THE ROLE OF RHOC

IN CHOLESTEROL TRAFFICKING

IN BREAST CANCER CELLS

by

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ABBREVIATIONS

ABCA1	ATP-binding cassette sub-family A member 1
ABCG1	ATP-binding cassette sub-family G member 1
ABCG4	ATP-binding cassette sub-family G member 4
ANOVA	Analysis of variance
ApoE	Apolipoprotein E
BMI	Body mass index
BSA	Bovine serum albumin
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
FMNL-2	Formin-like protein 2
GAP	GTPase activating protein
GDI	Guanine dissociation inhibitor
GDP	Guanosine diphosphate

GEF	Guanosine nucleotide exchange factor
GTP	Guanosine triphosphate
HDL	High density lipoprotein
HMGCR reductase	3-hydroxy-3-methylglutaryl-coenzyme A
IBC	Inflammatory breast cancer
IDL	Intermediate density lipoprotein
LDL	Low density lipoprotein
LDL-R	Low density lipoprotein receptor
LPDS	Lipoprotein-deficient serum
LXRα	Liver X receptor alpha
LXRβ	Liver X receptor beta
MAPK	Mitogen-activated protein kinase
MMTV-PyMT	Mouse mammary tumor virus-polyoma middle T
NMR	Nuclear magnetic resonance
PBS	Phosphate buffered saline
QPCR	Quantitative polymerase chain reaction
Ras	RAt sarcoma
RFP	Red fluorescent protein

Rho	Ras homologous
ROCK	Rho-associated protein kinase
RXR	Retinoid X receptor
SR-BI	Scavenger receptor class B type I
SREBP	Sterol regulatory element binding protein
VLDL	Very low density lipoprotein

ABSTRACT

Each year in the United States, greater than 280,000 women are diagnosed with breast cancer, making this cancer the most common cancer occurring in American women. Although there are many classifications of breast cancer, inflammatory breast cancer (IBC) has proven to be the most deadly. Some important risk factors that have been associated with the development of extremely aggressive breast cancers, such as IBC, include obesity and diet. The effect of obesity on breast cancer diagnosis and survival is especially evident in the United States, where the over-consumption of high-fat foods continues to contribute to obesity in the nation. With this observation, the relationship between breast cancer and cholesterol has been explored more and more over the last decade. The present study investigated the differences, in cholesterol storage and metabolism, between breast cancer and non-cancerous cell lines. The results of this study support observations seen in other reports which show that cancerous breast tissues display a dysregulation of cholesterol homeostasis, resulting in increased cholesterol content. Additionally, the findings show a connection between the breast cancer cells' dependence on extracellular cholesterol and the environment from which they were derived. Furthermore, the results of this study have allowed us to put forth a new model which highlights the idea that breast cancer cells are more likely to invade or migrate when they are not able to obtain necessary cholesterol from their extracellular environments. This

implication could serve to be important for determining novel methods of preventing breast cancer metastasis.

Chapter 1

INTRODUCTION

1.1 Breast Cancer

Breast cancer is the most common cancer in American women in the United States, where there is a 1 in 8 chance of a woman developing the disease in her lifetime. In 2011, it is estimated that greater than 280,000 women in the United States will be diagnosed with breast cancer. Of these women about 57,000 will have in situ breast cancer and approximately 230,000 women will have invasive breast cancer. Invasive breast cancers are those that are no longer confined to the area in which they started, but are invading surrounding tissues. In situ breast cancers are confined to the lobules or ducts where they started [American Cancer Society, 2011]. In 2010 more than 40,000 women died as a result of their breast cancer. Over a few decades, the incidence of breast cancer in the United States has been on a steady rise. Nevertheless, breast cancer mortality rates are slowly decreasing. The trends seen in incidence and mortality rates are currently attributed to advances in detection and treatment [Carlson, 2009].

The most common sign of breast cancer is a painless lump in the breast (Figure 1.1). Pain may sometimes accompany the cancer, but is less common. When breast cancer is suspected, physicians may use mammograms, ultrasound, and/or MRIs (Figure 1.1a) to have a better understanding of the disease, but a biopsy is

essential to confirm the disease [Günhan-Bilgen, 2002; American Cancer Society, 2011]. Once the nature of the cancer is established, doctors will decide on the best treatment option. Treatment may include surgery, chemotherapy, radiation therapy, and/or hormone therapy [Carlson, 2009]. No matter the type of cancer, treatment is usually aimed at controlling the disease, extending the life of the patient, and/or preserving the quality of life of the patient [Shenkier, 2004].

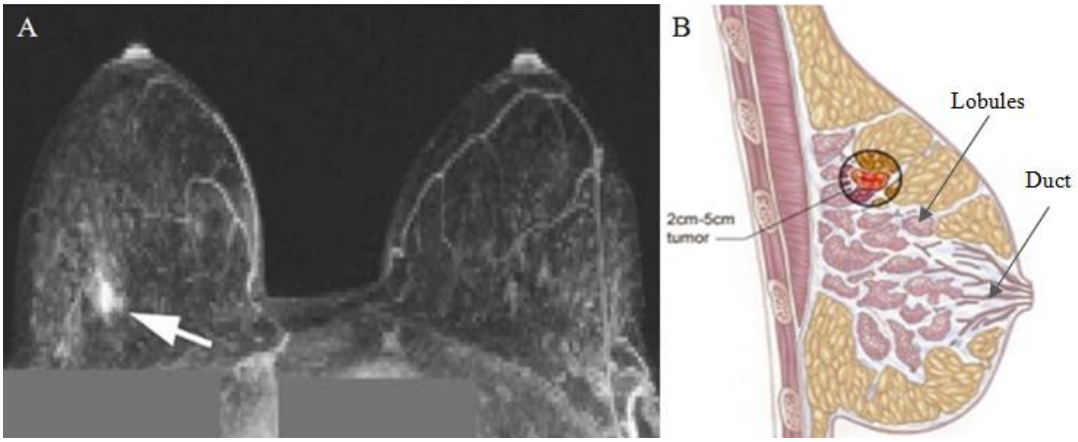

Figure 1.1 Common Breast Cancer Presentation (A) MRI of a solid breast tumor, depicted by arrow [adapted from Seewaldt, 2007]. (B) A classic sign of breast cancer is a hard, palpable lump [adapted from Akiavintage.com, 2011].

Breast cancer patient survival is dependent on many factors. The stage of breast cancer and the size of the tumor at diagnosis is a major determinant of patient survival. Generally speaking, smaller tumors detected at earlier stages are associated with much better survival rates than larger tumors. According to the American Cancer Society, the 5-year survival rate for patients with localized disease is 99%, but drops all the way down to 23% for patients with distant metastasis. Additionally, the 5-year

survival rate for patients with tumors less than 2.0 cm at diagnosis is 95%, whereas the 5-year survival rate for patients with tumors greater than 5.0 cm is 63% [American Cancer Society, 2011]. A trend between age and survival has also been noted. Women diagnosed at earlier ages have a lower 5-year survival rate than women who are diagnosed at older ages.

Many factors have been linked with the risk of developing breast cancer. Some general risk factors include age, family history of breast cancer occurring in younger ages, and exposure of the chest to irradiation therapy [Carlson, 2009]. Other risks may include the use of hormone therapy, early menarche, late menopause, and obesity [American Cancer Society, 2011]. Many studies have shown that obesity is a particular risk factor in postmenopausal patients. For instance, a study of nearly 500,000 women showed that the women with the highest BMI (body mass index) were over two times more likely to develop breast cancer than women with the lowest BMI [Lorincz, 2006]. Additionally, overall lifetime weight gain has been shown to be a factor in postmenopausal women who had never been treated with a hormone replacement therapy [Morimoto, 2002]. Finally, it has been determined that as a woman's BMI increases, there is a 3% greater risk in developing breast cancer for each $1kg/m^2$ [Endogenous Hormones and Breast Cancer Collaborative Group, 2003].

There are many different types of breast cancers which are most often classified by the origin and nature of the disease. Ductal carcinomas are the most common form of breast cancer [Li, 2003]. Ductal carcinomas may be classified as in situ or invasive, but they are derived from cells in the mammary ducts [Erbas, 2006]. Lobular carcinomas are the less common than ductal carcinomas, but may also present as in situ or invasive disease [Li, 2003]. Lobular carcinomas arise from cells in the

lobules of the breast [Foote, 1941]. Inflammatory Breast Cancer (IBC) is a more rare form of breast cancer. IBCs are mostly associated with ERBB2-overexpressing or basal-like cell-of-origin types [Van Laere, 2006]. Basal-like cancers are associated with a poor prognosis, and are classified as such when they have high expressions of laminin, fatty acid binding protein, keratin 5, and keratin 17. ErbB2-overexpressing breast cancers are associated with lower survival, and are found to overexpress ERBB2, GRB7, as well as other genes [Sorlie, 2001].

1.1.1 Inflammatory Breast Cancer

Inflammatory Breast Cancer (IBC) is an extremely lethal form of locally advanced breast cancer. IBC is estimated to affect less than 6% of breast cancer patients in the United States [Cristofanilli, 2003], although it is speculated that this figure is underestimated due to frequent patient misdiagnosis. This particular form of breast cancer is associated with an extremely poor prognosis. IBC has a 10-year disease free survival rate of less than 30%, as compared to an 80% 10-year disease free survival rate for non-IBCs [Cristofanilli, 2003]. The poor prognosis associated with IBC is due to its highly aggressive nature. IBC is particularly known for its extremely rapid onset and progression, insomuch that IBC is always classified as stage IIIB or IV at the time of diagnosis, where other forms of breast cancer are more likely to be diagnosed at stage I or II [Anderson, 2005]. Additionally, IBC tends to affect patients at a younger age that non IBCs overall [Anderson, 2005].

Inflammatory breast cancer has many other unique characteristics that distinguish it from other primary breast carcinomas. Patients who have IBC experience an array of symptoms quite distinct from those of most non-IBCs. Unlike non-IBC primary breast carcinomas, IBC is not usually accompanied by a palpable

mass. Common IBC symptoms include nipple retraction, tenderness, skin that is warm to the touch, "peau d' orange," swelling, thickening and reddening of the skin (Figure 1.2) [Cristofanilli, 2003]. The unique appearance of IBC is due to tumor emboli that travel through, invade, and block the dermal lymphatics. Although tumor emboli are sometimes seen in non-IBCs, the occurrence is especially prominent in IBCs. Additionally, IBC is particularly angiogenic in nature, and has a special capacity for vascular invasion [Kleer, 2000].

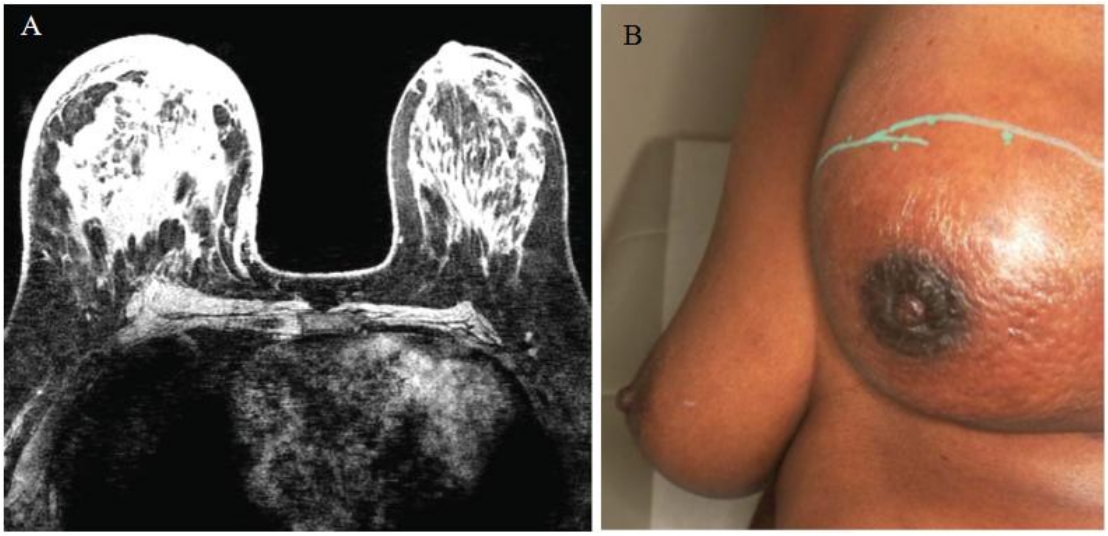

Figure 1.2 Inflammatory Breast Cancer Presentation (A) MRI of IBC (left breast) [adapted from Kidwell, 2007]. (B) Many of the classic symptoms of IBC: nipple retraction, peau d' orange, swelling and thickening of the skin, are visible [Kidwell, 2007].

Physicians diagnose IBC clinically by physical examination, and biologically by the presence of malignancy in the dermal lymphatics of skin biopsies [Kleer, 2000]. Additionally, mammograms and MRIs of IBCs generally display skin

thickening and dense breast tissue, rather than the classical tumor mass often seen in non-IBCs (Figure 1.2) [Günhan-Bilgen, 2002]. Once a patient is diagnosed with inflammatory breast cancer, treatment is aimed to contain the regional disease and prevent further metastasis [Kleer, 2000; Dagwood, 2010]. The aggressive nature of IBC calls for an equally aggressive treatment. Preferred treatment methods begin with systemic neoadjuvant chemotherapy, which is used to treat the cancer prior to surgery [Kleer, 2000, Cristofanilli, 2003]. After completion of chemotherapy, the patient may be given a mastectomy and then have radiation treatment. Alternatively, radiation may be given without a mastectomy, in an effort to control regional disease [Cristofanilli, 2003; Dagwood, 2011]. Although many advances have been made in IBC treatments, patient mortality is still extremely high.

1.2 Cholesterol Homeostasis

Cholesterol is an important lipid found in eukaryotic cell membranes, and is essential for eukaryotic life [Maxfield, 2010]. The unesterified form of this molecule, as seen in Figure 1.3, makes up about 25% of the lipids found in plasma membranes, and is responsible for membrane stability and fluidity [Beckerman, 2009]. Cholesterol also plays a role in membrane protein modulation, trafficking, endocytosis, and signal transduction [Esfhani, 1990]. Furthermore, cholesterol is an important precursor for hormones, vitamins, and bile salts [Berg, 2002].

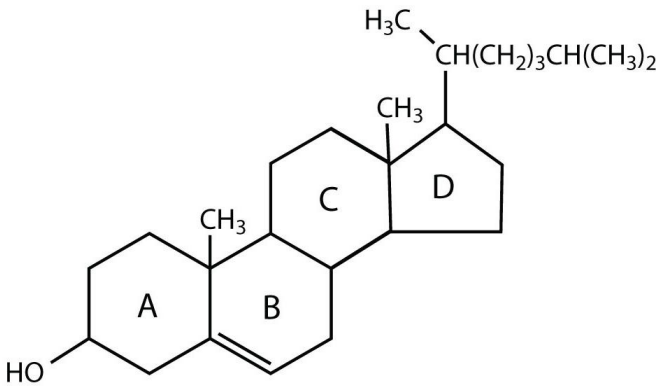

Figure 1.3 The Chemical Structure of Cholesterol. The polar OH group, orients with the polar head of phospholipids in the membrane. The non-polar hydrocarbon tail orients with the non-polar tail of the phospholipids. The four-fused carbon ring serves as the backbone for steroid molecules [Ball, 2011].

Cholesterol is such a significant molecule that it is one of the most highly regulated molecules inside the cell. Eukaryotic cells have many mechanisms that they use maintain intracellular cholesterol homeostasis. Cells can acquire cholesterol by synthesizing it *de novo* or by importing it from their extracellular environment. When intracellular cholesterol concentration becomes too high, normal eukaryotic cells will begin to export cholesterol to their extracellular environment [Pani, 2004].

1.2.1 Cholesterol: *de novo* Synthesis

The mevalonate pathway is a biosynthesis pathway that cells use to produce cholesterol, ubiquinone, dolichol, farnesol, and protein prenyl groups (Figure

1.4). When sterol levels are low, the mevalonate pathway is stimulated to produce cholesterol [Buhaescu, 2007]. Newly synthesized cholesterol is readily used in the extension of new membranes for growing and dividing cells. The relation between cholesterol synthesis and cell growth has been shown on numerous occasions. For instance, high cholesterogenic activity is seen in tissues with high rates of growth and proliferation [Esfahani, 1990]. Furthermore, studies have shown that inhibiting the mevalonate pathway has the ability to inhibit cell division [Wejde, 1992; Esfahani, 1990].

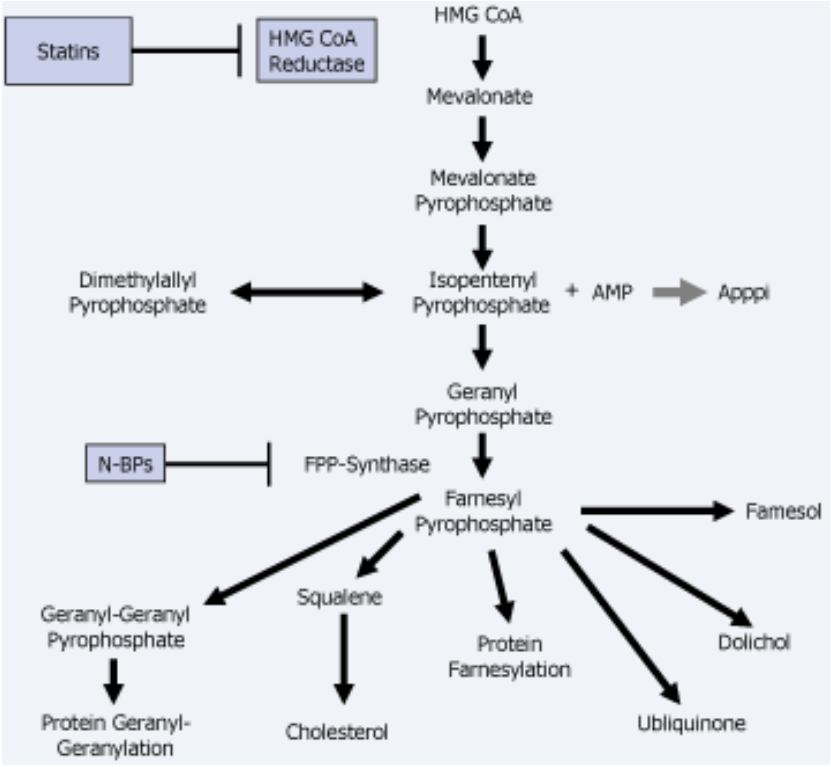

Figure 1.4 The Mevalonate Pathway. The rate-limiting reaction of the mevalonate pathway is the conversion of HMG CoA into mevalonate. This step is carried out by HMGCR, an enzyme that can be inhibited by statin drugs. The major products of the mevalonate pathway include cholesterol, protein prenyl groups, ubiquinone, and dolichol [Green, 2004].

The mevalonate pathway begins in the cytoplasm with the conversion of acetyl-CoA to acetoacetyl-CoA by acetoacetyl-CoA thiolase. Next, HMG-CoA synthase converts acetoacetyl-CoA into HMG-CoA. HMG-CoA is then transported into the ER where the rate-limiting enzyme, 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGCR), reduces HMG-CoA into mevalonate. Through an extensive sequence of reactions, mevalonate is then converted into free cholesterol. From the ER, these free cholesterol molecules are often immediately used in the plasma membrane. The cholesterol molecules may also be incorporated into lipid rafts in the Golgi, especially in cholesterol-rich domains called caveolae. The caveolae or other lipid rafts are then transported to the plasma membrane [Pani, 2004; Zhuang, 2005; Simmons, 2000].

Cholesterol production through the mevalonate pathway is mainly regulated by the activity of HMCGR, one of the most highly regulated enzymes currently studied. Low cholesterol levels are detected by the ER-membrane bound transcription factors, Sterol regulatory element binding proteins (SREBPs). Specifically, SREBP-1c and SREBP-2 contribute to cholesterol synthesis, and cause increase transcription of HMGCR and many other proteins involved in the mevalonate pathway [Brown, 1997; Buhaescu, 2007; Berg, 2002; Horton, 2002]. When cholesterol and other sterols accumulate in the cell, SREBP's are degraded and

HMGCR transcription is blocked. Furthermore, HMGCR can be degraded through proteolysis and ubiquitination, as a result of sterol accumulation. Finally, the activity of HMGCR is inhibited by phosphorylation [Berg, 2002]. It is clear that many different mechanisms are employed to maintain effective control of the mevalonate pathway through altering the activity of HMGCR.

1.2.2 Cholesterol: Receptor-mediated Uptake

In the body, cholesterol travels through the blood as a part of a lipoprotein molecule. There are 5 major types of lipoprotein particles that help to carry the hydrophobic cholesterol molecules through the bloodstream. As seen in Figure 1.5, these molecules include chylomicrons, very-low-density lipoproteins (VLDL), intermediate-density lipoproteins (IDL), low-density lipoproteins (LDL), and high-density lipoproteins (HDL) [Insel, 2010]. Chylomicrons are first formed in the intestines and eventually emptied into the bloodstream. In the bloodstream, the large chylomicrons are stripped of a large number of triglycerides, and significantly reduced in size. The smaller chylomicron molecules are then converted into vLDL by the liver. As the vLDL circulates through the blood, more triglycerides are removed and the molecule is converted in to a more dense form, IDL. Circulating IDL will begin acquiring cholesterol from circulating HDL, which is often the lipoprotein that accepts cholesterol from cells. The IDL can then be converted into LDL upon return to the liver [Insel, 2010].

Cells may use lipoproteins as a source of cholesterol, in addition to *de novo* synthesis. Most commonly, cells endocytose LDL molecules through their LDL receptor (LDL-R). Figure 1.5 shows that LDL molecules are the lipoproteins which contain the greatest percentage of cholesterol. These cholesterol-rich LDL particles

are used to transport hydrophobic cholesterol throughout the bloodstream, and other aqueous extracellular environments. As seen in Figure 1.6, LDL particles are spherical with an outer shell and an inner core, often containing over a thousand cholesterol molecules [Pani, 2004]. The outer shell is composed of a large Apolipoprotein B100 molecule, free cholesterol, and phospholipids. The outer shell is wrapped around a core of cholesterol esters and triglycerides [Orlova, 1999].

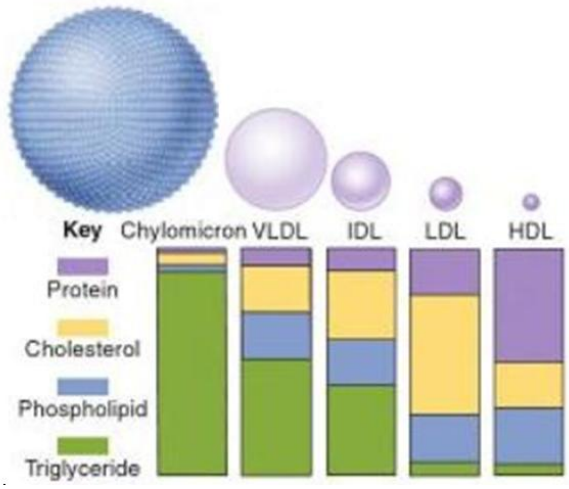

Figure 1.5 Lipoproteins. The common lipoproteins that help to carry cholesterol through the bloodstream, and the lymphatic system are chylomicron, very low-density lipoprotein (vLDL), intermediate-density lipoproteins (IDL), low-density lipoproteins (LDL), and high-density lipoproteins (HDL). LDL molecules are the most cholesterol-rich lipoprotein particles [adapted from Insel, 2010]

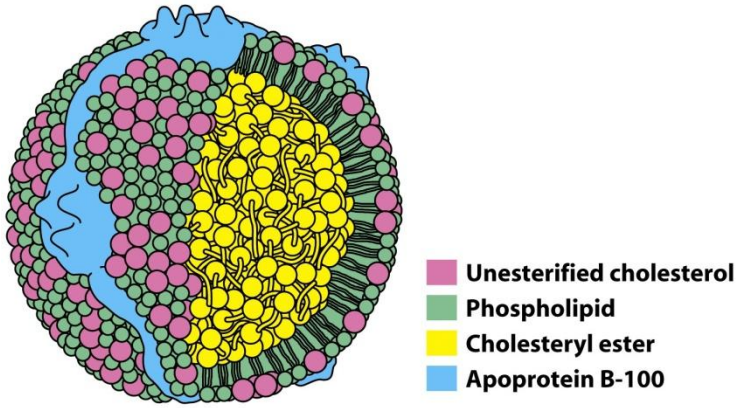

Figure 1.6 The Structure of an LDL Particle. The outer core of the LDL particle is made up of phospholipids, free cholesterol, and an Apolipoprotein B-100. The inner core contains cholesterol esters, and triglycerides [adapted from Berg, 2007].

The LDL receptor is a transmembrane protein located on the plasma membrane, usually in clathrin-coated pits. The LDL receptor has two well-known ligands Apolipoprotein E, and apolipoprotein B [Goldstein, 1985b]. LDL receptors recognize and bind to the LDL particles by direct contact to their Apolipoprotein B100 molecules. Once the LDL particle is bound, the LDL receptor is endocytosed and the LDL particle becomes enclosed in a coated endocytic vesicle (Figure 1.7). Next, the clathrin coat is removed and the vesicle fuses with and becomes an early endosome. In the early endosome, the receptor is separated from the LDL and recycled to the

plasma membrane [Pani, 2004]. Late endosomes, containing LDL particles then fuse with lysosomes, containing a wide array of enzymes. Inside the late endosome/lysosome the protein in the LDL molecule is broken down to single amino acids. Furthermore, the cholesterol esters are hydrolyzed by the acid lipases and released as free cholesterol. From here, the free cholesterol is transported out of the late endosome/lysosome with the help of NPC1 and NPC2 (Nieman-Pick type C1 and Nieman-Pick type C2) proteins, to different organelles such as the ER or Golgi [Hu, 2010]. The free cholesterol is often transported through the Golgi for use in the plasma membrane. Additionally, the free cholesterol can be reesterified in the ER for storage in the cell [Berg, 2002].

Similar to the *de novo* synthesis of cholesterol, uptake via LDL receptor is regulated by intracellular cholesterol concentration [Goldstein, 1985a; Goldstein 1986b]. Again, SREBPs have a role in the expression of LDL receptors. When cholesterol levels are low, SREBPs stimulate LDL receptor transcription. Conversely, when intracellular cholesterol is high, SREBP activity is inhibited, and LDL receptor production is decreased [Horton, 2002].

In humans, LDL receptor-mediated internalization is the most common method of cholesterol uptake, but steroidogenic cells can also use high-density lipoproteins (HDL) to obtain cholesterol. HDL receptor-mediated cholesterol uptake most often uses scavenger receptor class B type I (SR-BI), a cell surface receptor. SR-BI mediates the direct uptake of cholesterol esters from HDL molecules [Connelly, 1999]. Although the mechanism behind this process is not completely understood, it is certain that this process is quite unlike the LDL receptor process. For instance, the HDL molecule is tightly bound to SR-BI, but neither is internalized through the

endocytic pathway. Furthermore, the HDL molecule stays intact even though cholesterol esters are extracted from it, and transported into the cell. SR-BI is able to bind many types of lipoproteins, and is even able to mediate the import of cholesterol molecules that are not part of lipoproteins [Stangl, 1999]. While the details of SR-BI regulation are still being elucidated, levels of intracellular cholesterol and SREBP cleavage seem to have a role in regulating the expression of SR-BI [Trigatti, 2003].

In addition to this receptor-mediated uptake of cholesterol from HDL particles, caveolae have also been implicated in uptake of cholesterol esters from HDL [Simons, 2000]. Caveolae are cholesterol-rich microdomains in the plasma membrane that have been highly studied for their roles in signal transduction. These microdomains contain caveolin-1, 2, or 3 proteins which help to give caveolae their specific Ω-like shape, and act as a scaffolding domain for signal transduction [Simmons, 2000; Parton, 1996]. Caveolin-1 is has been shown to be highly interactive with cholesterol, and has been thought to play a role in cholesterol transport [Parton, 1996].

1.2.3 Cholesterol: Efflux

Accumulation of free cholesterol inside the cell is toxic [Simmons, 2000]. Therefore cells employ many ways of managing excess cholesterol and maintaining cholesterol homeostasis.

There are a couple of ways in which cholesterol travels bidirectionally depending on the concentration gradient. First, free cholesterol can diffuse from cell membranes to acceptor lipoproteins. More efficiently, SR-BI can aid in the efflux of

free cholesterol to proteins such as HDL and apolipoproteins. Both of these methods are used for cholesterol efflux, as well as influx [Yancey, 2003].

ATP-binding cassette transporter A1 (ABCA1) is a highly studied protein that has been linked to regulating cholesterol efflux. ABCA1 mediates unidirectional efflux of free cholesterol from cells, using ATP as a source of energy. Specifically, ABCA1 helps to transport free cholesterol to lipid-poor lipoproteins, such as HDL. Apolipoproteins such as Apolipoprotein E (ApoE) are often used to accept cholesterol from ABCA1-mediated transport, and subsequently deliver cholesterol to HDL [Yancey, 2003]. ABCA1 expression is induced by a few different transcription factors: Liver X receptors α (LXRα), LXRβ, nuclear orphan receptors, and/or retinoid X receptor (RXR). When intracellular cholesterol levels are high, these transcription factors work together to increase ABCA1 production [Wang, 2003; Yancey, 2003; Shultz, 200].

More recently, two more ATP-binding cassette transporters have been discovered to play a role in cholesterol efflux. ABCG1 and ABCG4 are proteins that have been shown to mediate cholesterol efflux to large HDL particles [Wang, 2004].

It is clear that many factors play a role in the essential process of cellular cholesterol homeostasis. The summation of the processes discussed is shown in Figure 1.7.

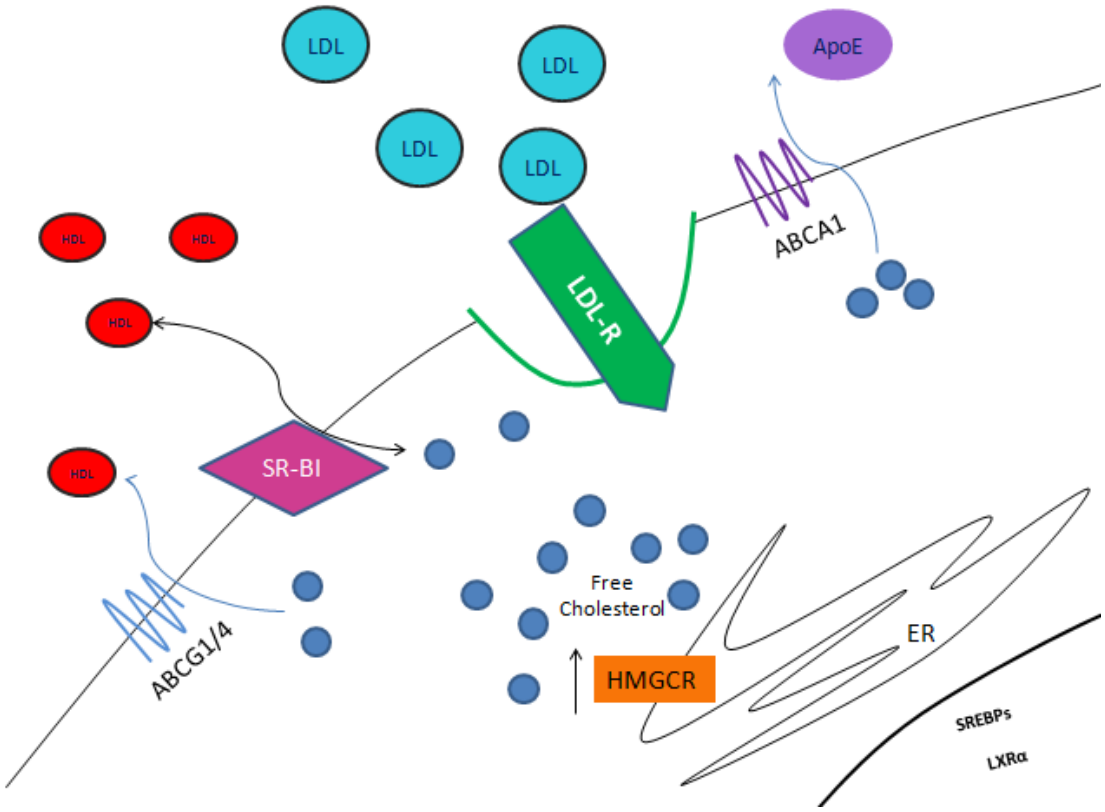

Figure 1.7 Intracellular Cholesterol Homeostasis. This image summarizes many of the processes involved in maintaining cholesterol homeostasis.

1.3 Cholesterol in Cancers

For greater than 100 years, scientists have been presented with evidence that a link exists between cancer and cholesterol. Although it is evident that this link exists, a clear understanding of the processes involved continue to evade the scientific community.

Many studies have been conducted to look at the possible relationship between circulating plasma cholesterol levels and cancer incidence. A number of studies have shown a correlation between low plasma cholesterol levels and a high overall cancer incidence [Williams, 1981; Wallace, 1982; Sherwin, 1987; Garcia-Palmieri, 1981; Cambien, 1980; Fiorenza, 2000]. Epidemiologic studies have shown that, leukemia is connected with low plasma cholesterol levels. Additionally, low plasma cholesterol is closely associated with lung cancer and colon cancer in males [Kritchevsky, 1992; Williams, 1981]. In females, cervical and breast cancer (in women ages 50 and under) have been strongly linked with low cholesterol serum levels [Kritchevsky, 1992; Vatten, 1990]. This observed relationship between low serum cholesterol and cancer incidence has caused scientists to support the proposition that malignant disease causes decreased circulating serum cholesterol levels [Jacobs, 2009].

Furthermore, many studies have shown that cholesterol levels are higher in solid tumors, of numerous cancer types, when compared with normal tissue [Dessi, 1994; Freeman, 2004]. The accumulation of cholesterol in solid tumors has been show in breast cancer, prostate cancer, oral cancer, gastrointestinal cancer, and others [Dessi, 1994; Freeman, 2004; Li, 2006; Mady, 2000]. Although the relationship between cholesterol accumulation and cancer progression is poorly understood, these findings suggest that malignant tissues are finding a benefit in accumulating cholesterol. The observed decrease in total serum cholesterol levels, in conjunction with elevated cholesterol content of malignant tissues, further point to the idea that malignant tissues collect and store cholesterol.

1.3.1 Cholesterol Homeostasis is Dysregulated in Many Cancers

As described above, intracellular cholesterol is tightly regulated, but when cells become malignant we often see a dysregulation in their cholesterol homeostasis [Li, 2006; Duncan, 2004]. One of the first studies showing a malfunction of cholesterol homeostasis mechanisms in cancerous tissues was completed in 1964. In this study, malignant liver tissue in mice, fed cholesterol-rich diets, showed an increase in cholesterol biosynthesis instead of the reduction that was so often seen in normal tissues [Siperstein, 1964]. Since then similar observations have been noted in many different malignant tissues [Esfahani, 1990]. In many cases, HMG-CoA reductase activity is up-regulated in malignant tissues [Wejde, 1992]. For instance, increased HMGCR activity has been shown in castrate-resistant prostate cell lines, as well as in colorectal cancer [Twiddy, 2003; Caruso, 2005]. High HMGCR activity results in increase production of mevalonate. Such an increase in mevalonate has been observed in lung, breast, leukemia, and many other cancerous cell lines [Hardwood, 1991; Duncan, 2004; El-Sohemy, 2005; Bennis, 2006; Li, 2006]. The reason for this upregulation of mevalonate is not completely understood. Nevertheless it has been hypothesized that mevalonate is increased in an effort to meet the metabolic needs of growing and proliferating cancer cells. Additionally, a study done in 2004 showed that administering mevalonate to mice growing MDA-MB-435-derived breast tumors helped to promote the growth of the tumors [Duncan, 2004]. The upregulation of HMGCR and mevalonate seen in these different types of cancers further supports the idea that cholesterol is an important aspect of cancer cell survival and proliferation.

1.3.2 Cholesterol in Breast Cancer

Environmental influence is strongly implicated in breast cancer. As mentioned in section 1.1, obesity has been linked with breast cancer development in women. Similar trends are being seen in both *in vivo and in vitro* studies.

Llaverias *et al.* used the MMTV-PyMT transgenic mouse model to study the effect of a cholesterol-rich diet on mammary tumor progression. Age-matched female mice fed high-cholesterol diets, containing 0.2% cholesterol developed mammary tumors that were significantly larger than mice fed "normal chow" diets, containing <0.03% cholesterol. Furthermore, high-cholesterol diets were associated with faster tumor onset, increased incidence, increased tumor burden, and higher tumor grade. Additionally, there was a trend of greater lung metastasis in mice fed high-cholesterol diets compared to mice fed normal diets. Finally, in accordance with data seen in human patients, mice fed high-cholesterol diets had lower plasma cholesterol levels than those fed normal diets. The lower plasma cholesterol levels directly correlated with mice that developed tumors. From this study, Llaverias *et al.* put forth the idea that cholesterol may act as a "rate-limiting factor of tumor progression." Interestingly, analysis of tumor cholesterol content via colorimetric assay did not reveal a significant difference between mice fed the two diets [Llaverias, 2011].

In a study using MDA-MB-231, Awad *et al.* found that treating these breast cancer cells with cholesterol caused an increase in migration of 67% [Awad, 2001]. High resolution-NMR spectroscopy was used to analyze lipophyllic compounds in tissue samples, and showed that higher concentrations of cholesterol and cholesterol esters were present in breast cancer tissue samples than in healthy breast tissue. This study found that the concentration of cholesterol esters especially

increased in “highly malignant samples” [Beckonert, 2003]. Another study showed that rat mammary tumors contained higher levels of cholesterol and cholesterol esters, when compared with normal mammary tissue. In addition, the rat mammary tumors displayed an increase in cholesterogenesis, compared to the normal rat mammary tissue [Rao, 1988]. These data highlight an obvious connection between breast cancer and its’ use of cholesterol, even though the exact reasons behind this connection are less obvious.

1.4 Rho GTPases

Rho GTPases (Ras homology guanosine triphosphatases) are a subfamily of small GTPase proteins. GTPases function as molecular switches that cycle between an active (GTP-bound) and inactive (GDP-bound) state [van Golen, 2010]. As shown in Figure 1.8, the GTPase cycle is regulated by guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs), and guanine dissociation inhibitors (GDIs). When the GTPase is active (GTP-bound) it is in a conformation which allows it to interact with downstream effector proteins. GAPs assist in GTPase inactivation by stimulating GTP hydrolysis to GDP. From here, a GEF aids in the release of the GDP from the GTPase [van Golen, 2010]. When the GDP is released the GTPase binds to GTP, which is much more abundant in the cell than GDP [van Golen, 2010; Hakoshima, 2003]. When the GTPase is bound to GDP, a GDI may bind and prevent the release of GDP, maintaining the GTPase inactivity. When the GTPase is inactive and bound by a GDI its membrane-association is blocked, and it remains in the cytoplasm until a GEF begins the cycle again [van Golen, 2010].

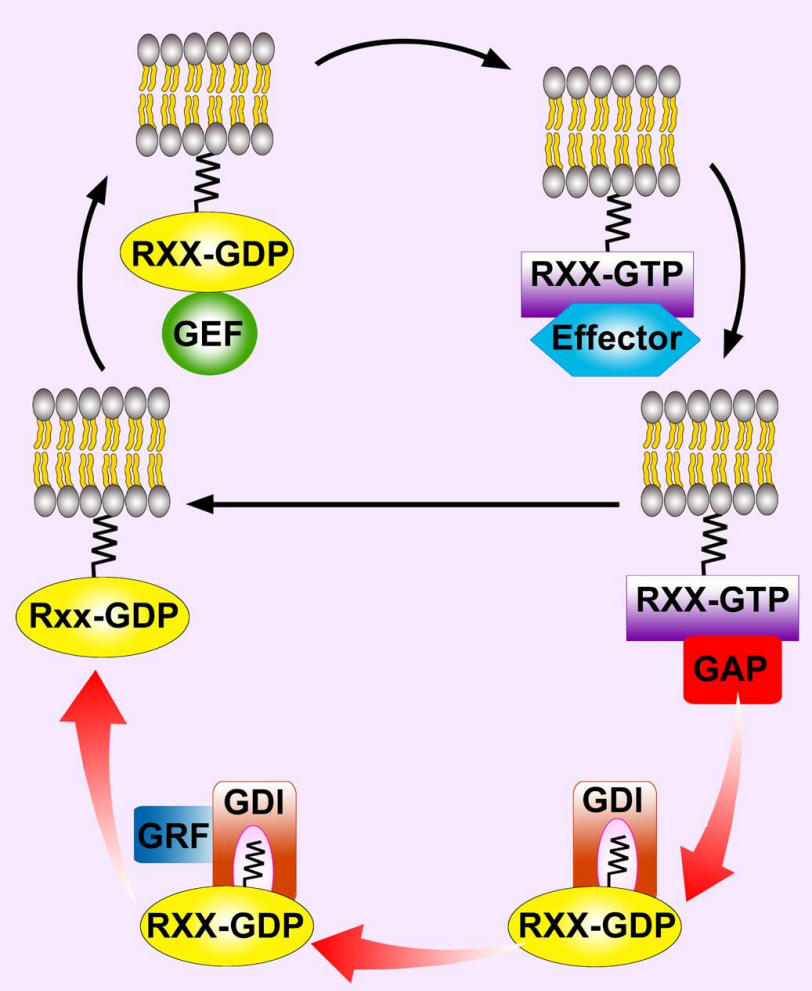

Figure 1.8 The Rho GTPase cycle. Active Rho is GTP-bound and attached to the membrane. GTPase activating protein (GAP) helps to stimulate hydrolysis of GTP to GDP which inactivates the Rho GTPase. Guanine dissociation inhibitor binds to the Rho GTPase and the attached prenyl group, and physically prevents the release of GDP, inhibiting reactivation of the Rho GTPase. A GDI-releasing factor (GRF) is then responsible to removing the GDI, and enabling the Rho GTPase to be reactivated again. Once the Rho GTPase is secured in the membrane by insertion of the prenyl group, a guanine exchange factor (GEF) stimulates GDP release. Once the GDP is released, GTP will bind the Rho GTPase and activate it [van Golen, 2010].

The Rho GTPase subfamily belongs to the Ras-superfamily. The Ras-superfamily is a large group of GTP-binding proteins, made up of greater than 150 proteins, which are divided into 5 subfamilies. The subfamilies are categorized by similarities in their amino acid sequence, as well as cellular functions that they exhibit [van Golen, 2010; Lacal 1993]. The Ras subfamily plays a role in regulating cell growth, while the Ran subfamily functions in nuclear transport. The Rab and Arf subfamilies operate in vesicle fusion. Finally, the Rho subfamily has a major role in regulating cytoskeletal functions. The Rho subfamily is made up of 22 proteins, which are further classified into subgroups, namely Rac, Rho, Cdc42, Rnd, RhoDF, RhoH, RhoUV, and RhoBTB [Jaffe, 2005; Boureux, 2002]. The Rho subclass consists of three highly homologous isoforms: RhoA, RhoB, and RhoC. RhoA and RhoC share a 91% homology in their amino acid sequence [van Golen, 2010]. Since they are so similar, it was originally presumed that they shared the same functions. Therefore, in initial experiments performed on Rho proteins, RhoA and RhoC were considered interchangeable, even though RhoA was most often the protein being analyzed. Recently, many studies have shown that RhoA and RhoC are undeniably different [van Golen, 2010]. RhoA has been shown to have functions in the contractile actin-myosin filament assembly, especially for the formation of stress fibers [Hall, 1998]. RhoB functions in cell motility, cell adhesion, cell survival, and cytokine trafficking [Sahai, 2002a; Wheeler, 2004]. RhoC functions in cell motility and invasion [van Golen, 2010].

1.4.1 RhoC GTPase

RhoC is a small monomeric protein, about 21 kDa in size, that has a large impact on cell biology [Etienne-Manneville, 2002; Farieda, 2006]. The RhoC protein can be partitioned into 3 major structural domains: the N-terminal G-domain, the effector binding domain, and the C-terminal membrane targeting domain [van Golen, 2010]. Most of the GTPase activity involves amino acids in the N-terminal G-domain portion of RhoC, including the switch I and switch II regions that undergo conformational changes between active and inactive states [Wheeler, 2004, van Golen, 2010]. The effector proteins interact with different amino acids in the effector binding domain, as well as the two switch domains, depending on the specific effector [Wheeler, 2004]. The C-terminal membrane targeting domain contains a hypervariable region where the most variation exists between RhoA, RhoB, and RhoC. Also, in the C-terminal domain is the CAAX motif that aids in post translational modification, which is necessary for membrane localization and activity of the protein [van Golen, 2010]. RhoC is postranslationally modified with the addition of a geranylgeranyl group on the C-terminus end [Wheeler, 2004]. Geranylgeranyl is a prenyl group that is produced in the mevalonate pathway, as seen in Figure 1.4. This geranylgeranyl group is responsible for RhoC's membrane association, and is blocked by Rho-GDI during inactive states [Wheeler, 2004; van Golen, 2010; Dias, 2007]. RhoC localizes to the plasma membrane when active, but the cytoplasm when inactive [Vega, 2011].

It is suspected that RhoC has many downstream effector proteins, but only a few have been discovered, to date. Kitzing *et al.* has found that Formin-like 2 (FMNL2) is a downstream effector protein of RhoC that effects ameboid cancer cell motility [Kitzing, 2010]. Another RhoC effector is Rho-associated protein kinase

(ROCK), which is understood to regulate actin-myosin contraction, and thought to be involved in cancer metastasis [Sahai, 2002b; Dias, 2007]. RhoC has also been shown to interact with mDia1 and 2, Rhotekin, Rhophilin, Citron Kinase, and more [Wheeler, 2004]. Finally, RhoC signaling though the MAPK pathway leads to motility and invasion [Van Golen, 2002]. All of the research analyzing RhoC points to its role in cytoskeletal arrangement, as well as cell motility [Wheeler, 2004].

Many studies have shown that RhoC is an important factor in cancers, especially cancers that are metastatic. RhoC overexpression has been seen in inflammatory breast cancer, pancreatic adenocarcinoma, ovarian carcinoma, melanoma, basal cell carcinoma, hepatocellular carcinoma, colorectal carcinoma, and more [van Golen, 201; Kleer, 2005]. In all of the above-mentioned diseases, RhoC overexpression seems to have the most influence on metastasis and invasion. For instance, Vega *et al.* showed that siRNA-inhibition of RhoC in prostate and breast cancer cells caused a decrease in the cells ability to migrate and invade [Vega, 2011]. Additionally, in 2005, Hakem *et al.* showed that RhoC was essential for cancer metastasis, but not initial tumor formation, in a RhoC knock-out mouse model [Hakem, 2005]. Furthermore, results from studies in the van Golen lab have supported the notion that interactions between RhoC and overexpressed caveolin-1, is possibly responsible for the invasive phenotype of inflammatory breast cancer [Joglekar, 2010]. Studies show that as cancers become increasingly metastatic, RhoC overexpression continues to increase. The relation of RhoC to ROCK activation helps to explain this trend since activity of the ROCK pathway is linked with cancer cell motion [Wheeler, 2004].

1.4.2 RhoC in Breast Cancer

It is clear that RhoC has an important role in breast cancer, especially those that are highly metastatic [van Golen, 2010]. In 2002, Kleer *et al.* found that RhoC expression showed a strong correlation with invasive breast carcinoma stage, and was especially useful in detecting small breast tumors with metastatic potential [Kleer, 2002]. van Golen *et al.* revealed that RhoC overexpression is seen in 90% of inflammatory breast cancer tumor samples, and in 38% of stage III breast cancer samples [van Golen, 1999]. A study of IBC tumor emboli, from Egyptian patients, showed that greater than 88% of patients had a high scoring overexpression of RhoC [Lo, 2009].

In the MDA-MB-231 breast cancer cell line, inhibition of RhoC synthesis via siRNA caused over a 70% decrease in invasion in an *in vitro* invasion assay. The same study showed that siRNA targeting RhoC caused a decrease in angiogenesis and tumor growth in an *in vivo* nude mouse model [Pillé, 2005]. Another study using retroviral ribozyme transgenes in MDA-MB-231 cells showed that knocking down RhoC lead to decreased invasiveness and migration *in vitro* retrovirus RhoC. Lang *et al.* found that Salvicine, a DNA topoisomerase II inhibitor, effectively decreased RhoC activity and inhibited its' plasma membrane association. Salvacine treatments, in mice with MDA-MB-435 cell xenografts, lead to decreased lung metastasis [Lang, 2005]. In invasive breast carcinoma cells, (SUM159) siRNA-induced inhibition of RhoC resulted in a decrease in the cells ability to invade through Matrigel. Additionally, when RhoC levels in these cells increased as a result of RhoA inhibition, their capacity to invade also increased [Simpson, 2004]. Another study linked RhoC expression with the production of angiogenic factors in mammary epithelial cells [van

Golen 2000a]. The results from these studies make it clear that overexpression of RhoC is closely related to its role in metastatic breast diseases.

1.5 Experimental Approach

There are many factors which contribute to the development, progression, and aggressiveness of breast cancer. The connection between RhoC overexpression and breast cancer metastasis has been clearly shown [van Golen, 2010]. Nevertheless, further understandings of the mechanisms behind this relationship are still to be uncovered. Additionally, the connection between cholesterol and breast cancer progression and migration is being made [Llaverias, 2011; Awad, 2001]. However, the understanding of how breast cancer cells are able to collect, and use cholesterol to benefit progression and migration is still lacking. We propose that RhoC overexpression is interconnected with the dysregulation of cholesterol homeostasis seen in breast cancer. ***Specifically, we hypothesize that RhoC helps to enhance cholesterol uptake by breast cancer cells, through its role in cytoskeletal arrangement.*** We begin by answering three questions: What differences do we see in cholesterol storage abilities of breast cancer cells and non-cancerous cells? What are the mechanisms in place that affects these differences? Is RhoC affected by, or have an effect on these mechanisms?

Chapter 2

BASELINE LIPID SIGNATURE OF BREAST CANCER, AND NON-CANCEROUS CELL LINES

2.1 Introduction

Dysregulation of cholesterol homeostasis is commonly seen in breast cancer [Llaverias, 2011; Rao, 1988]. In order to establish an understanding of the cholesterol storage mechanisms present in our cell lines, baseline lipid levels were first analyzed. We predicted that cancerous cell lines would have greater overall cholesterol levels than non-cancerous cell lines.

Cholesterol levels of four cell lines were assessed in order to understand differences between inflammatory breast cancer (IBC), non-inflammatory breast cancer (non-IBC), and non-cancerous cell lines. The inflammatory breast cancer cell lines examined was SUM 149. MDA-MB-231 and MDA-MB-435 were the two non-IBC cell lines studied. Finally, the non-tumorogenic mammary epithelial cell line, MCF10a, was also analyzed. Each cell line was stained with two stains that are commonly used to study cellular cholesterol composition.

Cholesterol is present in cells as cholesterol esters, and as unesterfied (free) cholesterol. When unesterified cholesterol is either made via the mevalonate pathway, or taken up from the extracellular environment, it can be used in membranes,

or converted into cholesterol esters for storage. Cholesterol esters are stored in lipid droplets within the cell [Brown, 1979; Brasaemle, 2007]. Lipid droplets are organelles which are used specifically for lipid storage. They are composed of a phospholipid monolayer that is incorporated with free cholesterol and proteins. This outer monolayer encloses a core of triglycerides and cholesterol esters.

We used two stains to help compare cholesterol utilization in breast cancer cells with non-cancerous cells. Oil Red O is a lipid stain often used to show cholesterol and triglyceride content in tissue samples, or adherent cells. Oil Red O is especially helpful in visualizing lipid droplets and areas of lipid accumulation. Although this stain is most often used to study lipid droplets in macrophages, or adipocytes, we were able to effectively stain for lipid droplets in our breast cancer cells [Stein, 2010; Gustafson, 2010]. To analyze overall content and distribution of free cholesterol within our cells, we used filipin. Filipin is a naturally fluorescent compound which binds to unesterified (free) cholesterol. This compound efficiently stains free cholesterol and aids in imaging unesterified cholesterol present in membranes.

2.2 Materials and Methods

2.2.1 Cell Culture

The inflammatory breast cancer line SUM149, was originally derived from a primary IBC tumor [Thomas, 2011]. SUM149 cells were maintained in Ham's F12 medium (cellgro by Mediatech, #10-080) supplemented with 5% (v/v) FBS (Atlanta Biologicals, #FP-0500-A), 1% L-Glutamine (Mediatech, #25-005), 1%

Penicillin/Streptomycin (Mediatech, #30-001), 1% antibiotic/antimycotic (Mediatech, #30-004), 1% Insulin/Transferrin/Selenium cocktail (Mediatech, #25-800), and 1µg/ml Hydrocortizone (Sigma-Aldrich, #H0888).

The breast cancer cell line MDA-MB-231 was derived from an adenocarcinoma pleural effusion [Neve, 2006]. MDA-MB-231 cells were grown in DMEM medium (Mediatech, #10-013), supplemented with 5% FBS (Atlanta Biologicals, #FP-0500-A), 1% Penicillin/Streptomycin (Mediatech, #30-001), and 750ug/ml Insulin (Sigma-Aldrich, #I6634). The breast cancer cell line MDA-MB-435 was originally derived from metastatic, ductal adenocarcinoma pleural effusion [Neve, 2006]. MDA-MB-435 cells were maintained in MEM medium (Mediatech, #10-022), supplemented with 5% FBS (Atlanta Biologicals, #FP-0500-A), 1% L-Glutamine (Mediatech, #25-005), 1% Penicillin/Streptomycin (Mediatech, #30-001), 1% sodium pyruvate (Mediatech, #25-000), and 2% MEM Non-essential amino acids (Mediatech, #25-025).

The non-tumorogenic mammary epithelial cell line, MCF10a, was maintained in 50:50 DMEM/F12 medium (Mediatech, #10-092) supplemented with 5% FBS (Atlanta Biologicals, #FP-0500-A), 5µg/ml Insulin (Sigma-Aldrich, #I6634), 0.5µg/ml Hydrocortisone (Sigma-Aldrich, #H0888), 50 µg/ml Bovine Pituitaary Extract (Gibco, #13028-014), 20ng/ml EGF (Sigma-Aldrich, #E4127), and 100ng/ml Cholera Toxin (Sigma-Aldrich, #C8052).

2.2.2 Oil Red O Staining and Imaging

Oil Red O staining was performed in 12-well plates on each cell line. Wells containing no cells, but media for each respective cell line, were used as background controls for the staining procedure. A main stock solution of Oil Red O was prepared

by adding 0.35g Oil Red O powder (Sigma-Aldrich, #O0625) to 100ml Isopropanol and stirring overnight. Before staining, a fresh working stock solution of Oil Red O was prepared using 60% of the main stock solution and 40% ddH_2O. After the working stock solution was mixed well, it was filtered using a 0.22µm syringe filter (Thermo Scientific Nalgene, #195-2520). Wells were washed with ice-cold 1X PBS (137mM NaCl, 2.7mM KCl, 8mM Na2HPO4, 1.46mM KH2PO4 pH 7.4), and fixed for 10 min with 4% paraformaldehyde in PBS. After fixing, wells were washed with ice-cold PBS, and stained with 0.2% Oil Red O working stock solution for 15 min. Finally, wells are washed with ddH_2O for 1 min, and stain was allowed to dry. Once stain was completely dry, 20X images were captured using a Nikon TMS inverted phase microscope. Duplicate images were taken using phase contrast to visualize and count the number of cells in each image, and brighfield to quantify the staining. Brightfield images were analyzed using ImageJ. This experiment was performed in triplicate. Samples were compared using Analysis of Variance (ANOVA) followed by Tukey's test.

2.2.3 Filipin Staining

Cells were plated in the four center wells of 8-well Lab-Tek™ II Chambered Coverglass over night. First, wells were washed 3 times in 1X PBS, pH 7.4. Next, cells were fixed in 4% paraformaldehyde (Electron Microscopy Sciences #15710) in PBS for 30 min. Wells were then washed again 3 times in PBS, and incubated with 200 µg/ml filipin solution in 10% BSA in PBS for 1 hour. Finally, cells were rinsed 3 times in PBS, and stored at 4°C with a drop of SlowFade® Antifade reagent (Invitrogen #S2828 Component A), in PBS for no more than two hours before

imaging. Cells were imaged using a Highspeed/Spectral Confocal Microscope: Zeiss 5 LIVE DUO.

2.3 Results

2.3.1 Cholesterol Accumulation in Breast Cancer Cells

Each untreated cell line was stained with Oil Red O and the relative stain intensity for each image was determined. Representative brightfield images of each cell line (Figure 2.1A-D) show an increase in Oil Red O staining in the cancerous cell lines compared to the non-tumorogenic cell line. Since different cell lines with different morphologies were used, relative stain intensity was quantified by dividing total stain area by the number of cells in each image. As hypothesized, the malignant cell lines stained more highly for cholesterol and triglycerides. SUM149, MDA-MB-231, and MDA-MB-435 cells display the greatest cholesterol and triglyceride staining with the average relative stain intensity being 911, 819, and 900 (arbitrary units) respectively (Figure 2.1E). On the other hand, MCF10a cells show a significantly lower level of relative cholesterol and triglyceride staining at 220 (arbitrary units). A replicate phase contrast image has been provided for better visualization of stained MCF10a cells (Figure 2.2).

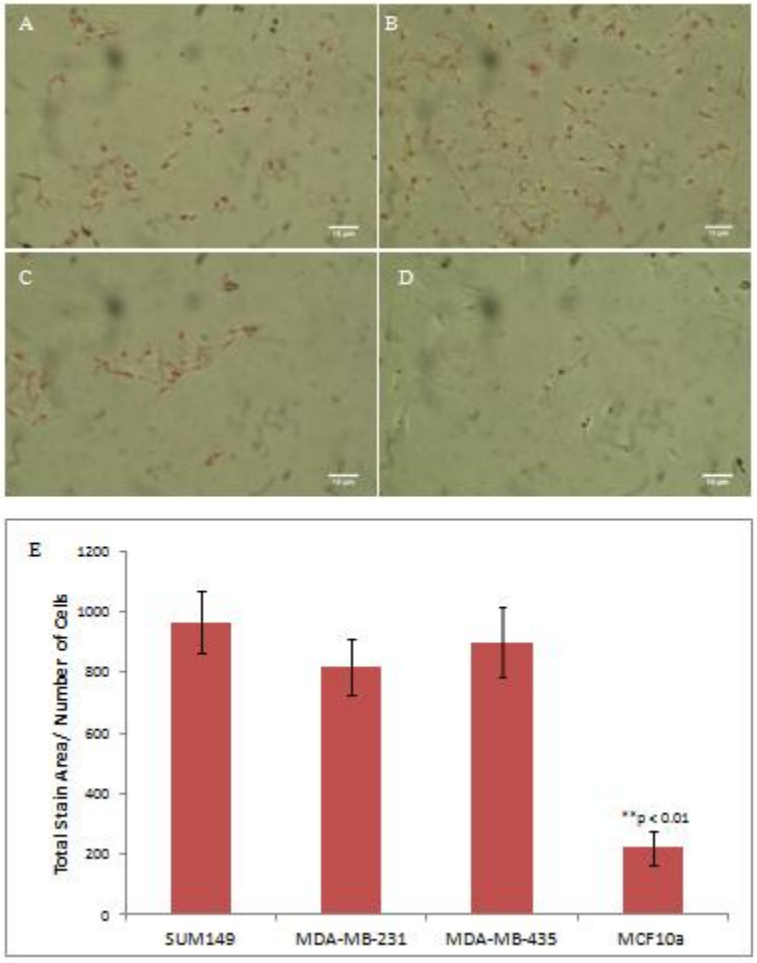

Figure 2.1 Oil Red O staining of untreated cells. Representative brightfield images of untreated SUM149 (A), MDA-MB-231 (B), MDA-MB-435 (C), and MCF10a (D) cells grown for 24 hours to allow attachment, and stained with Oil Red O. The scale bars represent 10µm. Each image was analyzed using ImageJ, and the total stain area was divided by the number of cells in each image. The graph shows that SUM149, MDA-MB-231, and MDA-MB-435 cells stain for cholesterol and triglycerides with Oil Red O significantly more than MCF10a cells ($p<0.01$, error bars are S.E.M.), using one-way ANOVA followed by Tukey's Honestly Significant Difference (HSD) test (E).

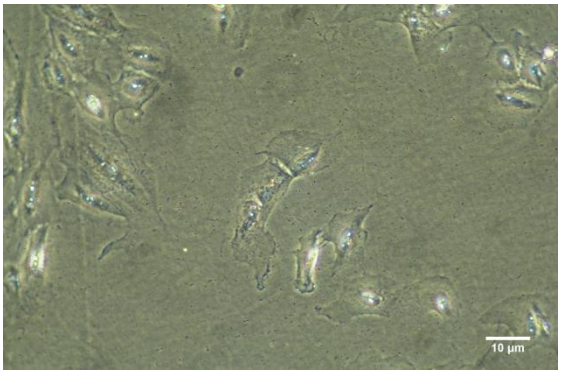

Figure 2.2 MCF10a cells in phase contrast. A duplicate phase contrast image of Figure 2.1d shows the presence of MCF10a cells, where the lack of Oil Red O staining makes them barely visible in the brightfield Figure 2.1d. The scale bar represents 10µm.

2.3.2 Free Cholesterol Content of Each Cell Line

Once the untreated cells were stained with filipin, cells were imaged using a Highspeed/Spectral Confocal Microscope: Zeiss 5 LIVE DUO. Z-stacks were taken of the cells to generate a 3D image. The Z-stacks were then analyzed using LSM Image Examiner. The average cell volume was divided by mean signal intensity, to determine relative staining intensity of each cell. A representative snapshot of a 3D image, used to determine the filipin fluorescent signal intensity for three MDA-MB 231 cells, is shown in Figure 2.3A. As seen in Figure 2.3B, MCF10a cells stain with the highest relative intensity for free cholesterol. Additionally, MDA-MB-435 cells have the next highest stain intensity, under MCF10a cells. Figure 2.3B also shows that MDA-MB- 231 and SUM149 cells have the lowest intensity of filipin staining.

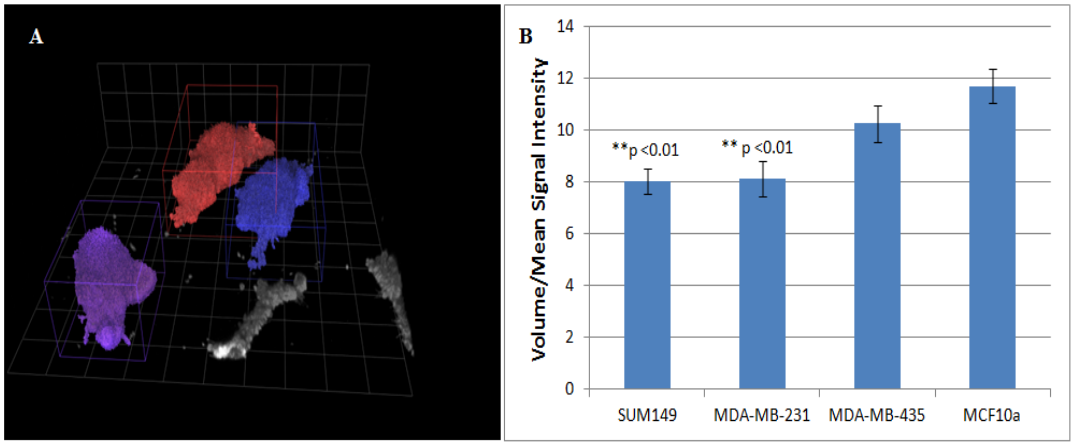

Figure 2.3 Filipin staining of untreated cells. A representative snapshot of a Z-stack of MDA-MB-435 cells stained with filipin. These cells were imaged on a Highspeed/Spectral Confocal Microscope: Zeiss 5 LIVE DUO as Z-stacks. The stain intensity of the three highlighted cells was determined using LSM Image Examiner (A). SUM149 and MDA-MB-231 cells display a significantly lower staining intensity than MCF10a cells (**p<0.01, error bars are S.E.M), using One-way ANOVA followed by Tukey's HSD test (B).

2.4 Conclusions

The high levels of Oil Red O staining, and the presence of lipid droplets in our cancerous cell lines are consistent with published data describing increased cholesterol content in breast cancer tissues [Sakai, 1992; Beckonert, 2003; Mady, 2000]. In addition, the findings from these two staining experiments help to shed light on the how each of these four breast cell lines are using cholesterol. Cells may allot internal cholesterol for storage as cholesterol ester, or for use in membranes as unesterified cholesterol [Esfhani, 1990]. This information, taken with the results from the two stains suggests that SUM149 cells, and MDA-MB-231 cells accumulate and store more cholesterol esters, and allot less free cholesterol to their membranes, than

MCF10a cells do. MCF10a cells are not accumulating cholesterol in lipid droplets, but are rather using their cholesterol in their membranes. Compared to the other cell lines, MDA-MB-435 cells seem to both store cholesterol, and allot a great deal of free cholesterol to their membranes.

An additional feature of the four cell lines being analyzed is that they exhibit varying degrees of invasiveness. Both SUM149 and MDA-MB-231 cells are highly mobile and invasive [Hoffmeyer, 2005; Wu, 2010]. MDA-MB-435 cells are moderately invasive, and MCF10a cells are non-invasive [Basu, 2006; Castelló-Cros 2009]. Typically, when cells are moving, or invading, they allot fewer resources for growth. Cholesterol is an essential membrane component for proliferating cells to use for the growth and expansion of their membranes [Esfahani, 1990]. The results of our studies imply that the more invasive SUM149 and MDA-MB-231 cells are able to store the excess cholesterol that they have accumulated, rather than directing all of their cholesterol to membrane production. The extra cholesterol being stored in these invasive cells may be attributed to their invasive natures. This notion is supported by a study of MMTV-PyMT transgenic mice fed diets high in cholesterol, showed that high levels of dietary cholesterol increased lung metastases resulting from mammary tumors [Llaverias, 2011]. The MDA-MB-435 cells, which are moderately invasive use an amount of cholesterol in their membranes that is similar to non-cancerous cells, and are able to store some as well. On the other hand, the non-invasive MCF10a cells seem to be taking advantage of the unesterified form of cholesterol to use in their membranes, possibly for proliferation. This information strongly suggests a role for cholesterol accumulation in invasive cancers.

There are substantial differences in cholesterol storage seen between breast cancer cell lines and non-cancerous cell lines. In order to further understand these differences observed, it is important to take a closer look at specific mechanisms involved in influx, storage, and efflux of cholesterol.

Chapter 3

ANALYSIS OF MECHANISMS INVOLVED IN CHOLESTEROL INFLUX, TRANSPORT, AND EFFLUX

3.1 Introduction

Breast cancer is one cancer that appears to be influenced by diet and environment [American Cancer Society, 2011]. There are large disparities present between breast cancer incidence in women of well developed countries, and women of less-developed countries. For instance, the incidence of breast cancer in North American women is much higher, than in Asian women [Coughlin, 2009 Awad, 2001]. One of the differences between these two groups of women is the over-abundance of cholesterol in the typical North American diet, compared to Asian diets [Awad, 2001]. This observation, and many others like it has led to cholesterol being one of the major components of the human diet studied in an effort define and understand its' particular role in breast cancer [Awad, 2001; Beckonert, 2003].

Results from the previous chapter confirmed the observation seen in other studies that breast cancer tissues contain higher levels of cholesterol than benign breast tissues [Sakai, 1992; Beckonert, 2003; Mady, 2000]. The overabundance of cholesterol is most likely due to alterations in cholesterol acquisition, efflux, or transport within the breast cancer cells. To gain further insight of exactly which

aspects of cholesterol transport are altered, it is important to take a closer look at different players that are involved in such mechanisms.

There are many proteins involved in increasing cholesterol when intracellular cholesterol levels are low. To start, HMGCR (3-hydroxy-3-methyl-glutaryl-CoA reductase) is the rate-limiting enzyme in the mevalonate pathway, which is responsible for *de novo* synthesis of cholesterol. HMGCR is normally stimulated when intracellular sterol levels are low [Buhaescu, 2007]. LDL-R (low-density lipoprotein receptor) and SR-BI help to increase intracellular cholesterol by facilitating transport from the extracellular environment [Pani, 2004; Stangl, 1999]. Normally, the production and activity of these proteins are all highly regulated by transcription factors which are affected by intracellular sterol concentrations [Brown, 1997; Stangl, 1999].

Under normal conditions, it is important for cells to be able to reduce their intracellular cholesterol levels if they are too high. ABCA1 is a protein that is partly responsible for retrograde movement of free cholesterol from the cell [Wang, 2004]. ABCA1 works by giving free cholesterol molecules to acceptor proteins such as Apolipoprotein E. LXRα is a transcription factor that is sensitive to high intracellular cholesterol levels, and stimulates ABCA1 transcription [Yancey, 2003]. Although there are many more proteins which are involved in modulating intracellular cholesterol, the above mentioned proteins have a central and critical impact on cholesterol levels.

In addition to these well known contributors to cholesterol homeostasis, we have proposed that RhoC, a small GTPase that directs cytoskeletal arrangement, may also be a factor in the ability of breast cancer cells to import and gather

cholesterol. As previously mentioned, caveolae aid in transporting cholesterol from the golgi to the plasma membrane, as well as from HDL particles in the extracellular environment into the cell. In addition, caveolae have been implicated as another site of localization of the LDL-R, besides the traditionally known clathrin-coated pits [Ness, 2003]. This information lead us to the idea that RhoC's particular role would involve enhancing trafficking cholesterol-rich caveolae to different destinations in the cell, through its ability to stimulate cytoskeleton formation.

An additional feature of the three cell lines studied in this phase, is that they have varying levels of active RhoC. SUM149 have extremely high levels of active RhoC and MDA-MB- 231 cells maintain moderate levels of RhoC activity (Figure 3.1). Moreover, although MCF10a cells express RhoC, the RhoC present is not active [per personal communication Dr. Kenneth van Golen, 2011]. This characteristic of our cell lines will allow us to gain additional information from our studies that may help highlight a role that RhoC may have in cholesterol distribution.

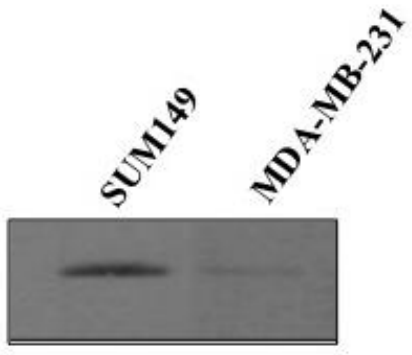

Figure 3.1 RhoC expression in SUM149 and MDA-MB-231 cells. This representative western blot depicts high RhoC expression in SUM149 cells, and moderate RhoC expression in MDA-MB-231 cells. It generously provided by Heather Lehman of the van Golen lab.

In order to analyze the cholesterol storage abilities of breast cancer cells compared with non-cancerous cells we studied SUM149, MDA-MB-231, and MCF10a (IBC, non-IBC, and non-tumorogenic cells, respectively) cell lines which displayed the most consistent lipid staining in our previous study. Using these cells we analyzed the effects of cholesterol depletion and cholesterol restoration on intracellular cholesterol levels. We also used the same techniques of cholesterol depletion and loading to evaluate changes in mRNA and protein expression of some of the key players involved in cholesterol acquisition and transport.

3.2 Materials and Methods

3.2.1 Preparation of LDL and LPDS

Fresh human serum was generously donated from the Blood Bank of Delmarva for the purpose of LDL isolation. The density of the serum was adjusted to 1.063g/ml using granulated NaBr (Fisher Scientific, # F255), and ultracentrifuged at 50,000 rpm for 22 hours at 15°C. Ultracentrifugation was performed using a Beckman 60Ti rotor (Beckman Coulter, # 200-7046), and Beckman Quick-seal® Polyallomer tubes (Beckman Coulter, #342414). After the first spin, the golden-colored top layer, containing of vLDL and LDL, was removed and density adjusted to 1.3 g/ml with NaBr. Density gradients were then prepared inside of ultracentrifuge tubes with a top layer of ddH_2O d= 1.0 g/ml, a middle layer of a NaBr solution d= 1.125 g/ml, and a bottom layer of lipoprotein solution d= 1.3 g/ml. The gradient was ultracentrifuged at 50,000 rpm for 60 min at 15°C. After this second spin, distinct separation of vLDL and LDL was visible. Both layers were carefully removed. The

LDL was dialyzed against three changes of Ca^{++}-free 1X PBS with 0.01% EDTA pH 7.4 at 4°C, and one change of Ca^{++}- free 1X PBS at 4°C. The integrity of the isolated LDL was confirmed by gel electrophoresis, on a 4-25% polyacrylamide gradient. For storage, LDL was aliquoted and frozen in 10% sucrose buffer [Rumsey, 1992]. Before cell treatment, thawed LDL was dialyzed against three changes of 1X PBS with 0.01% EDTA pH 7.4 to remove excess sucrose. After dialysis, LDL was sterile filtered using a 0.22µm syringe filter (Thermo Scientific Nalgene, #195-2520). The concentration of LDL was determined using a BCA protein assay kit (Thermo Scientific, #23225).

Lipoprotien-deficient serum (LPDS) was prepared from fetal bovine serum (FBS) (Atlanta Biologicals, #FP-0500-A), using a previously described protocol [Renaud, 1982]. First the FBS was density-adjusted to 1.21g/ml using granulated NaCl and NaBr. This FBS d=1.21 g/ml was then centrifuged for 48 hours at 10°C at 220,00 x g using a SW 41.Ti rotor (Beckman Coulter, #331336). After centrifugation, the top layer of lipoproteins was carefully removed beyond the noticeable gradient. The remaining LPDS was dialyzed in 4 changes of 1X PBS pH 7.4 at 4°C over the course of 24 hours. LPDS was then sterile-filtered using a 0.22µm filter, aliquoted, and stored at -20°C until use.

3.2.2 FBS, LPDS, and LDL Treatments

In an effort to standardize culture conditions for experiments, controls were performed using each cell line's respective base media and 5% FBS, with no other additive. Cells were washed with 1X PBS pH 7.4 and incubated in FBS-media for 24 hours prior to each experiment.

All LPDS treatments were performed as follows. Media containing 5% LPDS was prepared for each cell line in their respective base media. Cells were washed with 1X PBS before introduction of LPDS-medium. Cells were then incubated in LPDS-medium for 24 hours before the start of individual experiments.

LDL treatments were performed as follows. Cells were washed with 1X PBS and incubated in 5% LPDS medium for 24 hours. After 24 hours in LPDS, LDL was added at a concentration of 200ug/ml of media. Cells were incubated in LPDS media containing LDL for 12 hours prior to each experiment.

3.2.3 Oil Red O Staining

After cell treatments, Oil Red O staining procedures were conducted as previously mentioned in section 2.2.2.

3.2.4 qRT-PCR

3.2.4.1 RNA Isolation and Reverse Transcription

Total RNA was isolated from cells using RNeasy® Mini Kit (Quiagen #74104) according to the manufacture's protocol. Total RNA was DNase treated using an Ambion DNA™ Kit (Ambion #AM1906) according to the manufacturer's instructions. RNA quality and concentration was analyzed spectrophotometrically. RNA (1µg) was then reverse transcribed using Oligo (dT) Primer (Ambion #AM5730G), 10mM dNTP Mix (Promgea #C114B), and M-MLV Reverse Transcriptase (Invitrogen #28025-013) according to the manufacturer's instructions. If not used immediately, cDNA was stored at -20°C.

3.2.4.2 qRT-PCR

Real-time PCR experiments were carried out on reverse transcribed cDNA using primers (Table 3.1) at a final concentration of 0.4µM, and iTaq^{TM} SYBR$^{®}$ Green Supermix with ROX (BioRad, #172-5851) according to the manufacturer's instructions. Each sample was plated in triplicate wells, and amplification was carried out on an Applied Biosystems 7300 Real-Time PCR System. The thermal profile is as follows: Stage1- 1 repetition of 50°C for 2 minutes, Stage 2- 1 repetition of 95°C for 10 minutes, Stage 3 45 repetitions of 95°C for 15 seconds and 60°C for 1 minute, with a dissociation stage of 95°C for 15 seconds, 60°C for 1 min, and 95°C for 15 seconds. Dissociation curves were examined to detect non-specific product formation. Samples showing dissociation curves with multiple peaks were excluded from final results. Amplification efficiencies were calculated for each primer pair [Schmittgen, 2008], and primers resulting in less than 90% efficiency were excluded from final results. B2M, a gene expressed at similar levels of our proteins of interest, and minimally variant among breast cancer samples, was used as an internal control [McNeill, 2077]. B2M was chosen as the endogenous control over GAPDH because of its low variability and because its expression levels were more similar to the genes being measured (Table 3.2). Real-time PCR analysis was quantified using the $2^{-\Delta\Delta CT}$ method [Lo, 2008]. The real-time PCR experiments were performed three times. The results are reported as fold changes relative to FBS control cDNA for each cell line, after normalization to B2M internal control.

Table 3.1 QPCR primer sequences

Gene	Fwd Primer	Reverse Primer	Size
ABCA1	TGGCTTAGATTGGACAGCCCAAGA	AGCCAGACTTCTGTTGCTATGGGT	195
ApoE	GCCAATCACAGGCAGGAAGATGAA	ACCCAGCGCAGGTAATCCCAAA	192
B2M	TGTCTGGGTTTCATCCATCCGACA	TCACACGGCAGGCATACTCATCTT	168
HMGCR	TATGTGCTGCTTTGGCTGCATGTC	ATACCAAGGACACACAAGCTGGGA	83
LDL-R	TCAACACACAACAGCAGATGGCAC	AAGGCTAACCTGGCTGTCTAGCAA	140
LXRα	CATGCCTACGTCTCCATCCA	CGGAGGCTCACCAGTTTCAT	77
RhoC	AGCGGAAGCCTTGACTTCATCTCA	TCACCAGCTTCTTTCGGATTGCAG	112
SR-BI	CGAGTACCGCACCTTCCAGTT	ACCAGGATGTTGGGCATGAC	81

Table 3.2 Average Ct values for each gene

	SUM149	MDA-MB-231	MCF10a
ABCA1	25.7	23.1	26.2
ApoE	24.4	23.8	22.3
B2M	17.5	16.8	17.0
GAPDH	12.0	11.5	12.6
HMGCR	21.9	21.8	21.8
LDL-R	17.7	17.4	19.4
LXRα	18.8	20.8	21.9
RhoC	19.7	19.2	20.8
SR-BI	20.4	21.2	18.8

3.2.5 Western Blotting

Cells were grown to 90% confluence in 100-mm dishes. RIPA buffer (1X PBS, 150mM NaCl, 50mM Tris-HCl pH7.4, 2mM EDTA, 1% NP-40, 0.1% SDS, 10µl/ml phosphatase inhibitor (Thermo Scientific, #78420), and 5µl/ml protease inhibitor cocktail (Calbiochem, Gibstown, NJ)) was used to harvest protein from each cell line. A BCA Protein Assay kit (Thermo Scientific, #23225) was used to determine total protein concentration. Protein (30µg) was mixed with 2X Laemeli buffer (Sigma-Aldrich, #S3401), boiled for 3 minutes to induce denaturing, and separated by SDS-PAGE on a 12% polyacrylamide gel. Separated protein was then transferred to nitrocellulose membrane at 110V for 1 hour. The nitrocellulose membrane was then blocked using 5% milk (BioRad, #170-6404) in 1X PBST

(Phosphate-buffered saline with 0.05% Tween-20 (Fisher BioReagents, #BP337)) pH 7.4 overnight at 4°C, while rocking. Primary antibodies for ABCA1 (Abcam, #ab18180), HMGCR (Abcam, #ab98018), LDL-R (Abcam #ab30532), and β-actin (Cell Signalling, #4967L) were used according to the conditions found in Table 3.3. After incubation, membranes were washed twice in PBST for 5 min each and once in PBS for 5 minutes. Mouse (Cell Signalling, #7074), or Rabbit (GE Healthcare UK Limited, #NA934V) secondary antibodies, were added to the membrane at a concentration of 1:20,000 in 2.5% milk in PBST at room temperature for 2 hours. After incubation with secondary antibody, membrane was washed twice with PBST for 5 min and once with PBS. Following washing, protein was detected using Immobilon Western Chemiluminescent HRP Substrate (Milipore, #WBKLS0500), and X-Ray film. The developed film was scanned using a trans-luminescent scanner. Densitometry analysis was performed on the scanned images using ImageJ. Densitometry readings were normalized to β-actin signals as a loading control, to determine relative intensities.

Table 3.3 Working conditions for antibodies

Antibody	Dilution	Incubation conditions
1° ABCA1	undetermined	Rocking at 4°C overnight
1° β-actin	1:1000	Rocking at R.T. for 2 hours
1° HMGCR	undetermined	Rocking at 4°C overnight
1° LDL-R	1:300	Rocking at 4°C overnight

3.3 Results

3.3.1 The Effect of Extracellular Cholesterol Levels on Intracellular Cholesterol Accumulation in Breast Cancer and Non-cancerous Cells

Oil Red O staining of SUM149, MDA-MB- 231, and MCF10a cells treated with LPDS, LDL, or FBS as a control showed that there are major differences that exist between in each cell lines cholesterol and triglyceride content in response to changes in environmental cholesterol. Both SUM149 and MDA-MB-231 cells showed prominent staining with FBS as a control treatment, just as they had in the previous study when they were untreated (Figure 3.2A-C). MCF10a cells displayed very little staining with FBS, which is also consistent with the results described in Chapter 2 (Figure 3.2A and D). When SUM149 and MDA-MB-231 cells were treated with LPDS their stain intensity decreased (Figure3.2A, E, and F). Unlike the IBC cell line which showed a slight decrease in Oil Red O staining, MDA-MB-231 cholesterol and triglyceride content significantly decreased after incubating in LPDS for 24 hours (Figure 3.2A and F). The LPDS treatment had no significant effect on the non-cancerous MCF10a cells (Figure 3.2A and G).

After treatment with LDL, SUM 149 cells exhibited a significant increase in intracellular lipids from their LPDS-treated state (Figure 3.2 A, E, and G). Moreover, treatment of SUM149 cells with LDL for 12 hours, post 24-hour LPDS treatment, showed a trend of increasing Oil Red O staining beyond FBS controls, but was not significant. When MDA-MB-231 cells were treated with LDL, post LPDS treatment, they displayed a dramatic increase in lipid staining from their LPDS-treated condition, but lipid staining did not exceed that of FBS controls (Figure 3.2 A, C, F, and I). This effect may be due to the dramatic decrease in cholesterol and

triglycerides that MDA-MB-231 cells experience as a result of 24 hour treatment in LPDS. Finally, MCF10a cells showed a slight, but insignificant trend of increased Oil Red O staining after LDL treatment (Figure 3.2 A, D, and J).

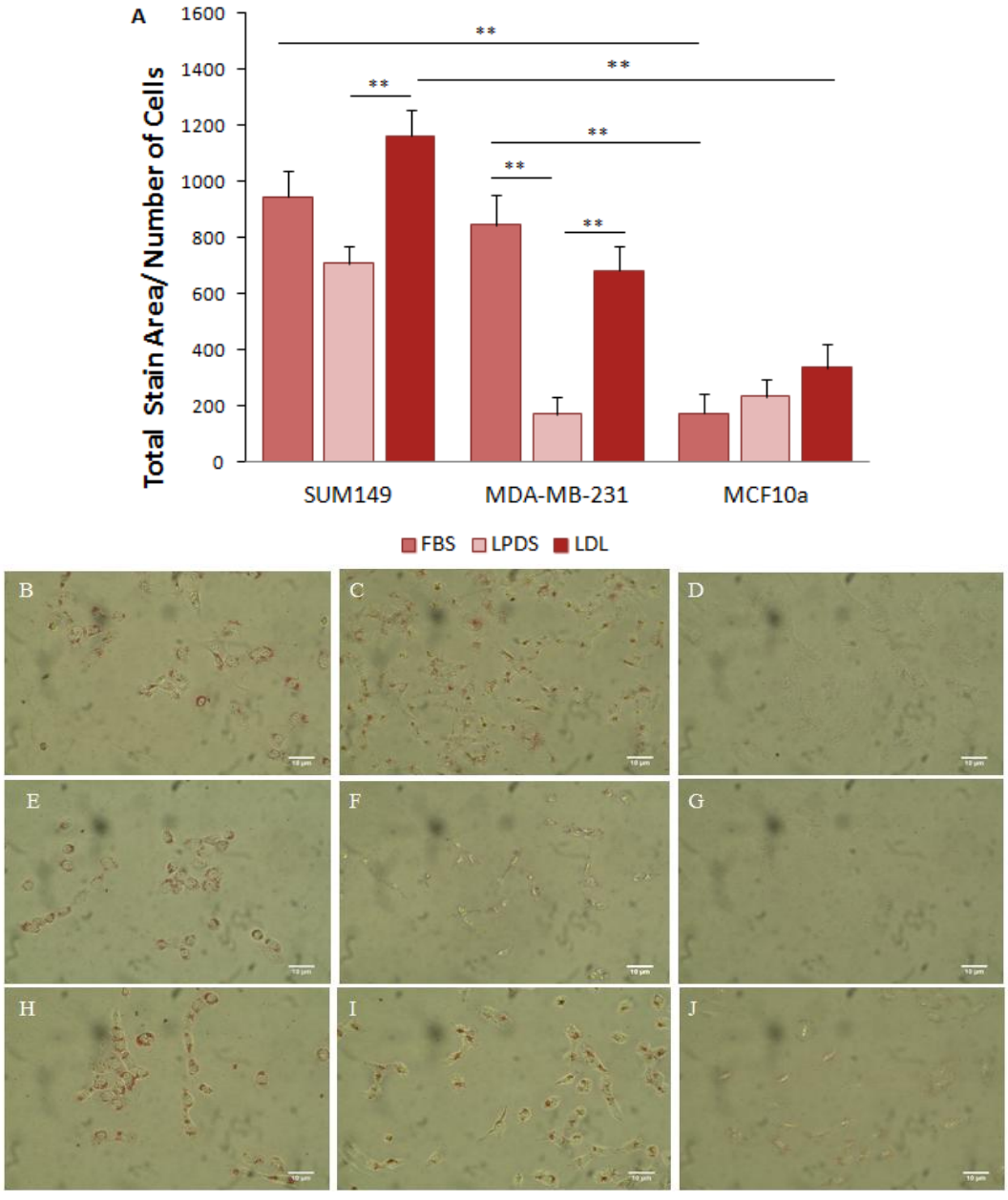

Figure 3.2 Oil Red O staining after treatment with FBS, LPDS, and LDL. (A) The relative stain intensity (total stain area/number of cells) was determined for each image using ImageJ. The graph depicts significant differences (**p<0.01). Statistical significance was determined by one-way ANOVA followed by Tukey's HSD test. The error bars represent S.E.M. Representative brightfield images for each treatment are provided: (B) SUM149 FBS control, (C) MDA-MB-231 FBS control, (D) MCF10a FBS control, (E) SUM149 LPDS, (F) MDA-MB-231 LPDS, (G) MCF10a LPDS, (H) SUM149 LDL, (I) MDA-MB-231 LDL, and (J) MCF10a LDL.

3.3.2 Analysis of the Effect of Extracellular Cholesterol Levels on mRNA Gene Expression

In the previous experiment we found significant differences in intracellular cholesterol content in response to changes in extracellular cholesterol. In order to understand the observed behaviors of each cell line, we analyzed the gene expression profiles of several transcripts that are involved in altering intracellular cholesterol levels, to look for changes that occur in response to varying extracellular cholesterol levels. In that respect, we used QPCR to determine changes in mRNA levels of ABCA1, ApoE, HMGCR, LDL-R, and RhoC that occur in each cell line after treatment with LPDS and LDL, as described before.

As seen in Figure 3.3A, the LPDS and LDL treatments resulted in significant changes in mRNA expression is some of the key genes involved in cholesterol acquisition. In inflammatory breast cancer cells, SUM149, mRNA expression of LDL-R significantly increased by 2.22 fold, after LPDS treatment ($p<0.01$). When cells were treated with LPDS for 24 hours, a significant 3.29 fold increase of HMCGR mRNA expression occurred ($p<0.01$). A trend for slight increases ABCA1 expression after LPDS and LDL treatment was seen in SUM149 cells, although not significant. In contrast, LXRα expression showed a significant decrease

after LDL expression. Additionally, relative mRNA expression of ApoE and RhoC remained unchanged after LPDS and LDL treatments.

The non-IBC cell line, MDA-MB-231 displayed the least variation in gene expression compared to SUM149 cells and MCF10a cells (Figures 3.3A and 3.3C respectively). There were no significant changes seen in ApoE, HMGCR, LDL-R, LXRα, or RhoC expression, after LPDS treatments. However, MDA-MB-231 cells showed a significant decrease, in ABCA1 expreesion, of 0.45 fold after LPDS treatment ($p<0.01$), and 0.53 after ($p<0.05$) LDL treatmens. MDA-MB-231 cells also displayes a significant 1.78 fold increase in ApoE expression ($p<0.05$), afer LDL treatment. Both HMGCR and LDL-R expression significantly decreased to 0.38, and 0.42 folds ($p<0.05$), respectively, as a result of LDL treatment. Finally, RhoC expression significantly decreased 0.71 fold after LDL treatment ($p<0.05$).

MFC10a, the non-tumorgenic mammary epithelial cells, maintained relatively stable expression of ABCA1, LXRα, and RhoC througout LPDS and LDL treatments. When treated with LPDS, ApoE expression increased 1.75 fold compared to the FBS control. Additionally, HMGCR showed a trend of increased expression after LPDS treatment. Moreover, LDL-R expression also displayed a increasing trend as a result of LPDS. After MCF10a cells were treated with LDL, relative ApoE expression rose dramatically by 5.07 fold ($p<0.05$). Finally, expression of HMGCR and LDL-R returned to levels similar to those seen in FBS control, afte LDL treatment.

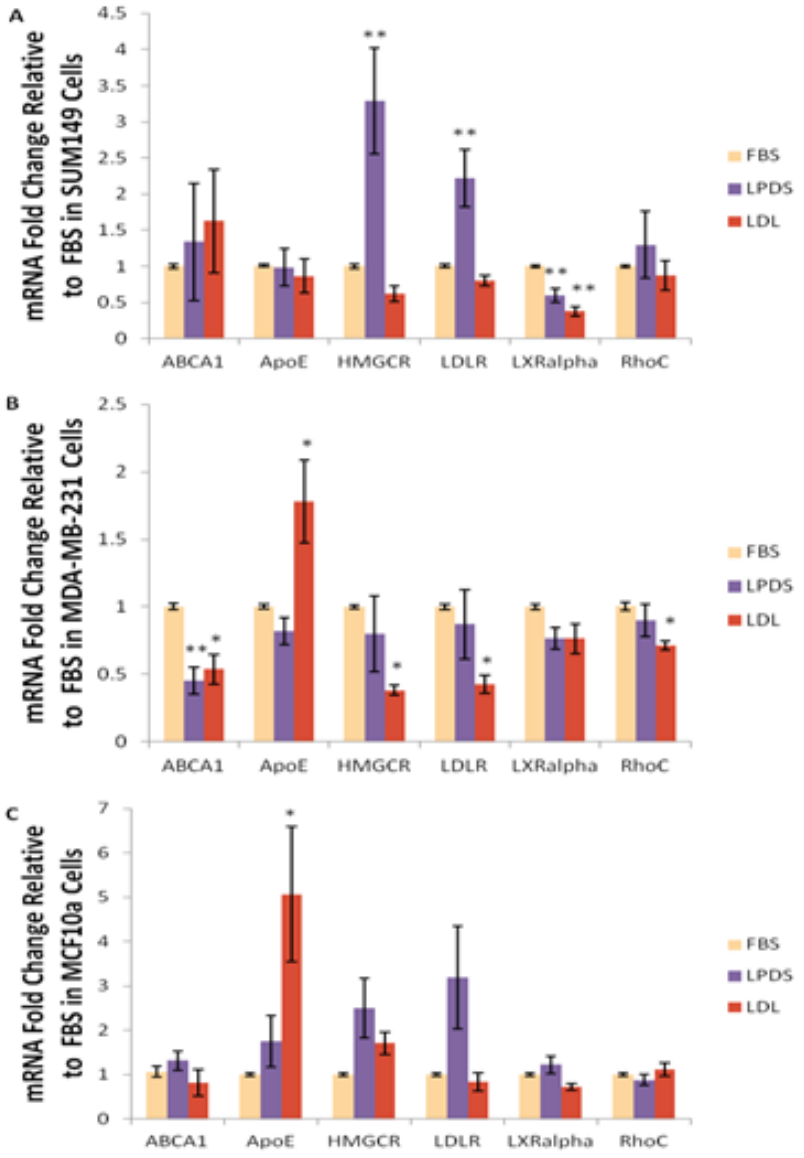

Figure 3.3 Relative mRNA fold change determined by QPCR analysis for (A) SUM149, (B) MDA-MB-231, and (C) MCF10a cells. The fold change for each gene is relative to FBS control mRNA levels. Statistical analysis was performed using one-way ANOVA, followed by Tukey's HSD test (*= $p<0.05$ and **= $p<0.01$). The error bars represent S.E.M.

3.3.3 Analysis of the Effect of Extracellular Cholesterol Levels on Protein Expression

Changes in LDL-R protein expression after treatment with LPDS, and LDL were similar to changes seen in mRNA by QPCR analysis. As seen in Figure 3.5 SUM149 expression of LDL-R rose dramatically as a result of LPDS treatment. This level of LDL-R protein did not greatly diminish after 12 hours of treatment with LDL. MDA-MB-231 cells reduced their LDL-R protein as a result of LPDS and LDL treatment. Finally, MCF10a cells showed a dramatic increase in LDL-R as a result of LPDS, just as the SUM149 cells did. Unlike the SUM149 cells, LDL-treated MCF10a cells had LDL-R levels that returned to baseline levels seen in FBS controls. The western blot analysis also shows us that SUM149 cells initially express the least LDL-R of each of the three cell lines. However, MDA-MB-231 cells begin with the highest expression of LDL-R in control FBS conditions. When LDL-R expression of SUM149 and MDA-MB-231 cells are normalized to expression seen in MCF10a cells, we find that MCF10a cells display a LDL-R expression, as a result of LPDS treatment, that is slightly greater than SUM149 and MDA-MB-231 cells (Figure 3.5). We also find that both SUM149 and MDA-MB-231 cells express more LDL-R than MCF10a cells, as a result of LDL treatment (Figure 3.5). In addition to western blotting for LDL-R, we also attempted to blot for ABCA1 and HMGCR. Nevertheless, these attempts have so far been unsuccessful due to banding patterns occurring at sizes inconsistent with those expected for the predicted molecular weight of these two proteins. Representative images of these western blots have been provided in Figure 3.6.

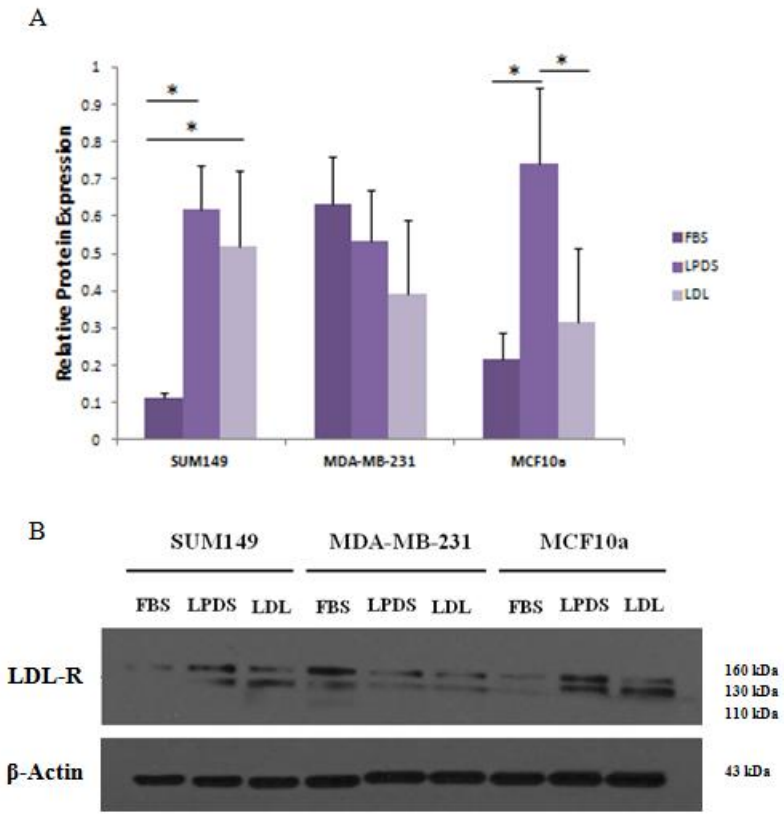

Figure 3.4 Western blot analysis of (A) LDL-R protein expression in SUM149, MDA-MB-231, and MCF10a cells as a result of FBS, LPDS, and LDL treatments. Data is presented as expression relative to β-Actin loading controls (*$p<0.05$). Error bars represent S.E.M. (B) A representative image of the western blot probing for LDL-R. The protein-specific bands show up at 160, 130, and at 110 kDa (in LDL treated SUM149 cells), according to the producer's specifications.

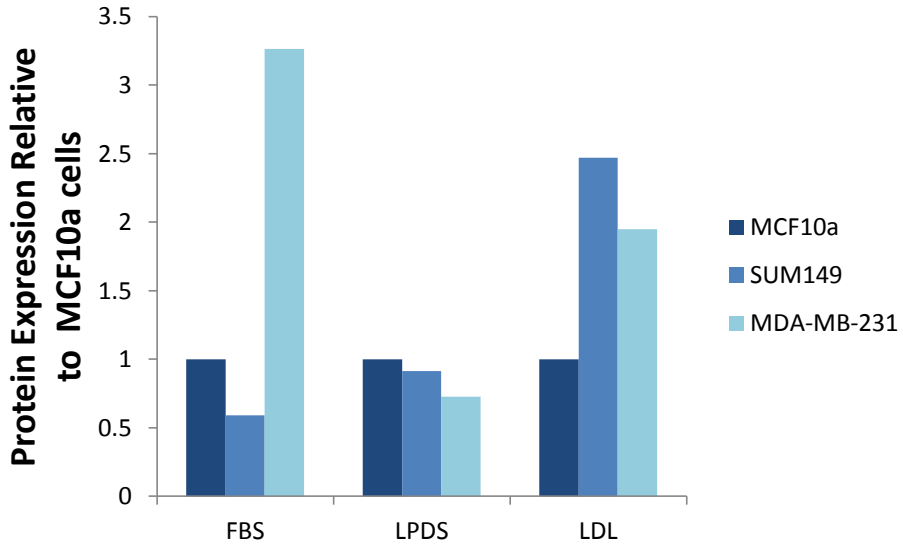

Figure 3.5 LDL-R expression normalized to MCF10a cells.

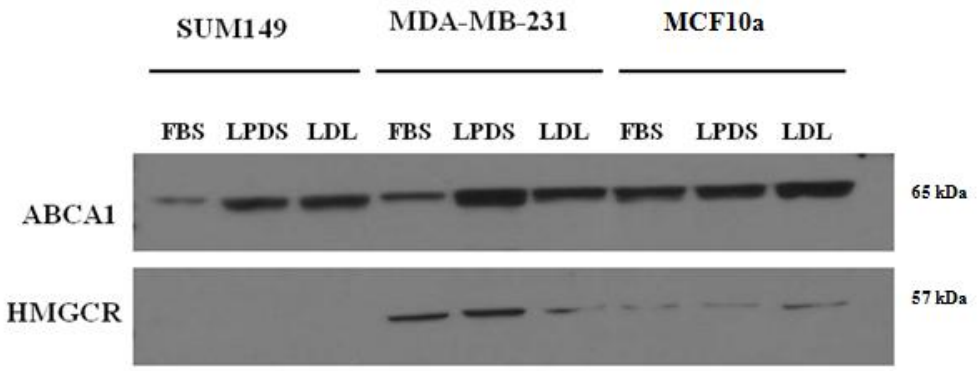

Figure 3.6 Representative western blots using an ABCA1 primary antibody, we expect a protein-specific band at 254 kDa, but continue to only recover a protein band at around 65 kDa. Using the HMGCR primary antibody, we would expect to see protein-specific bands at 97 kDa, but we have only ever been able to produce a non-specific band pattern around 57kDa.

3.4 Conclusion

We have found major differences in how each of these breast cell lines react to environments that are either rich in cholesterol, in the form of LDL, or lacking cholesterol. With Oil Red O staining (Figure3.2), we show that both breast cancer cell lines are less able to regulate their intracellular cholesterols levels than the non-cancerous cell line. This concept is consistent with information from other studies showing that cholesterol transport and storage is altered in many different cancers [Li, 2006; Duncan, 2004; Twiddy, 2011]. From Figure 3.2A, one might conclude that the breast cancer cell lines more readily release cholesterol as a result of being in a lipoprotein-deficient environment. However, when referring back to Figure 3.2D, we

see that MCF10a cells begin with basically no cholesterol or triglyceride accumulation, as detected by Oil Red O staining. Therefore, it would be difficult to show loss of lipid accumulation in MCF10a cells with the same methods.

Unlike MCF10a cells, both SUM149 and MDA-MB-231 cells exhibit a detectable loss of accumulated cholesterol and triglycerides as a result of LPDS treatment, but even between these two breast cancer cell lines there appears to be a difference in the amount of lipids that they lose. While the IBC cells do show a decreased lipid droplet content after LPDS treatment, this reduction is not significant. On the other hand, the non-IBC cells experience a dramatic decrease in lipid droplet content, when placed in a lipoprotein-deficient environment. The comparison between these two distinct forms of breast cancer cells suggest that unlike MDA-MB-231 cells, SUM149 cells posses specific mechanisms that enable them to maintain cholesterol and triglycerides which they have accumulated in lipid droplets.

Examination of how each cell line responds when treated with LDL after having been in a lipoprotein-deficient environment for 24 hours gives us further information as to how each cell line reacts to available cholesterol. We found that treating cells with LPDS for 24 hours before treating with LDL did not result in significant increases above FBS control intracellular cholesterol and triglyceride levels. Nevertheless, in SUM149 and MDA-MB-231 cells, we do see significant increase ($p<0.001$, Figure 3.2) in cellular cholesterol and triglyceride content as a result of LDL treatments, compared with LPDS treatments. SUM149 cells even begin to exceed the level of FBS controls, after 12 hours of LDL treatment. Since MDA-MB-231 cells have such a dramatic decrease in lipid droplets, they essentially have to increase *de novo* synthesis, or uptake a lot more extracellular LDL just to make it to

the level of FBS control cells. We expect that if either of these two cancer cell lines were treated with 200 µg/ml of LDL without having been first treated with LPDS for 24 hours, they would exhibit a level of cholesterol accumulation that would significantly exceed those of untreated cells. Although MCF10a cells exhibited a slight increase in cholesterol and triglyceride content after treatment with LDL, this increase was not significant when compared to the FBS control or the LPDS treated cells (Figure 3.2A). Again, this lack of response to extracellular cholesterol concentrations suggests that MCF10a cells are better equipped to regulate their intracellular cholesterol and triglyceride levels, compared to the two cancerous cell lines. Furthermore, this data is consistent with the notion that many cancers possess higher levels of intracellular cholesterol because the normal mechanisms for cholesterol accumulation are altered.

We found that both QPCR and Western blot analysis proved to be helpful in understanding why SUM149, MDA-MB-231, and MCF10a cell lines manage cholesterol in the manner they do. Starting with analysis of SUM149 cells, we see that when SUM149 cells are subject to a lipoprotein-deficient environment, they boost levels of HMGCR and LDL-R mRNA (Figure 3.2A). This result is also confirmed at the protein level, using western blot for both LDL-R (Figure3.3A). Both of these proteins are responsible for increasing intracellular cholesterol concentration when they are too low [Pani, 2004]. This observation shows us that the IBC cells respond to an increase and loss of cholesterol in an expected way. In addition, these findings are absolutely consitent with the data presented from Oil Red O staining of LPDS-treated SUM149 cells, where we saw that LPDS-treated SUM149 cells had only a slight decrease in lipid content compared to LPDS-treated MDA-MB-231 cells (Figure 3.2).

QPCR data showed that MDA-MB-231 cells treated with LPDS lacked significant changes in all of the genes analyzed, apart from ABCA1. (Figure3.3B). The lack of response seen in these breast cancer cells is also consistent with the Oil Red O staining of LPDS-treated MDA-MB-231 cells. We saw in Figure 3.2 that MDA-MB-231 cells experienced a dramatic decrease in intracellular lipid content as a result of LPDS treatment. This drastic loss of lipids can now be explained by the fact that neither HMGCR or LDL-R were upregulated to counteract the loss of cholesterol. This observation suggests that the cholesterol stores are being redistributed for use in the cell, rather then new cholesterol being produced or imported. It would be interesting to determine if this phenomenon in MDA-MB-231 cells is repeated in many other non-IBC cell lines. ABCA1 expression is significantly reduced after both LPDS and LDL treatments. The observation in MDA-MB-231 cells treated with LPDS further suggests an abnormal response to low extracellular cholesterol concentrations. The response of MDA-MB-231 cells to LDL treatment also suggests that other transproter proteins, besides ABCA1 are being used to reduce intracellular cholesterol. Further analysis of MDA-MB-231 gene expression shows a significant increase in ApoE after LDL treatment (Figure3.3B). ApoE is an lipoprotein that can be used as a cholesterol acceptor to help remove excess intracellular cholesterol [Yancey, 2003]. The upregulation of ApoE suggests that MDA-MB-231 cells begin to sense rising levels of cholesterol and put forth a greater effort to reduce them, than when they experience a loss in intracellular cholesterol. Additionally, significantly reduced expression of HMGCR, and LDL-R post LDL treatment supports the result seen by the upregulation of ApoE. The change in HMGCR and LDL-R expression

brings further light to the fact that these breast cancer cells were not able to completely return to a lipid content level equal to that of the FBS control cells. The sum of these findings ultimately suggests that homeostatic mechanisms present in MDA-MB-231 cells have completely deviated from the norm.

MCF10a mRNA and protein expression seems to most closely resemble that which we would expect in cells with limited ability to store cholesterol. For instance, after LPDS treatment, we see increasing trends in both HMGCR and LDL-R expression by QPCR (Figure3.3C). The upregulation of HMGCR and LDL-R appears to allow the cells to maintain appropriate intracellular cholesterol levels without being significantly affected by their environment (Fig3.2). When the cells are treated with LDL, HMGCR and LDL-R levels return back to those seen in FBS control cells (Figure3.3C) Additionally, in a more dramatic fashion than MDA-MB-231 cells, ApoE expression increased in MCF10a cells, which suggests that these cells exporting any extra cholesterol that they had accumulated as a result of LDL treatment. Relating back to the Oil Red O experiments we see that overall, that the changes in expression does not result in a great deal of lipid accumulation.

After analyzing the mRNA expression of RhoC, no significant difference is noted between treatments in SUM149 or MCF10a cells. This lack of response to altered extracellular cholesterol suggests that RhoC expression is not closely linked with cholesterol internalization in IBC, or non-tumorogenic cells. Nevertheless, MDA-MB-231 cells displayed a significant decrease in RhoC expression as a result of LDL treatment. This slight, but significant, decrease accompanied the more dramatic decreases seen in LDL-R and HMGCR expression, as a result of LDL treatment. This data might support the idea that RhoC activity is somehow involved in cholesterol

accumulation. If RhoC is involved, we might expect a decrease in RhoC expression to accompany a decrease in LDL-R expression. If cholesterol influx via LDL-R was downregulated, endocytosis of LDL-R would decrease, and there would be less of a need for RhoC to stimulate production of cytoskeletal tracks for the endocytic vessicles to travel on. However, no concrete statements can be made about this effect of LDL treatment on RhoC, at this time.

The data from the present study highlight the fact that breast cancer cells posess greater alteration in their cholesterol transport mechanisms than non-cancerous cells. The IBC cell line, SUM149 and MCF10a cells display mRNA expression profiles most closely resmbles what would be expected in normal cells. Even though the mRNA fold changes in SUM149 cells are similar to those seen in MCF10a cells, we see that the cholesterol influx and storage mechanisms in SUM149 cells favor an intracellular environment which is loaded with cholesterol. The non-IBC cell line, MDA-MB-231, not only presents with lipid accumlation in their untreated state, but also show signs of completely altered mechanisms of cholesterol acquisition which do not allow them to properly regulate their intracellular cholesterol levels. Unlike what is noted in MCF10a cells, the intracellular cholesterol levels of MDA-MB-231 cells appears to be most influenced by the environment that they are in. In conclusion, our results suggest both breast cancer cell lines are affected more by changes in extracellular cholesterol levels, while the non-cancerous cell line is not greatly affected by extracellular cholesterol concentrations.

Chapter 4

A ROLE FOR CHOLESTEROL ON BREAST CANCER CELL INVASION

4.1 Introduction

In our previous studies, we show that breast cancer cell lines not only accumulate intracellular cholesterol, but that the underlining mechanisms of cholesterol accumulation are altered compared to those seen in non-cancerous cells. These data support other studies which show that HMGCR and mevalonate expression are increased in cancerous cells [Twiddy, 2003; Caruso, 2005; El-Sohemy, 2005; Hardwood, 1991; Duncan, 2004; Li, 2006]. Additionally, our results lead to the idea that malignant breast tissues exploit, and maybe even depend on an excess of intracellular cholesterol. Nevertheless, there is still more to discover about the role that cholesterol may play in breast cancer development and progression. In order to explore a more functional aspect of cholesterol storage on breast cancer, we analyzed the effect that cholesterol depletion and loading has on the breast cancers cells ability to invade in vivo.

Additionally, we initially resolved to use three cell lines with varying degrees of RhoC activity in our studies, to help determine if there was a role for RhoC in cholesterol transport that could be ascertained. Again, SUM149 and MCF10a cells have high levels of endogenous active RhoC, where as MCF10a cells contain only inactive RhoC [per personal communication with Dr. Kenneth L. vanGolen]. Our studies of RhoC mRNA expression in the IBC, non-IBC, and non-cancerous cell lines

did not point to a clear role that RhoC may be playing in the ability of breast cancer cells to accrue cholesterol. Therefore, we have employed the use of a fast-cycling RhoC mutant (RhoC F30L-RFP), which continually cycles between active and inactive states. By transfecting MCF10 a cells with the RhoC F30L-RFP mutant, we hope to be able to determine if the expression of active RhoC in MCF10a cells has an effect on cholesterol accumulation.

4.2 Materials and Methods

4.2.1 Atorvastatin Timepoints

SUM149 and MDA-MB-231 cells were treated with 5 µM Atorvastatin calcium salt trihydrate (Sigma-Aldrich, #PZ0001), in normal media for 0, 24 and 48 hours in duplicate wells of 12-well plates. After completion of time course, cells were washed and stained with Oil Red O as previously described. Oil Red O stain was quantified by analyzing total stain area of images taken of 100% confluent cells, using ImageJ.

4.2.2 Invasion Assay

Invasion assays were performed using BD Matrigel™ Invasion Chamber 24-well plates with 8 µm pore PET (polyethylene terephathalate) inserts (BD Biosciences #354480). Invasion assays were carried out using SUM149 and MDA-MB-231 cells. Cells were treated for 24 hours before invasion assay was plated. Cells were treated with LPDS, LDL in LPDS, 5 µM Atorvastatin in LPDS, or left untreated in normal media. Cells were then

washed, trypsinized, and resuspended in serum free media at a density of 10,000 cells/ml. Suspended cells were plated in triplicate inserts that sat inside of wells with medium containing 15% FBS to act as a chemoattractant. After cell suspension was added to the insert, 5 µM Atorvastatin was added to cells which had been previously treated with 5 µM Atorvastatin, for a total of 48 hours. Cells were allowed to incubate for 22 hours in a tissue culture incubator at 37°C and 5% CO_2 atmosphere. After incubation period, non-invading cells were removed from the membrane using a moist cotton swab. The remaining, invading cells were then stained with 0.5 %crystal violet solution containing 20% methanol, washed with distilled water, and allowed to dry for 24 hours. The total number of invaded cells, for each well, was blindly counted.

4.2.2.1 Oil Red O Staining of Cells Treated for Invasion Assay

Oil Red O staining was used to determine the condition of SUM149 and MDA-MB-231 cells after treatment with LPDS, A+LPDS, and LDL+LPDS, at the time of invasion assay plating. Cell that had been plated using the same treatments, at the same time as those to be used in the invasion assay were stained with Oil Red O as previously described. Images of confluent cells were taken and analyzed using imageJ to compare total stain area between treatments.

4.2.3 MCF10a Transfections

MCF10a cells were transfected with pTagRFP fast-cycling RhoC (F30L-RFP) mutant DNA, and pTagRFP DNA as a control. The transfections were

performed using Fugene® HD Transfection Reagent (Promega, #E2311) according the manufacture's protocol. DNA was diluted to a concentration of 0.02 µg/ml in sterile ddH_2O. The diluted DNA was then mixed with the Fugene® HD Transfection Reagent at a ratio of 5:2, vortexed, and allowed to incubate at room temperature for 15 min. After the incubation period 100 µl of the transfection complex was added to cells grown to 80% confluence in 6-well plates. Cells were incubated for 24 hours in a tissue culture incubator at 37°C and 5% CO_2 atmosphere. Transfection efficiency was visually determined by the percentage of cells producing RFP, using a Nikon Eclipse TE-200U. Once the transfection efficiency was determined, cells were treated with 200 µg/ml LDL, or normal media. After 12 hours of treatment, cells were stained with Oil Red O as previously described.

4.3 Results

4.3.1 The Effect of Atorvastatin Treatment of Breast Cancer Cells in Normal Media

We treated the two breast cancer cell lines with Atorvastatin, a commercially available HMGCR inhibitor, in order to determine the effects of blocking the *de novo* synthesis of cholesterol through the mevalonate pathway. When SUM149 cells were treated with 5 µM Atorvastatin they display more lipid content by Oil Red O staining. Treatment with 5 µM Atorvastatin for 24 and 48 hours results in an equivalent increase in lipid staining (Figure 4.2A). When MDA-MB-231 breast cancer cells are treated with 5 µM Atorvastatin they display a slight increase in lipid staining after 24 hours, and another slight increase after 48 hours (Figure 4.2B). This increase in lipid staining is an intuitive result of Atorvastatin treatment, as we might

expect cells to increase their cholesterol uptake to compensate for the reduced production of cholesterol *de novo*. Additionally, the results of this experiment helped us to optimize our treatment methods for the invasion assays.

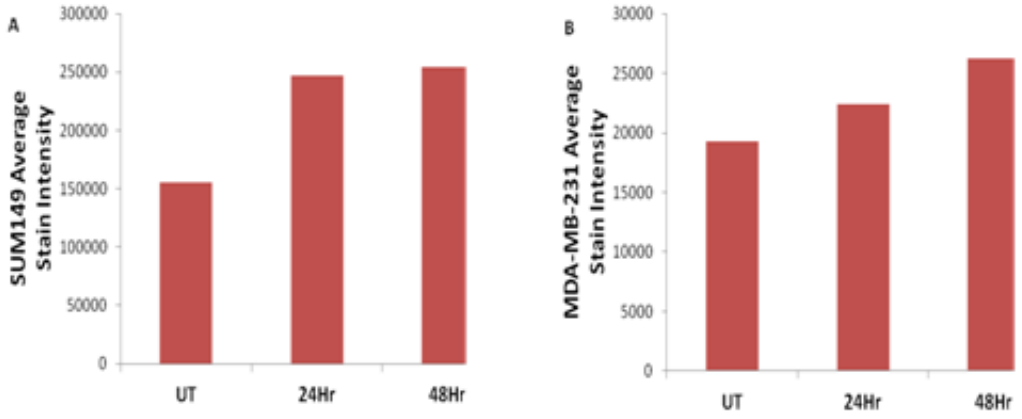

Figure 4.1 Atorvastatin time points. Average Stain Intensity of (A) SUM149, and (B) MDA-MB-231 cells, stained with Oil Red O, after 24 and 48 hours of treatment with 5µM Atorvastatin. Images of 100% confluent cells were taken using a Nikon TMS and analyzed on ImageJ to determine total stain intensity. In both cell lines, treatment with atorvastatin in normal media results in increased lipid staining.

4.3.2 The Effect of Atorvastatin, LPDS, and LDL Treatments on Breast Cancer Cell Invasion.

Oil Red O staining of cells treated with the same conditions, as those used in the invasion assay help to show the lipid profile of the cells as they are placed in the invasion assay. Since treating cells with 5 µM Atorvastatin in normal media resulted

in an increase of intracellular lipid storage, we decided to treat each cell line with 5 µM Atorvastatin in LPDS for this assay. Treating cells with 5 µM Atorvastatin in LPDS allowed us to reduce extracellular uptake, and *de* novo synthesis of cholesterol. As seen in Figure 4.2A and B, 24 hours of SUM149 and MDA-MB-231 cells treatment with LPDS and 5 µM Atorvastatin in LPDS (A+LPDS) both resulted in a decrease in Oil Red O staining intensity. Nevertheless, there was no major difference between cells treated with LPDS and A+LPDS. The similarity between LPDS and A+LPDS treatments can be explained by the fact that Atorvastatin blocks *de novo* synthesis of free cholesterol, which is often used in cell membranes. Since Oil Red O most readily stains lipid droplets, and accumulated lipids rather than the free cholesterol found in membranes, Oil Red O staining would not show any major change in membrane-associated cholesterol. Cells treated with LDL in LPDS (LDL+LPDS) displayed an increase in lipid content, by Oil Red O staining. These stains show us that we were able to effectively cause an increase and reduction of cholesterol in each breast cancer cell line before placing them into the invasion assay. However, as seen in Figure 4.2C, MCF10a cells did not display any major difference in Oil Red O staining after LPDS or A+LPDS treatments. The MCF10a cells displayed only a slight increase in Oil Red O staining as a result of LDL+LPDS treatments.

After 24 hours of the treatments described above, the ability of the cells to invade through Matrigel, relative to untreated cells was determined. Representative images are provided in Figure 4.3. SUM149 cells that were pre-treated with LPDS showed approximately 1.7 fold increase in invasion over the untreated control. SUM149 cells treated with A+LPDS were slightly less invasive than the untreated

cells (Figure 4.2D). In addition, when SUM149 cells were treated with LDL+LPDS, their invasive capacity remained similar to the untreated cells (Figure 4.2D). As shown in Figure 4.2E, MDA-MB-231 cells that had been previously treated in LPDS were nearly three times more invasive than untreated cells ($p<0.01$). Also, MDA-MB-231 cells treated with A+LPDS were twice as invasive as untreated cells. However, cells which had been pre-treated with LDL+LPDS were slightly less invasive than untreated MDA-MB-231 cells. As seen in Figure 4.3, MCF10a cells displayed very little invasion compared to the breast cancer cells. There were no significant differences in MCF10a invasion when treated with LPDS, A+LPDS, or LDL+LPDS compared to untreated cells (Figure 4.2F).

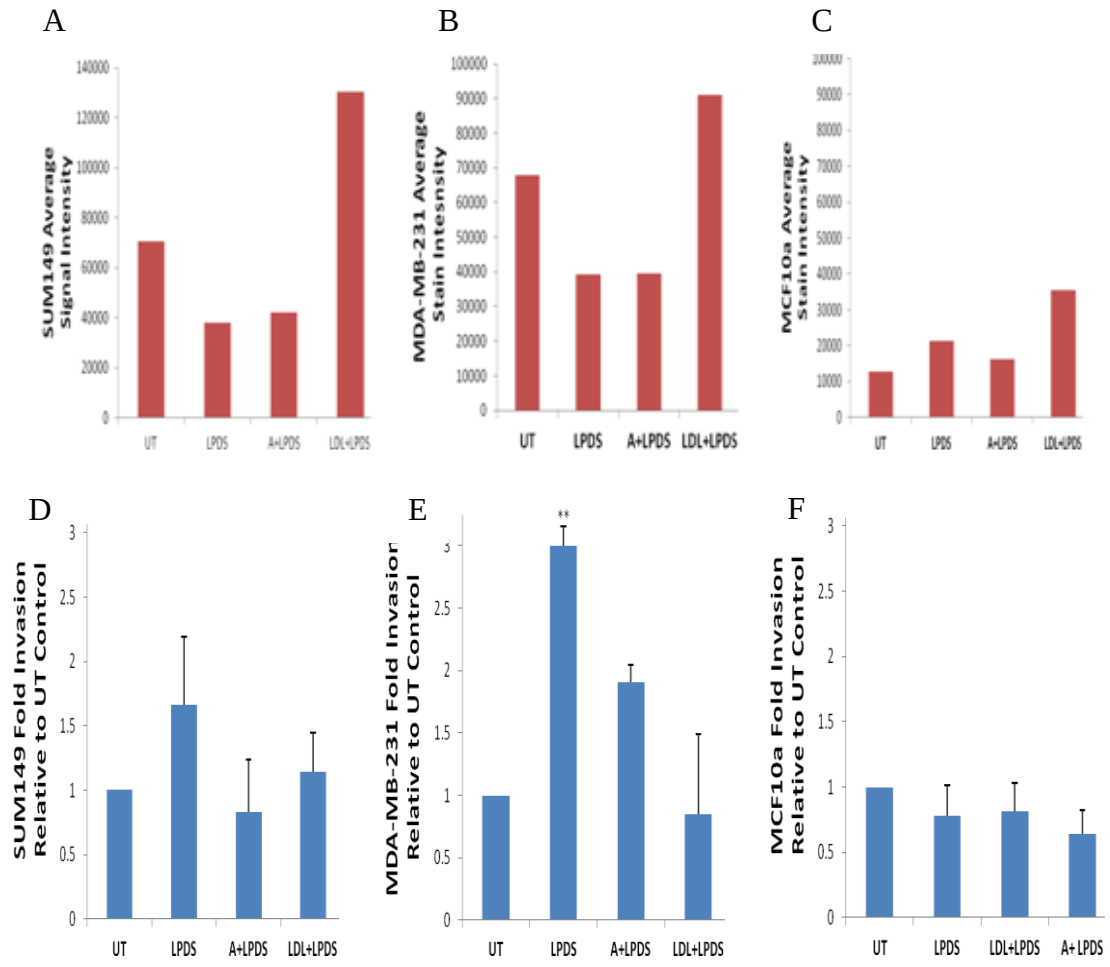

Figure 4.2 Breast cancer *in* vitro invasion Assays. Oil Red O staining of treatments prior to invasion assay for (A) SUM149, (B) MDA-MB-231, and (C) MCF10a cells. Number of invaded cells relative to untreated controls for (D) SUM149 and (E) MDA-MB-231(**p<0.01), and (F) MCF10a cells**.**

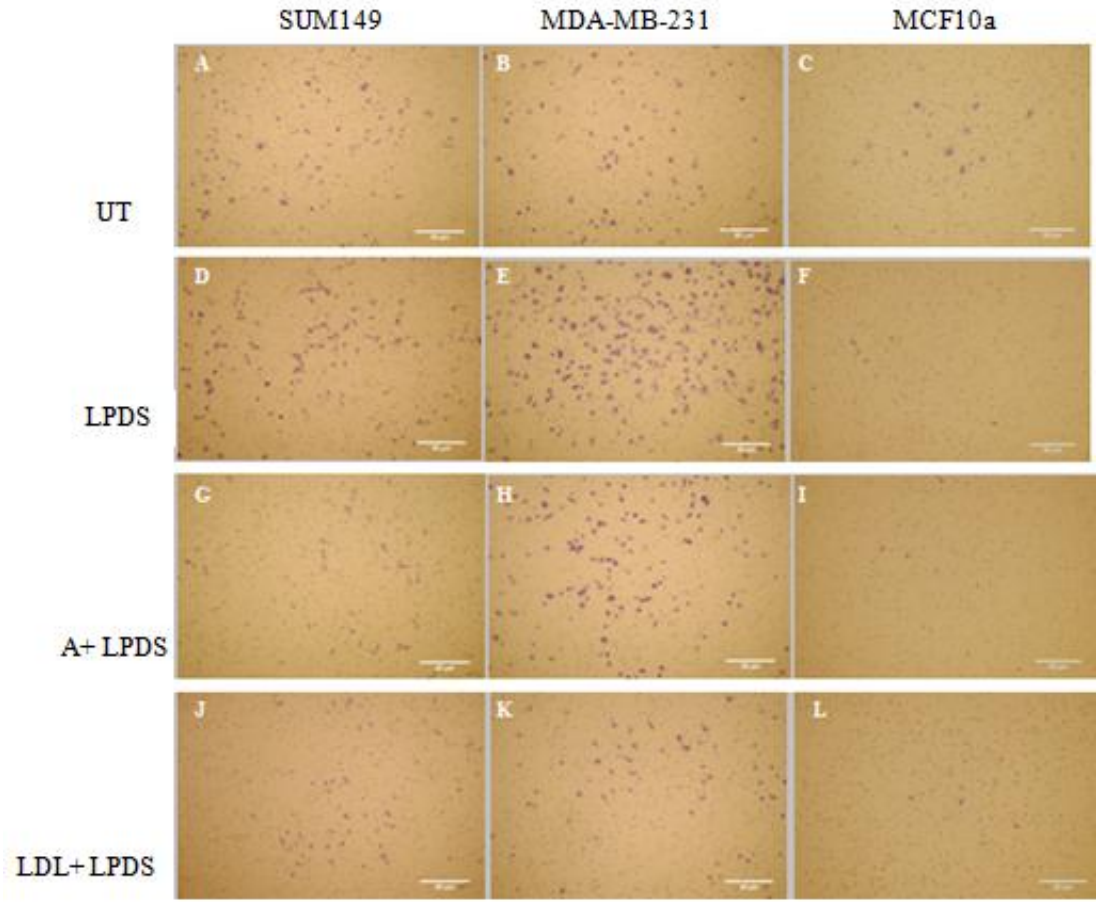

Figure 4.3 Representative images of invasion assays. (A) UT SUM149, (B) UT MDA-MB-231, (C) UT MCF10a, (D) LPDS treated SUM149, (E) LPDS treated MDA-MB-231, (F) LPDS treated MCf10a, (G) A+LPDS treated SUM149, (H) A+LPDS treated MDA-MB-231, (I) A+LPDS treated MCF10a, (J) LDL+LPDS treated SUM149, (K) LDL+LPDS treated MDA-MB-231, and (L) LDL+LPDS treated MCF10a invading cells were stained with crystal violet to aid in visualization. In both breast cancer cell lines, LPDS-treated cells displayed the most invasion. Images were taken using a Nikon Eclipes TE-200U. The scale bars represent 50µm.

4.3.3 MCF10a Transfection with RhoC F30L-RFP

In order to determine if RhoC activity had a direct effect on the cells ability to accumulate cholesterol, the non-tumorogenic cancer cell line which normally expresses inactive RhoC, MCF10a, was transfected with a fast cycling mutant RhoC (RhoC F30L-RFP). This mutant cycles through the active and inactive states of RhoC, and essentially amplifies the effect that RhoC has on a cell. After cells were incubated with the transfection reagent for 24 hours, and the transfection efficiency was determine by the presence of RFP, and cells were treated with 200µg/ml LDL for 12 hours. The cells were then stained with Oil Red O to determine if the presence of active RhoC had an effect of cholesterol uptake. Figure 4.4 shows that both the F30L-RFP mutants and the control RFP mutant displayed an increase in the uptake of LDL after being treated with 200µg/ml LDL. This observation suggests that the Fugene HD reagent used to transfect cells ultimately altered the cell membrane structure enough to allow them to take up a great deal of LDL, which was not the case in LDL-treated wild type cells.

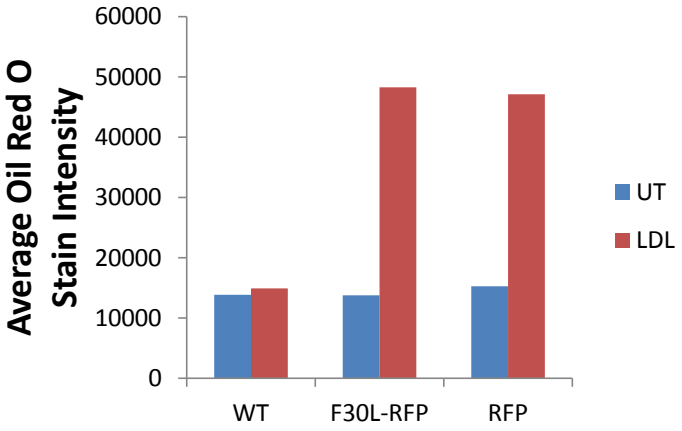

Figure 4.4 Oil Red O stain of MCF10a cells transfected with RhoC F30L-RFP to determine the effect of RhoC activity on the ability of cells to internalize cholesterol.

4.4 Conclusions

The results of the invasion assays were unlike what we initially expected. Results from the Llaverias *et al. in vitro* study showed that increasing dietary cholesterol results in greater mammary tumor metastasis [Llaverias, 2011]. However, in our MDA-MB-231 cells we see a trend that cells treated with LDL, increase their intracellular lipid content, and are less invasive through Matrigel, than untreated cells. We also see that MDA-MB-231 cells whose intracellular cholesterol levels have been depleted via LPDS or A+LPDS treatments are more invasive than untreated cells. The trend seen in these MDA-MB-231 cells may suggest the cell's requirement for a necessary dietary component, rather the direct effect that high levels of intracellular cholesterol has on the cells ability to invade. For instance, the cells which were treated with LPDS were in need of cholesterol, and therefore more willing to invade through the Matrigel towards the increasing concentrations of cholesterol, in order to

meet their dietary needs. In the same sense, the cells which were untreated or those treated with LDL were more content, and therefore less willing to migrate through the Matrigel to attain something that they had enough of.

When comparing invasion of both SUM149 and MDA-MB-231 cells which were treated with LPDS or A+LPDS, we see another interesting trend. Cells treated with A+LPDS are less invasive than LPDS-treated cells, although both treatments show similar patterns of lipid staining. This suggests that Atorvastatin-mediated inhibition of the mevalonate pathway has an effect on cells that goes beyond a simple intracellular cholesterol deficiency. This reduced invasion as a result of Atorvastatin treatment may be linked to other products of the mevalonate pathway. For instance, the prenyl groups geranylgeranyl and farnesyl are both products of the mevalonate pathway, and are both involved in posttranslational modification of small GTPases, such as Rho and Rac GTPases. The addition of these prenyl groups is a necessary feature of Rho GTPase activity. Therefore, the reduced invasion seen in A+LPDS treated cells, compared with LPDS, are most likely due to a decrease in small GTPase activity. This information is consistent with data showing that statin drugs have anti-invasive properties in many different types of cancer [Fritz, 2005; Issat, 2011; Hindler, 2006; Stamm, 2005]. This observation is additionally consistent with data describing the positive effect of RhoA and RhoC on invasive breast cancers [van Golen, 1999; Pille, 2005; Simpson, 2004].

In SUM149 cells, we see a similar, but less dramatic trend in cells treated with LPDS, compared to untreated cells. This trend is consistent with results presented in chapter 3, showing that LPDS-treated SUM149 cells exhibit a slight decrease in intracellular lipid composition compared to MDA-MB-231 cells (Figure

3.1). If we consider the increase in invasion to be a result of a need for extracellular cholesterol, we would expect SUM149 cells to have a less dramatic response to LPDS treatment, than MDA-MB-231 cells which displayed much more significant drop intracellular lipid content after LPDS treatment (Figure 3.1).

MCF10a transfection with RhoC F30L-RFP resulted in inconclusive data, due to similar findings in cells transfected with control RFP in which both transfections resulted in a substantial increase in Oil Red O staining after LDL treatment. The equivalent uptake of LDL in F30L-RFP and control RFP mutants was most likely due to increased permeability of the membrane as a result of the transfection procedure. A future attempt at expressing active RhoC in MCF10a cells would need to include a longer recovery period for the cells, after the transfection procedure, in order to return membrane permeability back to a normal state. After the cell membrane permeability has returned to normal, the cells could then be treated with LDL to determine if RhoC activity affects LDL uptake.

Chapter 5

DISCUSSION

Breast cancer is a major societal burden, claiming the lives of nearly 40,000 women each year, in the United States [American Cancer Society, 2011]. There are many classes of breast cancer, but among the most deadly is inflammatory breast cancer (IBC), which usually takes the life of its victim within 4 years of their diagnosis [Cristofanilli, 2003]. Many studies have shown that obesity and some dietary factors can increase the risk of a woman getting breast cancer [Lorincz, 2006]. Through this information, and other studies, cholesterol has been implicated to be interconnected with breast cancer [Llaverias, 2011; Awad, 2001; Rao, 1988; El-Sohemy, 2005]. Understanding details of the relationship between cholesterol and breast cancer is necessary for determining possible advancements in patient care and treatment. Nevertheless, proper understandings of such details remain out of reach.

The findings of the present study not only reaffirm observations seen in other studies, but also bring novel information that helps to understand underlining mechanisms of cholesterol accumulation by breast cancer cells. Our initial studies using Oil Red O showed an abundance of visible lipid droplets in both breast cancer cell lines. This evaluation is consistent with high-resolution NMR readings which found that more malignant tissues contained greater amounts of cholesterol esters than normal breast tissues [Beckonert, 2003]. The presence of high concentrations of cholesterol esters in extremely malignant tissues indicates the storage of cholesterol as

esters, inside of lipid droplets. This sort of cholesterol storage in breast cancer is being observed more often, but the question of what benefit these cells have in storing cholesterol still remains. One may speculate that breast cancer cells maintain cholesterol stores in an effort to provide for increased cellular proliferation. Future studies to analyze this hypothesis may include proliferation assays of breast cancer cells that have been depleted of their intracellular stores of cholesterol.

Our results from treating cells with LPDS and LDL give new insights into the way each cell line prefers to amass cholesterol. While both the IBC and non-IBC cell line display an intracellular cholesterol content that is dependent on the extracellular environment, the SUM149 cell lines are more responsive to altered extracellular cholesterol concentrations. The results from these experiments suggest that IBC cells have not only developed mechanisms which encourage uptake and over-storage of extracellular cholesterol, but that they have also capitalized on *de novo* synthesis of cholesterol to minimize the depletion of those stores. This characteristic seems essential to IBC cells which are known for their presence in the dermal lymphatics. The dermal lymphatics, while abundant in cholesterol-rich lipoprotein molecules, often undergo fluctuations in composition which are affected by diet as well as the transfer of fluids [Sloop, 1987]. The ability of these IBC cells to maintain their cholesterol stores would essentially help them survive the environment of the dermal lymphatics.

On the other hand, we found that MDA-MB-231 cells were not capable of maintaining their intracellular cholesterol stores, when placed in an environment depleted of lipoproteins. This finding suggests that MDA-MB-231 cells rely heavily on their extracellular environment for their cholesterol loading. This characteristic of

MDA-MB-231 cells is consistent with the fact that this cell line was originally derived from a malignant pleural effusion. A study of the cholesterol content in pleural effusions showed that malignant pleural effusions contain high concentrations of cholesterol (an average of 94mg/dl) [Hamm, 1987]. This study suggests that these cancer cells were better equipped for an environment with consistently high concentrations of cholesterol, such as a malignant pleural effusion. Nevertheless, it would be necessary to repeat the experiment with multiple cell lines of IBC and non-IBC cell lines in order to see if the observation holds true for each cancer type regardless of the cell line.

Although we see this clear link between cholesterol deposition and the environment that these two cancers were derived from, it is unclear whether the phenotype of these cells denotes the origin. Did the mutations which caused these cells to become cancerous also cause them to seek out environments which best fit their abilities? Did the cells which found themselves in these particular environments develop further characteristics that allowed them to out-survive other cancerous cells present? Future studies aimed at analyzing mutations, in regulatory components of cholesterol metabolism, transport, and storage, in breast cancers from multiple origins may help to answer these questions.

The trends from our studies on the effect of cholesterol depletion, and loading on breast cancer cells implicate a new role for cholesterol concentration in breast cancer cell invasion. Our studies suggest that malignant breast cells which have been programmed to accrue intracellular cholesterol are more willing to seek out cholesterol by invasion, when their needs are not being met in their current environments. This observation provides an understanding of why we often see

greater cholesterol accumulation in more metastatic cancers. Rather than the extra cholesterol increasing their ability to invade and metastasize, the invasive properties are a result of their need for cholesterol. In the Llaverias *et al*. study, we saw that malignant rat mammary metastasis increased as a result of a cholesterol-enriched diet [Llaverias, 2011]. This method for introducing more cholesterol to the breast tumor results in a systemic cholesterol increase, providing higher concentrations of lipoproteins in the circulatory system. Malignant cells in need of cholesterol would be more likely to invade to reach the circulatory systems which are rich in cholesterol. The information from our invasion studies, taken with this understanding of the metastasis seen as a result of increased dietary cholesterol may be used to develop a method to help prevent cancer metastasis. For instance, it may be possible to prevent breast cancer metastasis by reducing circulating cholesterol levels, while increasing regional cholesterol to the sight of the tumor. We might expect this type of treatment to be advantageous if one is trying to prevent metastasis from occurring in a patient while simultaneously treating with chemotherapy, radiation, or before surgery to remove the tumor. In this setting, statins could also be employed as a way of decreasing the cells ability to invade, by its role in inhibiting Rho GTPase activation [Collison, 2003, Klawitter, 2010]. Future studies to test this idea would include an animal model for breast tumors where the simultaneous effect of systemic cholesterol reduction and regional cholesterol treatment would be analyzed to determine the effect on tumor metastasis over a period of time.

REFERENCES

American Cancer Society. 2011 Breast Cancer Facts & Figures 2011-2012. Atlanta: American Cancer Society.

Anderson WF, Schairer C, Chen BE, Hance KW, Levine PH. 2005. Epidemiology of inflammatory breast cancer (IBC). Breast Dis 22:9-23.

Awad AB, Williams H, Fink CS. 2001. Phytosterols reduce in vitro metastatic ability of MDA-MB-231 human breast cancer cells. Nutr Cancer 40(2):157-64.

Ball DW, Hill JW, Scott RJ. 2011. The Basics of General, Organic, and Biological Chemistry. http://catalog.flatworldknowledge.com/catalog/editions/p421791

Basu GD, Liang WS, Stephan DA, Wegener LT, et al. 2006. A novel role for cyclooxygenase-2 in regulating vascular channelnformation by human breast cancer cells. Breast cancer research 8(6)

Beckerman M. 2009. Cellular Signalling in Health and Disease. Springer Science+Business Media, LLC, 233 Spring Street, New York, NY 10013, USA.

Beckonert O, Monnerjahn J, Bonk U, Leibfritz D. 2003. Visualizing metabolic changes in breast-cancer tissue using 1H-NMR spectroscopy and self-organizing maps. NMR Biomed 16(1):1-11.

Bennis F, Favre F, Le Gaillard F, and Soula G. 2006. Importance of mevalonate-derived products in the control of HMG-CoA reductase activity and growth of human lung adenocarcinoma cell line a549. International Journal of Cancer 55(4):640-645

Berg JM, Tymoczko JL, Stryer L. 2002 Biochemistry, 5th edition. W.H. Freeman and Co., New York, NY 10013

Berg JM, Tymoczko JL, Stryer L. 2007 Biochemistry, 6th edition. W.H. Freeman and Co., New York, NY 10013

Boureux A, Vignal E, Faure S, Fort P. 2007. Evolution of the Rho family of ras-like GTPases in eukaryotes. Mol Biol Evol 24(1):203-16.

Brasaemle DL. 2007. The perlipin family of structural lipid droplet proteins: stabilization of lipid droplets and control of lipolysis. Journal of Lipid Research. 48:2548-2559.

Brown MS, Goldstein JL. 1979. Reversible accumulation of cholesteryl esters in macrophages incubated with acetylated lipoproteins. J. Cell Biology. 82:597-613

Brown MS, Goldstein JL. 1985a. The LDL receptor and HMG-CoA reductase--two membrane molecules that regulate cholesterol homeostasis. Curr Top Cell Regul 26:3-15.

Brown MS, Goldstein JL. 1985b. The receptor model for transport of cholesterol in plasma. Ann N Y Acad Sci 454:178-82.

Brown MS, Goldstein JL. 1997. The SREBP pathway: regulation of cholesterol metabolism by proteolysis of a membrane-bound transcription factor. Cell 89(3):331-40.

Buhaescu I, Izzedine H. 2007. Mevalonate Pathway: a review of clinical implications. Clinical Biochemistry. 40: 575-584

Buhaescu I, Izzedine H. 2007. Mevalonate pathway: a review of clinical and therapeutical implications. Clinical Biochemistry 40 (2007) 575–584

Cambien F, Ducimetiere P, Richard J. 1980. Total serum cholesterol and cancer mortality in a middle-aged male population. Am J Epidemiol. 112:388–94

Carlson RW, Allred DC, Anderson BO, Burstein HJ, Carter WB, Edge SB, Erban JK, Farrar WB, Goldstein LJ, Gradishar WJ et al. 2009. Breast cancer. Clinical practice guidelines in oncology. J Natl Compr Canc Netw 7(2):122-92.

Caruso MG, Notarnicola M. 2005. Biochemical changes of mevalonate pathway in human colorectal cancer. Anticancer Res 25(5):3393-7.

Castelló-Cros R, Khan DR, Simons J, Valianou M, Cukierman E. 2009. Staged stromal extracellular 3D matrices differentially regulate breast cancer cell responses through PI3K and beta1-integrins. BMC Cancer 9:94

Chang S, Parker SL, Pham T, Buzdar AU, Hursting SD. 1998. Inflammatory breast carcinoma incidence and survival: the surveillance, epidemiology, and end results program of the National Cancer Institute, 1975-1992. Cancer 82(12):2366-72.

Collisson EA, Kleer C, Wu M, De A, Gambhir SS, Merajver SD, Kolodney MS. 2003. Atorvastatin prevents RhoC isoprenylation, invasion, and metastasis in human melanoma cells. Mol Cancer Ther 2(10):941-8.

Connelly MA, Klein SM, Azhar S, Abumrad NA, Williams DL. 1999. Comparison of class B scavenger receptors, CD36 and scavenger receptor BI (SR-BI), shows that both receptors mediate high density lipoprotein-cholesteryl ester selective uptake but SR-BI exhibits a unique enhancement of cholesteryl ester uptake. J Biol Chem 274(1):41-7.

Coughlin SS, Ekwueme DU. 2009. Breast cancer as a global health concern. Cancer Epidemiology 33(5):315-318

Cristofanilli M, Buzdar AU, Hortobágyi GN. 2003. Update on the management of inflammatory breast cancer. Oncologist 8(2):141-8.

Cristofanilli M. 2008. Inflammatory breast cancer: defining a new entity. Semin Oncol 35(1):6.

Dawood S, Merajver SD, Viens P, Vermeulen PB, Swain SM, Buchholz TA, Dirix LY, Levine PH, Lucci A, Krishnamurthy S et al. 2011. International expert panel on inflammatory breast cancer: consensus statement for standardized diagnosis and treatment. Ann Oncol 22(3):515-23.

Dawood S. 2010. Biology and management of inflammatory breast cancer. Expert Rev Anticancer Ther 10(2):209-20.

Demierre MF, Higgins PD, Gruber SB, Hawk E, Lippman SM. 2005. Statins and cancer prevention. Nat Rev Cancer 5(12):930-42.

Dias SM, Cerione RA. 2007. X-ray crystal structures reveal two activated states for RhoC. Biochemistry 46(22):6547-58.

Duncan RE, El-Sohemy A, Archer MC. 2004. Mevalonate promotes the growth of tumors derived from human cancer cells in vivo and stimulates proliferation in vitro with enhanced cyclin-dependent kinase-2 activity. J Biol Chem 279(32):33079-84.

Duncan RE, El-Sohemy A, Archer MC. 2005b. Statins and cancer development. Cancer Epidemiol Biomarkers Prev 14(8):1897-8.

El-Sohemy A, Archer MC. 2000. Inhibition of N-methyl-N-nitrosourea- and 7,12-dimethylbenz[a] anthracene-induced rat mammary tumorigenesis by dietary cholesterol is independent of Ha-Ras mutations. Carcinogenesis 21(4):827-31.

Endogenous Hormones and Breast Cancer Collaborative Group. 2003. Body mass index, serum sex hormones, and breast cancer risk in postmenopausal women. Journal of the National Cancer Institute 95(16):118-126.

Erbas B, Provenzano E, Armes J, Gertig D. 2006. The natural history of ductal carcinoma in situ of the breast: a review. Breast Cancer Res Treat 97(2):135-44.

Esfahani M, Swaney J. 1990. Advances in cholesterol research. The Teleford Press, Inc. P.O.B 287 Caldwell, NJ 07006, USA

Etienne-Manneville S, Hall A. 2002. Rho GTPases in cell biology. Nature 420(6916):629-35.

Fagherazzi G, Fabre A, Boutron-Ruault MC, Clavel-Chapelon F. 2010. Serum cholesterol level, use of a cholesterol-lowering drug, and breast cancer: results from the prospective E3N cohort. Eur J Cancer Prev 19(2):120-5.

Farieda A, Fariedb LS, Kimuraa H, et al. 2006. RhoA and RhoC proteins promote both cell proliferation and cell invasion of human oesophageal squamous cell

carcinoma cell lines in vitro and in vivo. European Journal of Cancer. 42(10):1455-1465

Fiorenza AM, Branchi A, Sommariva D. 2000. Serum lipoprotein profile in patients with cancer. A comparison with non-cancer subjects. Int J Clin Lab Res 30(3):141-5.

Foote FW, Stewart FW. 1941. Lobular Carcinoma in situ. American Journal of Pathology. 17:491-497

Freeman MR, Solomon KR. 2004. Cholesterol and Prostate Cancer. Journal of Cellular Biochemistry. 91:54-69.

Fritz G. 2005. HMG-CoA reductase inhibitors (statins) as anticancer drugs (review). Int J Oncol 27(5):1401-9.

Garcia-Palmieri MR, Sorlie PD, Costas R, Jr., Havlik RJ. 1981. An apparent inverse relationship between serum cholesterol and cancer mortality in Puerto Rico. Am J Epidemiol 114:29–40.

Goldstein JL, Brown MS, Anderson RG, Russell DW, Schneider WJ. 1985a. Receptor-mediated endocytosis: concepts emerging from the LDL receptor system. Annu Rev Cell Biol 1:1-39.

Goldstein JL, Brown MS. 1985b. The LDL receptor and the regulation of cellular cholesterol metabolism. J Cell Sci Suppl 3:131-7.

Green JR. 2004. Bisphosphonates: Preclinical Review. Oncologist. 9:3-13.

Günhan-Bilgen I, Ustün EE, Memiş A. 2002. Inflammatory breast carcinoma: mammographic, ultrasonographic, clinical, and pathologic findings in 142 cases. Radiology 223(3):829-38.

Gustafson B, Smith U. 2010 Activation of Canonical Wingless-type MMTV Integration Site Family (Wnt) Signaling in Mature Adipocytes Increases β-Catenin Levels and Leads to Cell Dedifferentiation and Insulin Resistance. J Biol Chem. 285(18):14031–14041.

Hakem A, Sanchez-Sweatman O, You-Ten A, Duncan G, Wakeham A, Khokha R, Mak TW. 2005. RhoC is dispensable for embryogenesis and tumor initiation but essential for metastasis. Genes Dev 19(17):1974-9.

Hakoshima T, Shimizu T, Maesaki R. 2003. Structural basis of the Rho GTPase signaling. J Biochem 134(3):327-31.

Hall A. 1998. Rho GTPases and the actin cytoskeleton. Science 279(5350):509-14.

Hamm H, Brohan U, Bohmer R, Missmahl HP. 1987. Cholesterol in pleural effusions. A diagnostic aid. Chest 92(2):296-302.

Harwood HJ, Alvarez IM, Noyes WD, Stacpoole PW. 1991. In vivo regulation of human leukocyte 3-hydroxy-3-methylglutaryl coenzyme A reductase: increased enzyme protein concentration and catalytic efficiency in human leukemia and lymphoma. J Lipid Res 32(8):1237-52

Hindler K, Cleeland CS, Rivera E, Collard CD. 2006. The role of statins in cancer therapy. The Oncologist 11:306-315

Hoffmeyer MR, Wall KM, Sharmawardhane SF. 2005. In vitro analysis of the invasive phenotype of SUM 149, an inflammatory breast cancer cell line. Cancer cell international 5(11)

Horton JD, Goldstein JL, Brown MS. 2002. SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver. J Clin Invest 109(9):1125-31.

Hu J, Zhang Z, Shen W, Azhar S. 2010. Cellular cholesterol delivery, intracellular processing and utilization for biosynthesis of steroid hormones. Nutrition & Metabolism 7:47

Hu J, La Vecchia C, de Groh M, Negri E, Morrison H, Mery L, Group tCCRER. 2011. Dietary cholesterol intake and cancer. Ann Oncol.

Insel P, Turner RE, Ross D. 2010. Discovering nutrition Third Edition. Jones and Bartlett Publishers. Sudbury, MA 01776, USA

International Collaborative Group. Circulating cholesterol level and risk of death from cancer in men aged 40 to 69 years. JAMA 1982; 248:2853–9.

Issat T, Nowis D, Bil J, Winiarska M, Jakobisiak M, Golab J. 2011. Antitumor effects of the combination of cholesterol reducing drugs. Oncol Rep 26(1):169-76.

Jacobs EJ, Gapstur SM. 2009. Cholesterol and cancer: answers and new questions. Cancer Epidemiol Biomarkers Prev 18(11):2805-6.

Jaffe AB, Hall A. 2005. Rho GTPases: biochemistry and biology. Annu Rev Cell Dev Biol 21:247-69.

Joglekar MS, van Golen K. 2010. Role of Caveolin-1 and PDGFR in Inflammatory Breast Cancer. Cancer Res 70(24 Suppl.):327s

Kidwell KA. 2007. Inflammatory breast cancer: A race against time. JAAPA 20(9): 40-44.

Kitahara CM, Berrington de González A, Freedman ND, Huxley R, Mok Y, Jee SH, Samet JM. 2011. Total cholesterol and cancer risk in a large prospective study in Korea. J Clin Oncol 29(12):1592-8.

Kitzing TM, Wang Y, Pertz O, Copeland JW, Grosse R. 2010. Formin-like 2 drives amoeboid invasive cell motility downstream of RhoC. Oncogene 29(16):2441-8.

Klawitter J, Shokati T, Moll V, Christians U. 2010. Effects of lovastatin on breast cancer cells: a proteo-metabonomic study. Breast Cancer Res 12(2):R16.

Kleer CG, Griffith KA, Sabel MS, Gallagher G, van Golen KL, Wu ZF, Merajver SD. 2005. RhoC-GTPase is a novel tissue biomarker associated with biologically aggressive carcinomas of the breast. Breast Cancer Res Treat 93(2):101-10.

Kleer CG, van Golen KL, Braun T, Merajver SD. 2001. Persistent E-cadherin expression in inflammatory breast cancer. Mod Pathol 14(5):458-64.

Kleer CG, van Golen KL, Merajver SD. 2000. Molecular biology of breast cancer metastasis. Inflammatory breast cancer: clinical syndrome and molecular determinants. Breast Cancer Res 2(6):423-9.

Kleer CG, van Golen KL, Zhang Y, Wu ZF, Rubin MA, Merajver SD. 2002a. Characterization of RhoC expression in benign and malignant breast disease: a potential new marker for small breast carcinomas with metastatic ability. Am J Pathol 160(2):579-84.

Kritchevsky SB, Kritchevsky D. 1992. Serum cholesterol and cancer risk: an epidemiologic perspective. Annu Rev Nutr. 1992;12:391-416.

Lacal JC, McCormick F. 1993. Ras Superfamily of GTPases. CRC Press, Inc. Boca Raton, FL 33431, USA.

Lerebours F, Bieche I, Lidereau R. 2005. Update on inflammatory breast cancer. Breast Cancer Res 7(2):52-8.

Li CI, Anderson BO, Daling JR, Moe RE. 2003a. Trends in incidence rates of invasive lobular and ductal breast carcinoma. JAMA 289(11):1421-4.

Li YC, Park MJ, Ye SK, Kim CW, Kim YN. 2006b. Elevated levels of cholesterol-rich lipid rafts in cancer cells are correlated with apoptosis sensitivity induced by cholesterol-depleting agents. Am J Pathol 168(4):1107-18; quiz 1404-5.

Llaverias G, Danilo C, Mercier I, Daumer K, Capozza F, Williams TM, Sotgia F, Lisanti MP, Frank PG. 2011. Role of cholesterol in the development and progression of breast cancer. Am J Pathol 178(1):402-12.

Lo AC, Georgopoulos A, Kleer CG, Banerjee M, Omar S, Khaled H, Eissa S, Hablas A, Omar HG, Douglas JA et al. 2009. Analysis of RhoC expression and lymphovascular emboli in inflammatory vs non-inflammatory breast cancers in Egyptian patients. Breast 18(1):55-9.

Lo AC, Kleer CG, Banerjee M, Omar S, Khaled H, Eissa S, Hablas A, Douglas JA, Alford SH, Merajver SD et al. 2008. Molecular epidemiologic features of inflammatory breast cancer: a comparison between Egyptian and US patients. Breast Cancer Res Treat 112(1):141-7.

Lorincz AM, Sukumar S. 2006. Molecular links between obesity and breast cancer. Endocr Relat Cancer 13(2):279-92.

Mady EA. 2000. Association between estradiol, estrogen receptors, total lipids, triglycerides, and cholesterol in patients with benign and malignant breast tumors. J Steroid Biochem Mol Biol 75(4-5):323-8.

Maxfield FR, van Meer G. 2010. Cholesterol, the central lipid of mammalian cells. Curr Opin Cell Biol 22(4):422-9.

McNeill RE, Miller N, Kerin MJ. 2007. Evaluation and validation of candidate endogenous control genes for real-time quantitative PCR studies of breast cancer. BMC Mol Biol 8:107.

Morimoto LM, White E, Chen Z, Chlebowski RT, Hays J, Kuller L, Lopez AM, Manson J, Margolis KL, Muti PC et al. 2002. Obesity, body size, and risk of postmenopausal breast cancer: the Women's Health Initiative (United States). Cancer Causes Control 13(8):741-51.

Neve RM, Chin K, Fridlyand J, Yeh J, Baehner FL, Fevr T, Clark L, Bayani N, Coppe JP, Tong F et al. 2006. A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes. Cancer Cell 10(6):515-27.

Ness GC, Kohlruss N, Gertz KR. 2003. Association of the low-density lipoprotein receptor with caveolae in hamster and rate liver. Biochemical and Biophysical Research communications. 303:177-181

Orlova EV, Sherman MB, Chiu W, Mowri H, Smith LC, Gotto AM. 1999. Three-dimensional structure of low density lipoproteins by electron cryomicroscopy. Proc Natl Acad Sci U S A 96(15):8420-5.

Pani A, Dessi S. 2004 Cell growth and cholesterol esters. Kluwer Academic {lenum Publishers, 233 Spring Street, New York, NY 10013, U.S.A

Parton RG. 1996. Caveolae and Caveolins. Current Opinion in Cell Biology 8:542-548

Pillé JY, Denoyelle C, Varet J, Bertrand JR, Soria J, Opolon P, Lu H, Pritchard LL, Vannier JP, Malvy C et al. 2005. Anti-RhoA and anti-RhoC siRNAs inhibit the proliferation and invasiveness of MDA-MB-231 breast cancer cells in vitro and in vivo. Mol Ther 11(2):267-74.

Rao KN, Melhem MF, Gabriel HF, Eskander ED, Kazanecki ME, Amenta JS. 1988. Lipid composition and de novo cholesterogenesis in normal and neoplastic rat mammary tissues. J Natl Cancer Inst 80(15):1248-53.

Renaud JF, Scanu AM, Kazazoglou T, Lombet A, Romey G, Lazdunski M. 1982. Normal serum and lipoprotein-deficient serum give different expressions of excitability, corresponding to different stages of differentiation, in chicken cardiac cells in culture. Proc Natl Acad Sci U S A 79(24):7768-72.

Rumsey SG, Galeano NF, Arad Y, Deckelbaum RJ. 1992. Cryopreservation with sucrose maintains normal physical and biological properties of human plasma low density lipoproteins Journal of Lipid Research 33:1551-1561

Sahai E, Marshall CJ. 2002a. RHO-GTPases and cancer. Nat Rev Cancer 2(2):133-42.

Sahai E, Marshall CJ. 2002b. ROCK and Dia have opposing effects on adherens junctions downstream of Rho. Nat Cell Biol 4(6):408-15.

Sakai K, Okuyama H, Yura J, Takeyama H, Shinagawa N, Tsuruga N, Kato K, Miura K, Kawase K, Tsujimura T. 1992. Composition and turnover of phospholipids and neutral lipids in human breast cancer and reference tissues. Carcinogenesis 13(4):579-84.

Schmittgen TD, Livak KJ. 2008. Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc 3(6):1101-8.

Schultz JR, Tu H, Luk A, Repa JJ, Medina JC, Li L, Schwendner S, Wang S, Thoolen M, Mangelsdorf DJ et al. 2000. Role of LXRs in control of lipogenesis. Genes Dev 14(22):2831-8.

Seewaldt VL, Scott V. 2007. Rapid Progression of Basal-Type Breast Cancer. N Engl J Med 356(13):e12

Shenkier T, Weir L, Levine M, Olivotto I, Whelan T, Reyno L, Cancer SCoCPGftCaToB. 2004. Clinical practice guidelines for the care and treatment of breast cancer: 15. Treatment for women with stage III or locally advanced breast cancer. CMAJ 170(6):983-94.

Sherwin RW,Wentworth DN, Cutler JA, Hulley SB, Kuller LH, StamlerJ. 1987. Serum cholesterol levels and cancer mortality in 361,662 men screened for the Multiple Risk Factor Intervention Trial. JAMA. 257:943–8

Simons K, Ikonen E. 2000. How cells handle cholesterol. Science 290(5497):1721-6.

Simpson KJ, Dugan AS, Mercurio AM. 2004. Functional analysis of the contribution of RhoA and RhoC GTPases to invasive breast carcinoma. Cancer Res 64(23):8694-701.

Siperstein MD, Fagan VM. 1964. Deletion of the Cholesterol-negative Feedback System in Liver Tumors. Cancer Res 24:1108-1115.

Sloop CH, Dory L, Roheim PS. 1987. Interstitial fluid lipoproteins. J Lipid Res 28(3):225-37.

Srlie T, Perou CM, Tibshirani R, et al. 2001. Gene expression patterns of breast carcinoma distinguish tumor subclasses with clinical implications. PNAS 98(19):10869-10874

Stamm JA, Ornstein DL. 2005. The role of statins in cancer prevention and treatment. Oncology (Williston Park) 19(6):739-50; discussion 753-4.

Stangl H, Hyatt M, Hobbs HH. 1999. Transport of lipids from high and low density lipoproteins via scavenger receptor-BI. J Biol Chem 274(46):32692-8.

Stein S, Lohmann C, Schäfer N, Hofmann J, Rohrer L, Besler C, Rothgiesser KM, Becher B, Hottiger MO, Borén J et al. 2010. SIRT1 decreases Lox-1-mediated foam cell formation in atherogenesis. Eur Heart J 31(18):2301-9.

Thomas ZI, Gibson W, Sexton JZ, Aird KM, Ingram SM, Aldrich A, Lyerly HK, Devi GR, Williams KP. 2011. Targeting GLI1 expression in human inflammatory breast cancer cells enhances apoptosis and attenuates migration. Br J Cancer 104(10):1575-86.

Trigatti BL, Krieger M, Rigotti A. 2003. Influence of the HDL receptor SR-BI on lipoprotein metabolism and atherosclerosis. Arterioscler Thromb Vasc Biol 23(10):1732-8.

Twiddy AL, Leon CG, Wasan KM. 2011. Cholesterol as a potential target for castration-resistant prostate cancer. Pharm Res 28(3):423-37.

Valero V, Buzdar AU, Hortobagyi GN. 1996. Locally Advanced Breast Cancer. Oncologist 1(1 & 2):8-17.

van Golen KL (ed.) The Rho GTPases in cancer. Springer. 2010; v-vii

van Golen KL, Bao LW, Pan Q, Miller FR, Wu ZF, Merajver SD. 2002b. Mitogen activated protein kinase pathway is involved in RhoC GTPase induced motility, invasion and angiogenesis in inflammatory breast cancer. Clin Exp Metastasis 19(4):301-11.

van Golen KL, Davies S, Wu ZF, Wang Y, Bucana CD, Root H, Chandrasekharappa S, Strawderman M, Ethier SP, Merajver SD. 1999. A novel putative low-affinity insulin-like growth factor-binding protein, LIBC (lost in inflammatory breast cancer), and RhoC GTPase correlate with the inflammatory breast cancer phenotype. Clin Cancer Res 5(9):2511-9.

van Golen KL, Wu ZF, Qiao XT, Bao L, Merajver SD. 2000a. RhoC GTPase overexpression modulates induction of angiogenic factors in breast cells. Neoplasia 2(5):418-25.

Van Laere SJ, Van den Eynden GG, Van der Auwera I, Vandenberghe M, van Dam P, Van Marck EA, van Golen KL, Vermeulen PB, Dirix LY. 2006. Identification of cell-of-origin breast tumor subtypes in inflammatory breast cancer by gene expression profiling. Breast Cancer Res Treat 95(3):243-55.

Vatten LJ, Foss OP, Kvinnsland S. 1991. Overall survival of breast cancer patients in relation to preclinically determined total serum cholesterol, body mass index, height and cigarette smoking: a population-based study. Eur J Cancer 27(5):641-6.

Vatten LJ, Foss OP. 1990. Total serum cholesterol and triglycerides and risk of breast cancer: a prospective study of 24,329 Norwegian women. Cancer Res 50(8):2341-6.

Vega FM, Fruhwirth G, Ng T, Ridley AJ. 2011. RhoA and RhoC have distinct roles in migration and invasion by acting through different targets. J Cell Biol 193(4):655-65.

Wallace RB, Rost C, Burmeister LF, Pomrehn PR. 1982 Cancer incidence in humans: relationship to plasma lipids and relative weight. J Natl Cancer Inst 68:915–18.

Wang N, Lan D, Chen W, Matsuura F, Tall AR. 2004. ATP-binding cassette transporters G1 and G4 mediate cellular cholesterol efflux to high-density lipoproteins. Proc Natl Acad Sci U S A 101(26):9774-9.

Wang N, Tall AR. 2003. Regulation and Mechanisms of ATP-Binding Cassette Transporter A1-Mediated Cellular Cholesterol Efflux. Arterioscler Thromb Vasc Biol 23:1178-1184

Wasan Laboratory. Vancouver, British Columbia. Retrieved from http://www.wasanlab.com/research.html in Oct 2011.

Wejde J, Blegen H, Larsson O. Requirement for mevalonate in the control of proliferation of human breast cancer cells. Anticancer Res. 1992 Mar-Apr;12(2):317-24.

Wheeler AP, Ridley AJ. 2004. Why three Rho proteins? RhoA, RhoB, RhoC, and cell motility. Exp Cell Res 301(1):43-9.

Williams PR. 1981. Cancer Incidence by levels of cholesterol. JAMA, the journal of the American Medical Association. 245(3):247

Wu M, Wu ZF, Rosenthal DT, Rhee EM, Merajver SD. 2010. Characterization of the roles of RHOC and RHOA GTPases in invasion, motility, and matrix adhesion in inflammatory and aggressive breast cancers. Cancer 116(11 Suppl):2768-82.

Yancey PG, Bortnick AE, Kellner-Weibel G, de la Llera-Moya M, Phillips MC, Rothblat GH. 2003. Importance of different pathways of cellular cholesterol efflux. Arterioscler Thromb Vasc Biol 23(5):712-9.

Zhuang L, Kim J, Adam RM, Solomon KR, Freeman MR. 2005. Cholesterol targeting alters lipid raft composition and cell survival in prostate cancer cells and xenografts. J Clin Invest 115(4):959-68.