the plate was put in the oven to dry. Cells were treated with 10 mM Tris (pH=10) to solubilize the SRB. Soluble material (200µl) was transferred to a 96 well plate and absorbance at 540 nm was measured using a Spectro plate reader. The height of the bars in figure 1 represents the mean of triplicate data for 3 experiments and error bars reflect the standard deviation of the mean. Statistic analysis (ANOVA) was carried out using the Graphpad Prism 4.0 software and significant differences called at $p \leq 0.05$.

Western Blots

To determine the presence of protein in breast cancer cell lines, western blot analysis were carried out. Cells were seeded in 10 cm² cell culture plates and grown until they became 80% confluent. Cells were then washed with PBS and solubilized with 200µl of electrophoresis sample buffer (recipe in appendix C). Culture wells were scraped and a sample was collected, sonicated (3x5 sec pulses) and boiled for analysis. The cell samples were quantified using a Bradford Protein assay (Thermo Scientific, Rockford, IL) and samples (30-50 µg protein) were loaded onto 8% SDS-PAGE gels (BioRad, Hercules, CA). The electrophoresis was run for 1.5-2 hours at 80-120V. Resolved proteins were transferred to a nitrocellulose membrane (BioRad, Hercules, CA) overnight in 4°C at 30V. Blotted proteins were stained with ponceau stain before probing to ensure protein was present and the cells did not degrade during the electrophoresis process. Membranes were then blocked using 5% non-fat dry milk in tris buffer saline with 20-Tween (TBS-T). Membranes were washed three times with TBS-T for 5 minutes each prior to probing with primary antibodies. Primary antibodies were diluted in primary antibody dilution buffer (1g BSA in 20 mls of TBS-T). The following antibodies with dilutions indicated in parentheses were used for these evaluations: cytokeratin 6 (1:500) (Novous Biologicals, Littleton, CO), vimentin (1:5000), and smooth muscle actin (1:500) (Abcam, Cambridge, MA, USA). The membranes were incubated at 4°C

overnight with gentle agitation. Antibody bound membranes were washed three times with TBS-T (5 minutes each time). Antibody labeled proteins were identified using secondary antibody conjugated to horseradish-peroxidase. Membranes were incubated for 1 hour, at room temperature with secondary antibodies (1:10,000) in blocking buffer and washed 3 times with TBS-T. A chemiluminescent reagent (Thermo Scientific Rockford, IL) was then added to initiate chemiluminescence and incubated at room temperature for 5 minutes. Membranes were exposed to film from 1 second to 10 minutes, depending on the signal strength of the antibodies. Films (blue ultra autorad purchased from ICS Bioexpress, Kaysville, UT) were developed on a 100 PLUS x-ray film processor.

Immunocytochemistry

Another method to detect protein in cells is immunocytochemistry. The advantage of immunocytochemistry is that the location and the intensity of the protein within the cells can be located. Immunocytochemistry also provides a general idea of the fraction of cells that express the protein. In this procedure, cells were seeded on prewashed coverslips. Coverslips of 18x18mm in dimension and #1 thickness were washed with a biodegradable detergent and stored in 80% ethanol. On the day of use, the coverslips were rinsed in 5 separate containers of (15 mega ohm) water. The coverslips were then placed in a 6-well plate. Cells were seeded at 1×10^5 cells per well. When cells on the coverslips reached 50% confluency, they were washed once with PBS and fixed in ice cold methanol. Fixed cells were stored in a -20°C freezer until ready to use. The exception was when smooth muscle actin was probed. In case of SMA staining, cells on coverslips were fixed in 2% paraformaldehyde. The coverslips were washed three times with PBS-T. They were incubated in 1% non-fat dry milk in PBS-T for one hour in 37°C . The blocking buffer was aspirated off and 200 μl of diluted primary antibodies were added to the coverslips. Vimentin antibodies (Developmental Studies Hybridoma Bank, Iowa City, IA) and smooth muscle actin (Abcam, Cambridge, MA, USA) were used at 1:200 dilutions. The cells on coverslips were incubated for 1-2 hours at 37°C in a humidified chamber. Coverslips were washed three times with PBS-T for 5 minutes each. Secondary antibodies were added in the same manner as the primary antibodies.

Coverslips were incubated in a humidified chamber in 37°C for 1 hour. The coverslips were washed 3 times with PBS-T and mounted on a slide. No Hoechst staining was done, because it seems to interfere with the primary and secondary antibodies. A fluorescent microscope was used to evaluate fluorescent cells and photo images captured using a Nikon eclipse TE300 camera and MetaMorph software. The presence or absence of cells was recorded as well as the intensity and location of the fluorescence. These data were then compared with results of the western blot analysis and flow cytometry. In some cases, different antibodies were required between technique due to the intrinsic limitation of the utility of the antibodies.

Flow Cytometry

A third method to analyze proteins in cells is utilizing flow cytometry. Specifically, I used this method to analyze those proteins that were positive in western blot assay, vimentin, SMA and CK6. In addition, I evaluated cell surface markers, CD24 and CD49f, which are commonly used to categorize mammary epithelial cells into stem, progenitor and differentiated subpopulations.

Vimentin, smooth muscle actin, cytokeratin 6

For intracellular and permeabilized fixed staining, an intracellular FCM system kit (Santa Cruz Santa Cruz, CA) was used. The kit included FCM fixation buffer, FCM permeabilization buffer and FCM buffer. From here on out, these reagents were in permeabilized fixed cells to assess intracellular expression. The same protocol was followed here as with CD24 until the cells were fixed. The cells were resuspended in fixation buffer at 1ml per 10^6 cells and incubated at room temperature on a rotator for 30 minutes. The cells were spun down and the supernatant was decanted. The cells were again washed with PBS once. 10 million cells were permeabilized by adding 1 ml of ice cold permeabilization buffer in drop wise manner while vortexing. The cells were incubated at room temperature on a rotator for 5 minutes. The cells were resuspended in wash buffer at a final concentration of 1×10^6 cells/ml, after being washed once with PBS. The cells were aliquoted into 100 μ l and primary antibodies were added based on

manufacturers' recommendations; 20 μ l of vimentin (Santa Cruz, Santa Cruz, CA) and 5 μ l of the SMA (Epitomics, Burlingame, CA) and CK6 (Novous Biologicals, Littleton, CO). The cells were incubated on ice and covered for 30 minutes. If necessary, secondary antibodies were added and the cells were incubated for an additional 15 minutes. The cells were centrifuged and resuspended in 500 μ l of wash buffer. The isotype control (Epitomics, Burlingame, CA) was a rabbit IgG. The isotype is the control for all three antibodies, although vimentin was raised in the mouse and the other two in rabbit, they are all IgG antibodies.

CD24

CD24 antibody was incubated with cells reaching between 70%-90% confluency. Cells were released from the plates using 0.25% trypsin in EDTA (Mediatech, Inc Manassas, VA) for 3-5 min at 4°C. Proteolysis was terminated by adding 8.5mls of serum containing media. The number of cells was determined using a hemocytometer. The cells were spun down and resuspended in staining buffer to give 1x10⁶ cells/ml. The cells were aliquoted 100-200 μ l per tube and blocked for 20 minutes with either anti-Fc receptor (Santa Cruz Santa Cruz, CA) or rabbit serum, depending on whether the cells were mouse or human. The primary antibodies were added and incubated on ice for 30 minutes. If primary antibodies were not labeled with a fluorescent probe, then a secondary antibody tagged with FITC was added and incubated for an additional 15 minutes. The cells were spun down and washed 2 times with a staining buffer.

CD49f

For CD49f, the cells needed to be alive and have time to recover overnight in normal media, from being trypsinized before being probed. Live, recovered cells were added to sterile, non-coated petri dishes and rocked over night, to prevent their attachment. The cells were collected and counted the next day following a wash twice in PBS with 0.5% BSA. The cells were resuspended to a final concentration of 1x10⁶cells/ml. The cells were blocked for 15 minutes at room temperature with either rabbit serum or Fc receptor (Santa Cruz Santa Cruz, CA). Primary CD49f antibodies

were added, ($20\mu\text{l}/10^6$ cells) and incubated for 30 minutes on ice. The cells were washed twice with PBS with 0.5% BSA and resuspended with 500 μl of staining buffer. All cells were analyzed on a BD FACSCalibur flow cytometer; data collected with CellQuest software and analyzed using the FlowJo software (Tree Star Inc Ashland, OR).

Results

Treatment with Estradiol, Tamoxifen and ICI 182,780 had no effect on the UMD227 cell viability and proliferation.

We know that tissue sections of tumors which developed from transplant of UMD227 cells in mammary glands of mice contained a low number of ER⁺ cells. Because ER⁺ breast cancer is known to be responsive to ER antagonists, I tested the responsiveness of UMD227s to estradiol 17 β (estradiol) and two ER antagonists, tamoxifen, a well know selective estrogen receptor modulator (SERM) and ICI 182,780S (Fluvestrant) a well characterized selective estrogen receptor down regulator (SERD). Estradiol 17 β (E2) is a key endogenous estrogen in the body which binds to ER to promote cell proliferation in both normal and tumorigenic cells in the breast. Figure 3 represents results of the SRB assay of the UMD227 cells treated with estradiol, ICI and tamoxifen. The first bar represent cell numbers following the E2 treatment. Compared to the ethanol group, there is no significant difference ($P>0.05$ for treatment group). The stock solution of estradiol, tamoxifen and ICI all contain ethanol. E2 error bars are relatively large from 0.001 to 10 nM concentrations, so the ability to detect a low level response from E2 addition was not possible. Nonetheless, the lack of response to estradiol 17 β supports our previous *in vitro* and *in vivo* data. In addition, unpublished observations from our laboratory indicate that E2 does not alter the cell cycle dynamics of UMD227s (A. Skildum, unpublished data) and cells form tumors with similar incidence and latency, in male and female syngeneic mice following transplantation.

The second bar represents the cell numbers following ICI treatment on the UMD227 cells, and shows there is no overall effect. The proliferation rate slightly increases as the concentration decreases, peaking between 0.01 nM and 1 nM. Only at 0.1 nM ICI was a significant increase in proliferation of UMD227 cells. The third bar

represents the number of UMD227 cells following tamoxifen treatment. Which, like ICI, The growth is not inhibited by tamoxifen.

In conclusion, UMD227 cells are not sensitive to E2 or estrogen antagonists. As the only know effect of ICI is inhibitory, we believe statistical significance found for ICI increasing in UMD227 cell growth is due to the high variability intrinsic to this assay. This data corroborates with other observations in our laboratory on the lack of E2 sensitivity on the UMD227s growth *in vivo* and *in vitro*.

UMD227s do not express protein found in luminal breast cells.

Western blots were done to see if the UMD227s expressed certain epithelial markers that included luminal cell markers (CK18 and MUC-1) and mesenchymal/myoepithelial cell markers (CK6, vimentin and SMA) (fig 4). There was no evidence of CK18 or MUC1 found in UMD227's (data not shown). UMD227 cells do express CK6, SMA and vimentin, which indicates that they have mesenchymal like characteristics. The negative results for CK18 and MUC-1 in the UMD227's indicate that these cells are not of a luminal cell type of breast cancer.

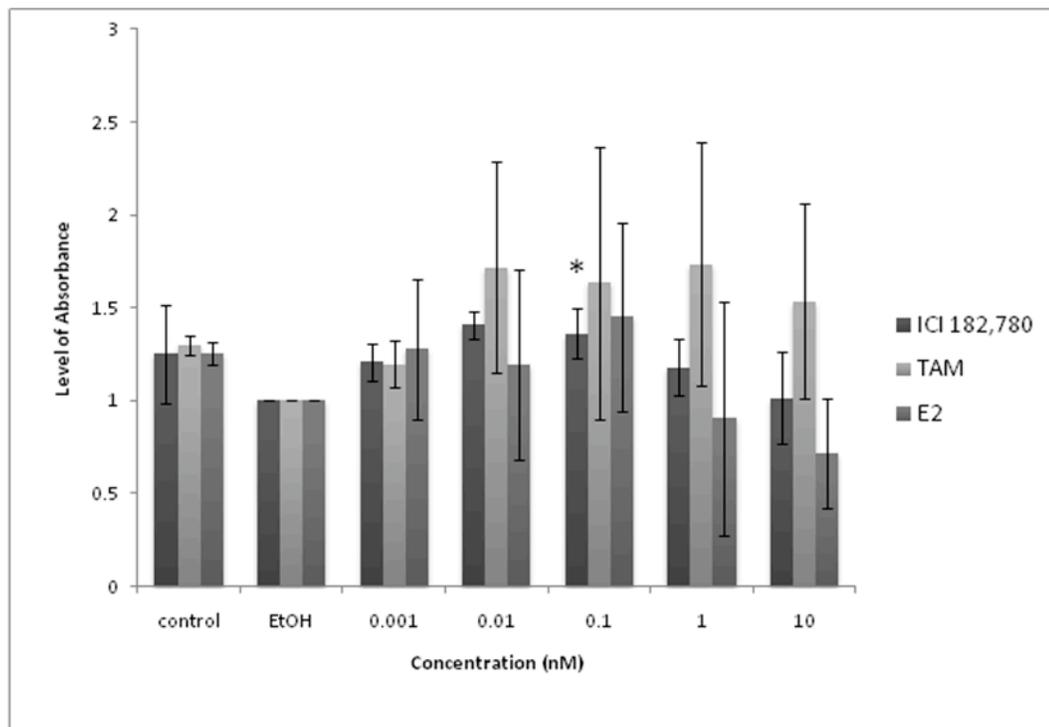


Figure 3. UMD227 cells were not responsive to estrogens or antiestrogens *in vitro*. UMD227 cells were treated with 1×10^{-12} to 1×10^{-8} M estradiol 17β (E2) or antiestrogens, ICI 182,870 (ICI) and tamoxifen (TAM). Summary of 3 experiments carried out in triplicates/experiment. The absorbance level is on the y-axis and reflects the number of cells in the well following 72 hrs of incubation. The bars represent the mean and the error bar are the standard deviation of 3 experiments which were run in triplicate/experiment. Treatment groups are compared to the ethanol control. Data were analyzed using ANOVA and significant established at p value < 0.05 as depicted by asterisk.

Immunocytochemistry further demonstrates of the presence of vimentin and smooth muscle actin in the UMD227 cell line.

The proteins that were found expressed in the UMD227 cells using western blots analysis, were further analyzed using immunocytochemistry (ICC). I wanted to determine where in the cell the proteins were being expressed as well as the intensity of the expression. In UMD227 cells, no signal was detected with the CK6 antibodies (no data shown) but ICC showed vimentin and SMA expressed in the cytoskeleton (See fig.5). The ICC supports the finding in the western blot that SMA and vimentin are expressed in the UMD227 cells. The control human cell line, MDA-MB-231 cells expressed vimentin in their cytoskeleton whereas the control human cell line, MCF-7 cells had no evidence of vimentin in their cytoskeleton (See fig 4). These results come

with the understanding that the vimentin antibodies are not the same antibody used in the western blots. The vimentin used in ICC was a gift from Dr. Jon Holy, which he acquired from Developmental Studies Hybridoma Bank, Iowa City, IA.

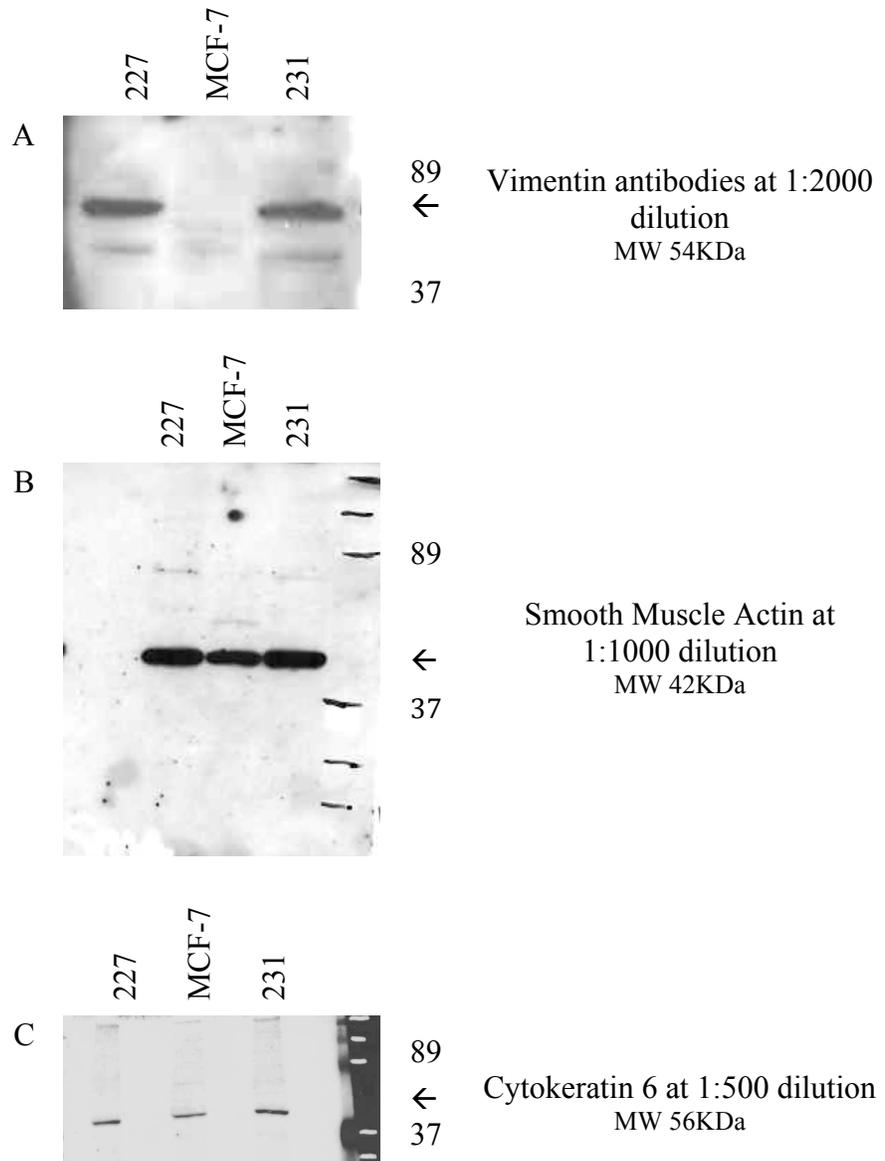


Figure 4. (A) Shows vimentin present in 227s and MDA-MB-231s, and not the MCF-7s. (B) SMA appears to be present in all 3 cell lines as well as CK6 in (C).

Unfortunately, the CK6 antibody appears not to work for the ICC assay, even though it was specific for western blots and ICC. No reactivity was found in the control cell line, MDA-MB-231's, which could indicate the CK6 antibodies, were not working under the conditions carried out. Previous published data demonstrate CK6 expression in MBA-MD-231 cells (Trask, Band et al. 1990). ICC analysis indicates that the MCF-7 was negative for SMA, which is a contradiction to the results from the western blot data. In spite of these ambiguities, these data indicate that the UMD227 cells are more similar to MBA-MD-231 cells than MCF-7 cells and can be of either myoepithelial or mesenchymal like or of progenitor cell lineage.

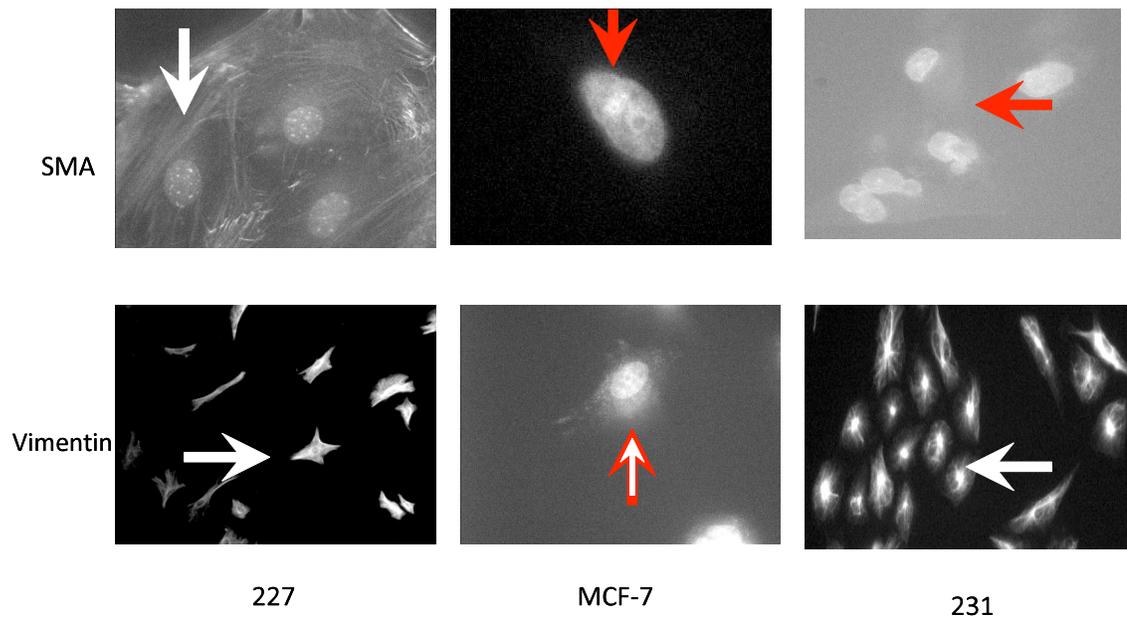
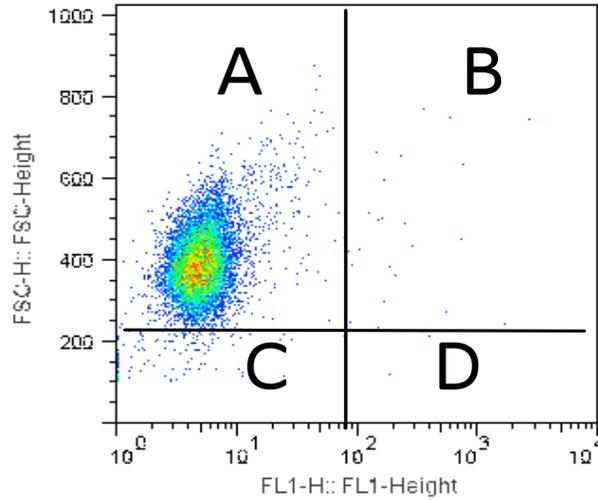


Figure 5. Immunocytochemistry was done to further support the results found with Western Blots. The top half of the panel shows smooth muscle actin (SMA) staining of the UMD227 cells compared to MCF-7 and MDA-MB-231 (using oil immersion magnification for all 3 cell lines). The bottom half of the panel shows the vimentin staining of the same cell lines. For vimentin staining, MCF-7 cells were photographed using oil immersion at 60x magnification, whereas MDA-MB-231 and UMD227 cells were at 20x magnification. The white arrows indicated the staining of the extracellular filaments in the cells. The red arrows point to the cells that were negative for either SMA or vimentin. Red and white arrow points to the background noise of the MCF-7 staining of vimentin.

Table 3. A summary of the flow cytometry data. Quadrant A represents the negative or low expressing population. Quadrant B represents the positive or high expressing population (see inserted picture). 3 experiments were done using the same antibodies and cell lines. The table summarizes the number of cells found in quadrant A and B of each experiment. The total number does not include the cells found in quadrant C and D. As those cells are regarded as possible fragments, based on forward scatter or size of cells.



MBA-MD-231			MCF-7			UMD227-R2		
Experiment 1			Experiment 1			Experiment 1		
Antibodies	Quadrant A	Quadrant B	Antibodies	Quadrant		Antibodies	Quadrant	
Isotype	94.68	1.01	Isotype	A	Quadrant B	Isotype	A	Quadrant B
SMA	0.28	93.09	SMA	91.67	1.13	SMA	97.38	0.16
CK6	1.11	90.52	CK6	1.3	91.54	CK6	0.59	96.96
Vimentin	89.86	1.73	CK6	35.17	58.52	CK6	14.48	82.68
			Vimentin	92.95	0.19	Vimentin	97.82	0.33
Experiment 2			Experiment 2			Experiment 2		
Antibodies	Quadrant A	Quadrant B	Antibodies	Quadrant		Antibodies	Quadrant	
Isotype	94.08	4.51	Isotype	A	Quadrant B	Isotype	A	Quadrant B
SMA	0.36	97.88	SMA	90.97	2.5	SMA	93.16	0.72
CK6	1.65	96.6	SMA	1.34	92.25	SMA	1.86	92.66
Vimentin	88.92	9.77	CK6	29.25	62.3	CK6	2.25	92.97
			Vimentin	92.37	0.89	Vimentin	94.61	0.35
Experiment 3			Experiment 3			Experiment 3		
Antibodies	Quadrant A	Quadrant B	Antibodies	Quadrant		Antibodies	Quadrant	
Isotype	92.68	4.01	Isotype	A	Quadrant B	Isotype	A	Quadrant B
SMA	2.31	93.35	Isotype	54.55	40.23	Isotype	89.22	0.28
CK6	2.58	93.77	SMA	2.91	90.54	SMA	3.58	86.19
Vimentin	91.41	5.18	CK6	81.85	12.35	CK6	21.89	68.14
			Vimentin	95.13	0.09	Vimentin	90.54	1.17
Average			Average			Average		
Isotype	93.81	3.18	Isotype	79.06	14.62	Isotype	93.25	0.39
SMA	0.98	94.77	SMA	1.85	91.44	SMA	2.01	91.94
CK6	1.78	93.63	CK6	48.76	44.39	CK6	12.87	81.6
Vimentin	90.06	5.56	Vimentin	93.48	0.39	Vimentin	94.32	0.62

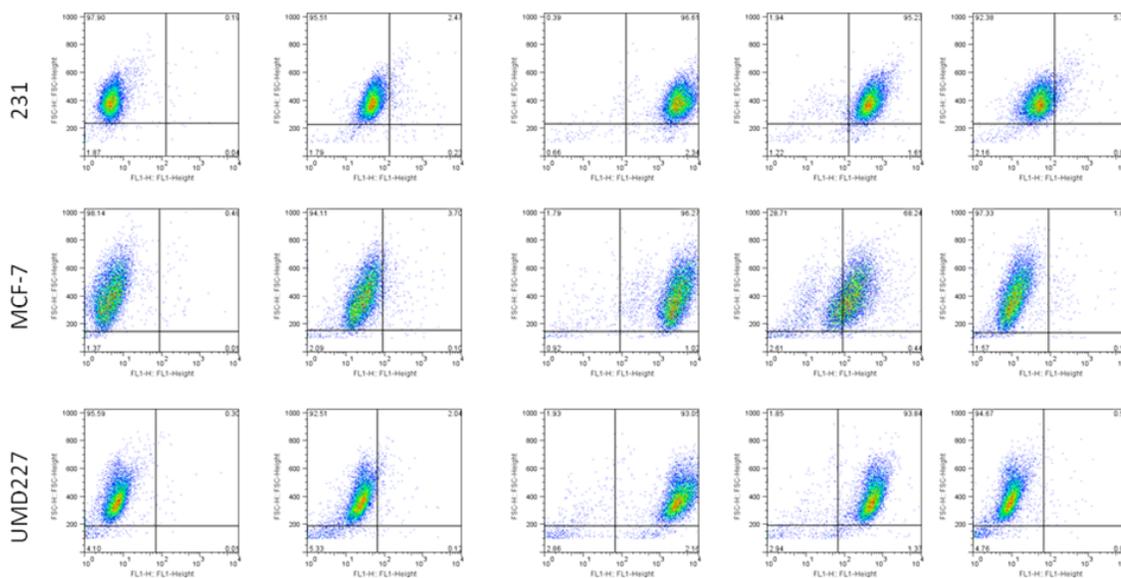


Figure 6. The results of the flow analysis of the MDA-MB-231, MCF-7 and 227 cell lines. The columns are the antibodies that were analyzed, except the control group which no antibodies were added. The quadrant lines were placed based on the isotype results. Forward scatter is on the y-axis and the intensity of the fluorescent of the antibodies is on the x-axis.

The flow cytometry show that the UMD227 cell line expresses markers characteristic of epithelial-mesenchymal transition and/or of myoepithelial cells.

The flow analysis was done to further test SMA, CK6 and vimentin in UMD227 cells. Flow cytometric analysis revealed that the epithelial markers, SMA and CK6, were highly expressed in the UMD227 population as well as the MDA-MB-231 and MCF-7 cell lines. When compared to the isotype control, an average (n=3) of 91% and 81% of the UMD227 cell population expressed SMA and CK6 respectively. As a comparison, about 90% of the MDA-MB-231 cell population expressed SMA and CK6, and 90% of the MCF-7 cell population expressed SMA and less than 50% of the cells expressed CK6. Table 3 summarizes the population of cells that expressed the antibodies at a negative/low level and positive/high level from each experiment. Figure 6 shows the results of one of the experiments with all 3 cell lines and the 5 different antibody treatments. As with the western blot data, flow cytometric analysis for SMA revealed expression in MCF-7. The expression of vimentin was not above background in all 3 cell lines including MDA-MB-231 which my western blot data previously shown to express this protein. A different vimentin antibody was then purchased for flow cytometry

specifically. There were a total of 3 different vimentin antibodies used from 3 different companies. The western blot and ICC gives strong support of the presence of vimentin in UMD227 cells and MDA-MB-231 cells, regardless of the failure of the antibody used for the flow cytometry data.

To further categorize the UMD227's; two commonly used surface markers that are used to distinguish epithelial cell subpopulation of stem, progenitor and mature differentiated cells were evaluated; CD24 and CD49f. Past studies have shown that CD24 provides information about the invasiveness of a tumor in humans (Al-Hajj, Wicha et al. 2003). Also, cells expressing low levels of CD24 are known to produce tumors when transplanted in a mouse, in small numbers suggesting they delineate a tumor cell populations (Al-Hajj, Wicha et al. 2003). Other studies have shown that CD24 is a luminal cell marker in humans and a pan epithelial marker in mice (Stingl, Eirew et al. 2006). The evaluation of the UMD227 cells for CD24, if expressed at high levels, depicts luminal cell and, if expressed at low levels, depicts myoepithelial cells. Because there are no single CD24 antibody that binds both mouse and human protein, I incorporated a positive murine cell line, 4T1 cells (Kruger, Kaplan et al. 2006). 4T1 cells are a known epithelial cell line isolated from a Balb/c mouse mammary gland tumor, (according to the ATCC website, <http://www.atcc.org/>). UMD227 and 4T1 cells stained with CD24, for 4T1 cells were positive for CD24 as expected (Kruger, Kaplan et al. 2006) and UMD227 cells appear mostly or entirely negative for CD24 staining (Figure 7).

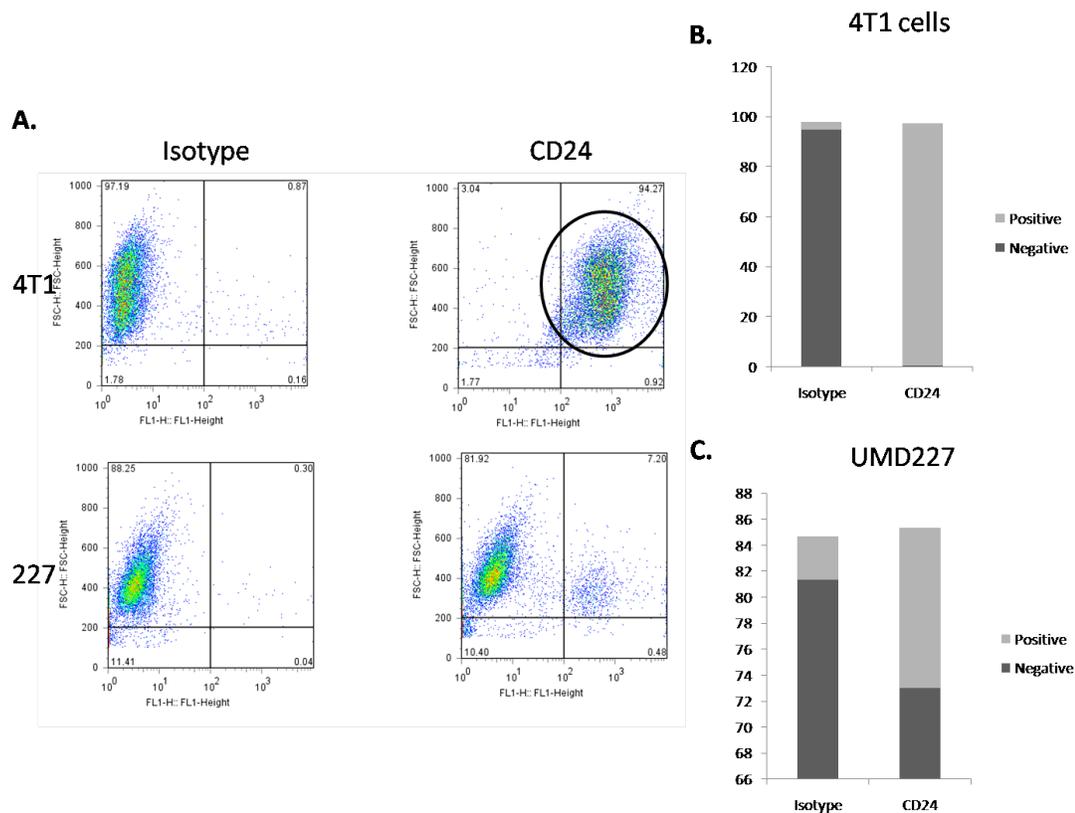


Figure 7. Flow cytometry analysis confirming the effectiveness of the CD24 antibodies, using 4T1 mice cell line as a control. A. Shows the results of the 4T1 expressing 94% of the population positive for CD24. B and C is the histogram representation of 4T1 and UMD 227 cells flow cytometry results using isotype and CD24. The results indicates that the CD24 antibodies work.

Stingl (2006) also looked at how cells from the mammary gland could be separated based on combined expression levels of CD24 and CD49f (refer to table 4). The data presented in this study suggest that a very small subpopulation of UMD227 expressing CD49f. The fraction of cells which express the cell surface marker, CD24, is small for both MDA-MB-231 and UMD227 cells. While most of MCF-7 cells expressed CD24 (see fig.8). The MDA-MB-231 cells were positive for CD49f whereas MCF-7 cells had a moderate fraction of the population that expressed CD49f. The CD24 and CD49f results indicate that the UMD227 cells have a small sub-population of cells expressing these markers and that the general population of cells is not of epithelial-like in their make-up.

Table 4. The cell population identification based on the expression of CD49f and CD24 in murine mammary gland (Stingl J et al, 2006).

CD49f ^{hi} CD24 ^{med}	Mammary stem cells
CD49f ^{lo} CD24 ^{med/lo}	Myoepithelial cells
CD49f ^{lo} CD24 ^{hi}	Luminal cells

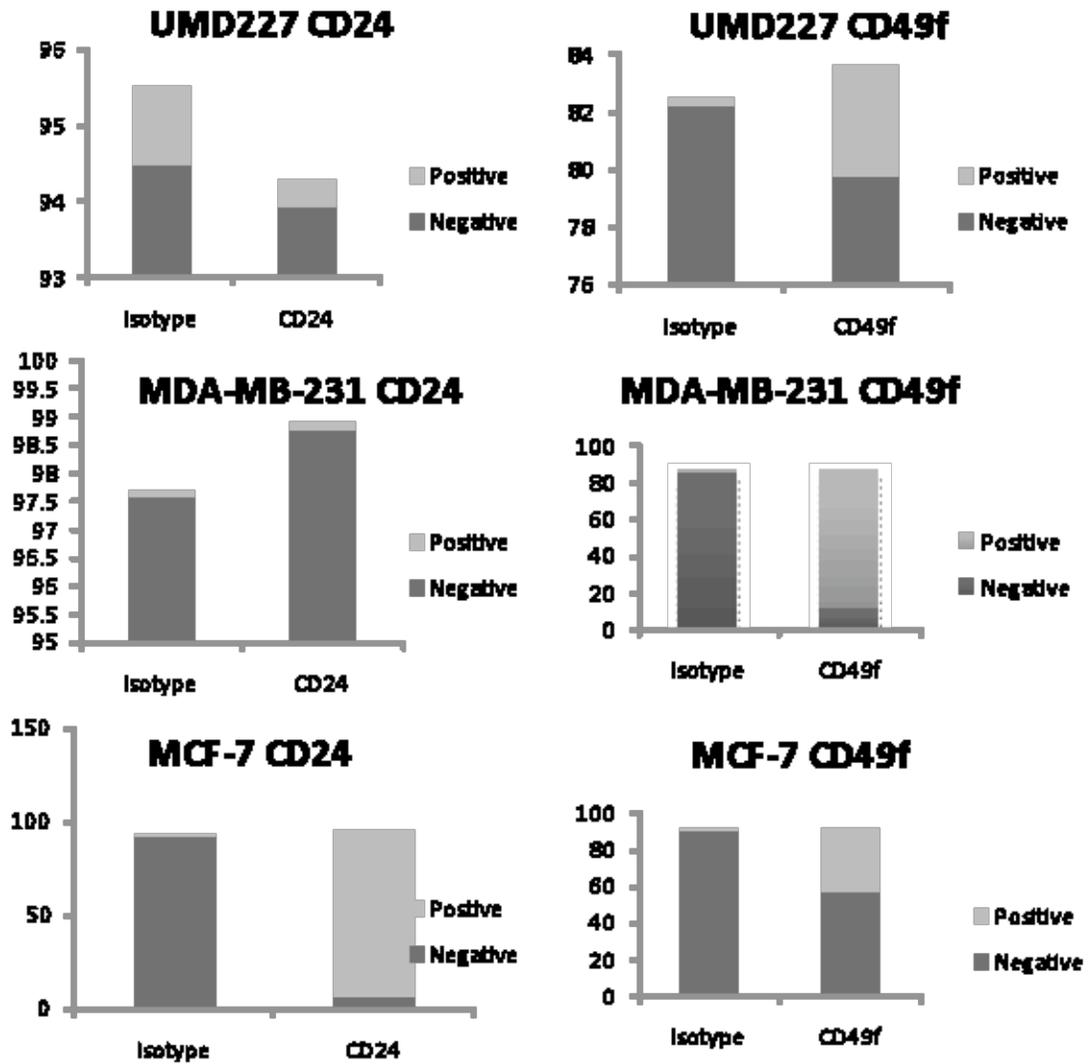


Figure 8. Histogram representing the flow cytometry results using the CD24 and CD49f antibodies on all 3 cell lines.

Discussion

The goal of this study was to characterize the UMD227 cell line and the hypothesis that these cells emulate a basal cancer cell phenotype. To accomplish this goal, I generated a molecular profile and compared it to two well characterized human breast cancer cell lines, MCF-7 and MDA-MD-231. Breast cancer is heterogeneous and although there are many human breast cancer cell lines that represent this heterogeneous nature including the basal-like phenotype, there are very few murine breast cancer cell lines available

Most cells in UMD227 tumors are ER- but a low number are ER+. One goal of my thesis was to determine whether *in vitro* growth in UMD227 cells was influenced by estradiol 17β or estrogen antagonists, or antiestrogen. The results demonstrate a lack of estrogen sensitivity. This concurs with *in vivo* data for which transplanted UMD227 cells grew equally well in males as in females with similar incidence and latency. One treatment concentration of ICI suggests a stimulation effect but this seems to be an artifact due to assay related factors; there are no reports of mitogen response of this pure antiestrogen and such an effect was not seen at higher and lower concentration of ICI, in the SRB assay. Clinically, human breast cancers with low level expression of ER are often *de novo* resistant to estrogen antagonists. Our *in vitro* results especially important as in some cases of breast cancer, tamoxifen can act as agonist, which is not the case with UMD227 cells. These data concur with additional evaluations done in our laboratory, including flow cytometric cell cycle analysis of UMD227 cells, which also did not identify effects of estradiol, tamoxifen and ICI on distribution of cells in cell cycle phases (A.Skildum, unpublished data).

In addition, due to their low expression of ER and insensitivity to estrogen, the data suggest that the UMD227 cells share features with basal-like cancer cells (Irvin and Carey 2008), as well as cells undergoing EMT. Tumors that are devoid of ER are typically of basal cell type (Molyneux, Regan et al. 2007). Western blot analyses of UMD227 cells indicate that low expression for epithelial growth factor receptor (EGFR) and lack of HER2 overexpression (A. Gingery, unpublished data). This further supports the characterizations UMD227 cells as basal-like.

Also supporting the basal-like molecular phenotype of UMD227 cells is that of the cell shape was found to be spindle shaped or spindloid, *in vitro* as well as when making up a tumor. The literature classifies spindloid tumors into epithelial (carcinomas) versus mesenchymal (sarcomas) types by the expression of cytokeratins (Thiery 2002). CK6 expressing epithelial cells in the mammary gland are the non-luminal cells including myoepithelial cells and basal cells. CK6 expression indicates that the UMD227 cells can be classified as epitheloid and therefore the cancer type is similar to human breast cancer known as spindloid carcinomas.

I chose two different types of human breast cancer cell lines for which to compare molecular features of UMD227 cells, because tumors from murine mammary cell lines are readily available to the research community. Murine cancer cell lines are important tools for investigating the role of immune cells in tumorigenesis, as human cell lines can only be xenografted-the host is always immunocompromised, which diminishes risk of tissue rejection. The majority of the breast cancer cell lines are of human origin and many have well characterized *in vitro* and *in vivo* traits. MCF-7 cells are of luminal type expressing ER⁺/PR⁺ and are non-invasive cells that grow in colonies. Meanwhile, the MDA-MB-231 cells are ER⁻/PR⁻ and express markers of EMT typical of myoepithelial/mesenchymal cells, and are aggressive and invasive (Hendrix, Seftor et al. 1996). In addition, the morphology of MDA-MB-231 cells is spindloid, similar to UMD227 cells and tumors (Figure 9). UMD227 cells share similarities to MCF-7 as they are both SMA positive and expression of CD49f are negative/low. But UMD227 cells also share features of MDA-MB-231 as they have low to no expression of ER and PR (A. Skildum et al, unpublished data), and positively express the EMT markers, vimentin and SMA. The epithelial marker, CK6 classifies them as epithelial cell, and yet the general population is low/negative for CD24. The fact that UMD227 cells express normal myoepithelial cells typically express CD24 although at lower levels than luminal cells. However, myoepithelial progenitor and stem cells are CD24 low or negative and CK6 positive. Table 4 provides a summary comparison of UMD227 cells with MCF-7 and MDA-MB-231 cells.

Table 5. A characteristic profile summary of all three cell lines, based on the results found in this study.

	MCF-7	MDA-MB-231	UMD227
ER/PR	+/+ *	-/- *	Low/- **
Vimentin	-	+	+
CK6	+ med	+hi	+hi
SMA	+	+	+
CD24	+hi	-	Very small amount
CD49f	low	hi+	-
Morphology	Non-spindled shape	Spindle shaped	Spindled shaped

*Information found on the ATCC website: www.atcc.org

** Findings from A. Skildum and O. Zhdankin (unpublished data)

In summary, this investigation demonstrates that the UMD227 cells have properties that are seen in myoepithelial cells (vimentin, SMA, CK6 expression, and spindloid morphology) and epithelial-mesenchymal transition (vimentin, SMA, absence of luminal markers CK18 and MUC-1). EMT cells normally function during embryogenesis and wound healing (Mani, Guo et al. 2008). But it is also thought that EMT plays a major role in aggressiveness of the tumor and tumor metastasis (Mani, Guo et al. 2008). To further investigate the extent of EMT in UMD227 cells, additional specific EMT markers such as fibronectin, or N-cadherin and the elevated expression of EMT transcriptional regulators, Slug and Twist (Yang, Bielenberg et al. 2009) (Hollier, Evans et al. 2009) could be evaluated.

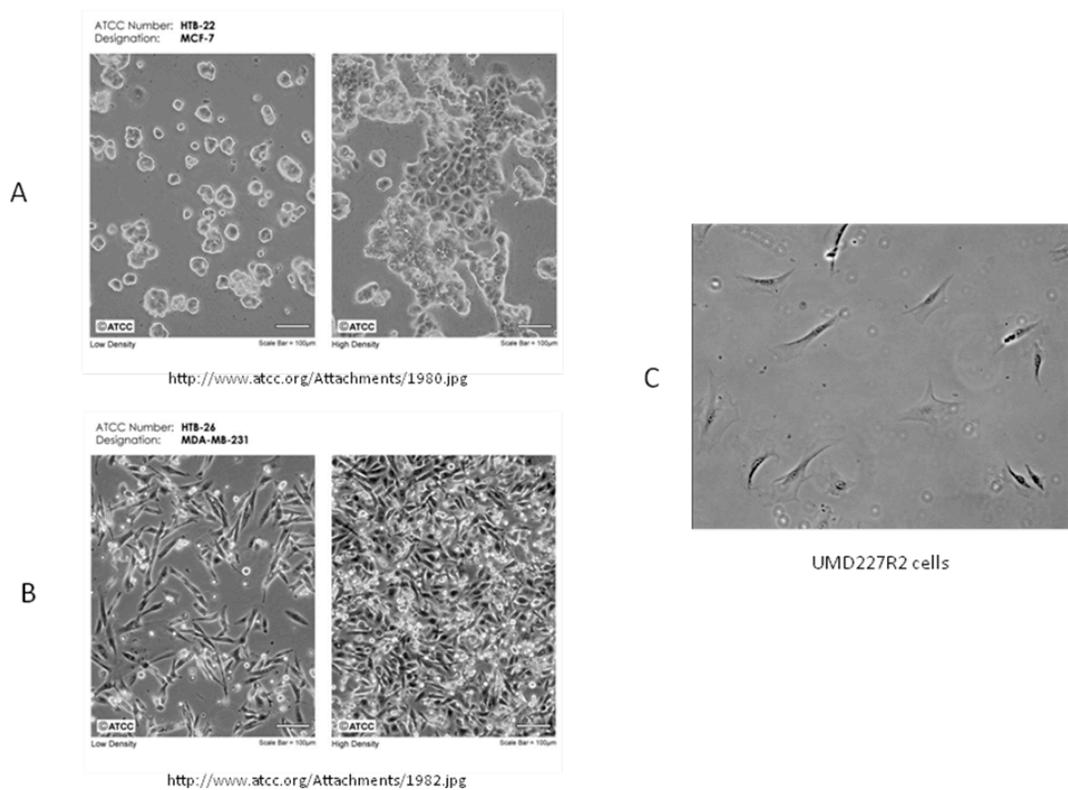


Figure 9. Morphology pictures of MCF-7, MDA-MB-231 and UMD227R2 cells. A) MCF-7 cells grow in clusters and the shapes are more circular. B) MDA-MB-231 cells are long and spindled in shape. C) UMD227 cells are more similar to the 231s in respect to the MFC-7 cells.

Conclusion

As a consequence of having a large capacity to remodel and regrow, mammary glands are vulnerable to cancer development. Because of that, the relevance of this study was to provide another tool to study the second leader of cancer death in women today; breast cancer. The evidence gathered classifies the UMD227 cells in the basal-like cell category, but does not clearly tell us whether the UMD227 cells are progenitor myoepithelial or differentiated myoepithelial cells. They are positive for CK6 which indicates epithelial origin, but CK6 is also expressed by progenitor cells. The UMD227 cells were also positive for SMA, a characteristic of EMT cells and/or myoepithelial cells. UMD227 cells are unique tools for modeling basal-like cancer.

Future Studies

The purpose of this study was to characterize the cancer cell line, UMD227 that was isolated from a murine mammary tumor. These characterizations indicate the UMD227 cell line reflects a basal-like tumor type that can be insensitive to estrogen modulation. Further characterizations would help define the classification of UMD227 cells thereby better defining the utility of this cell line for investigating cancer biology, prevention and therapy. Data generated in this thesis provides the foundation for further investigation of flow cytometry markers as a method for defining the tumor repopulating cells. The tumor repopulating cells within a dish is defined as those cells that contribute to the formation of a tumor and upon transplant of the bulk of the tumor consist of cells that are phenotypically different and not capable of forming tumors. It has been proposed that these cells within the bulk of a tumor which are capable of metastasis. Although therapeutics are often good at removing the cells which make up the bulk of the tumor, often some cells persist. It is thought that the tumor repopulating cells are highly resistant to conventional therapeutics, specifically basal tumors. Future goals include dual labeling of cells to distinguish the CD49f and CD24 expression levels to help further define the UMD227 cells.

Caveat

Vimentin is mainly found in cells that are highly invasive and metastatic (Hendrix, Seftor et al. 1996), such as the MDA-MD-231. MCF-7 cell line is considered to be non-metastatic and feebly invasive. Surprisingly, the MCF-7 cell lines were found to express SMA. There is no literature found supporting this finding in which SMA was evaluated in these cells and not found. MCF-7 also was found to express CK6 on the western blots. However, CK6 has several isoforms during mammary development; CK6a is found in the body cells and luminal epithelial cells ((Takahashi, Paladini et al. 1995). This could explain why we observe CK6 in MCF-7 cells. In addition, compared to earlier published results, the sensitivity of the detection kits likely makes it possible to see even small amounts of protein, e.g. SMA.

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Appendix A: Abbreviations

227	UMD227R2
231	MDA-MB-231
NRL-TGF- α	neu-related lipocalin transforming growth factor- alpha
TEB	terminal end buds
TDLU	terminal ductal lobuloalveolar
ER	estrogen receptor
PR	progesterone receptor
RTK	receptor tyrosine kinase
EGF	epithelial growth factor
TGF- α	transforming growth factor- alpha
MAPK	mitogen activated protein kinase
SMA	smooth muscle actin
CK6	cytokeratin 6
CK18	cytokeratin 18
MUC-1	mucin 1
SRB	sulforhodamine B colorimetric assay
PBS	phosphate buffer saline
TBS-T	tris buffer saline-tween 20
FITC	fluorescein isothiocyanate
BSA	bovine serum albumin
SERM	selective estrogen receptor modulator
ATCC	American type tissue collection

Appendix B: Summary of where the antibodies were purchased for Western Blot, Immunocytochemistry and Flow Cytometry.

Vimentin	SMA	CK6	
Abcam	Abcam	Novous Biologicals	Western Blot
DSHB *	Abcam	Abcam/Novous	Immunocytochemistry
Santa Cruz	Epitomics	Novous Biologicals	Flow Cytometry

*Developmental Studies Hybridoma Bank

Appendix C: Recipes

Stain buffer recipe:

Dulbecco's PBS

1% FCS

0.09% sodium azide

pH 7.4-7.6

Sample buffer recipe:

7.4 ml water

4.0 ml 50% glycerol

2.0 ml 1M Tris pH6.8

2.0 ml 10% SDS