Effects of Branched Chain Amino Acid Transaminase 1 in Claudin-low Breast Cancer

By Lisa Reynen

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ABSTRACT

Effects of Branched Chain Amino Acid Transaminase 1 in Claudin-low Breast Cancer

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Dr. Roger Moorehead

Breast cancer can be classified into five distinct subtypes. One subtype, claudin-low breast cancer, accounts for approximately 7% of the breast cancer cases and these tumors are notoriously aggressive. RNA sequencing of human claudin-low breast cancers by other groups and RNA sequencing of a murine claudin-low breast cancer cell line by our group has revealed that Bcat1 is up-regulated in this breast cancer subtype. Bcat1 regulates the metabolism of branched chain amino acids. Based on this data we hypothesized that the expression of Bcat1 is driving the aggressive nature of this cancer subtype. BCAT1 was confirmed to be up-regulated in our murine claudin-low breast cancer cell line at the mRNA and protein level. However, our study demonstrates that BCAT1 does not influence proliferation, survival, or migration. However, it does appear that the reverse BCAT1 reaction may be favoured in our murine claudin-low breast cancer cell line.
ACKNOWLEDGMENTS

I would like to thank all those who helped me throughout my research by providing guidance and support.

To my advisor, Dr. Roger Moorehead, I cannot put into words how grateful I am for the opportunity to do research under your guidance, I consider myself truly lucky to have had the privilege of working with you for the past two years. I am forever appreciative of your patience and support.

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I would like to thank my parents for all of their support and encouragement.

Finally, I would like to thank Rebecca Jagroop, for listening to me whine for two years.
DECLARATION OF WORK PERFORMED

I declare that all of the work described in this thesis, with the exception of the items shown below, was performed by me.

Dr. Rob Jones compiled and analyzed the human claudin-low breast cancer tumor data collected from the University of North Carolina Microarray Database

Dr. Roger Moorehead conducted qRT-PCR on human cell lines, MCF-7 and MDA-MB-231, for expression levels of Beql mRNA.

The Metabolomics Innovation Centre of the University of Alberta conducted liquid chromatography – mass spectrometry for analysis of conditioned media samples.

Some reagents used in western blotting and immunofluorescence were created by current and former members of the Moorehead lab including: Paige Corner, Dr. Rob Jones, and Katrina Watson
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<td>ATM</td>
<td>Ataxia-telangiesctasia mutated [gene]</td>
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<td>BCAA</td>
<td>Branched chain amino acid</td>
</tr>
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<td>BCAT1</td>
<td>Branched chain amino acid transaminase 1</td>
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<tr>
<td>BCAT2</td>
<td>Branched chain amino acid transaminase 2</td>
</tr>
<tr>
<td>BRCA</td>
<td>Breast cancer [gene]</td>
</tr>
<tr>
<td>BrdU</td>
<td>Bromodeoxyuridine</td>
</tr>
<tr>
<td>DOT1L</td>
<td>Disruptor of telomeric silencing 1</td>
</tr>
<tr>
<td>ECAR</td>
<td>Extracellular acidification rate</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial to mesenchymal transition</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen receptor</td>
</tr>
<tr>
<td>FCCP</td>
<td>Carbonylcyanide p-trifluoromethoxyphenylhydrazone</td>
</tr>
<tr>
<td>HER2</td>
<td>Human epidermal growth factor receptor 2</td>
</tr>
<tr>
<td>HRT</td>
<td>Hormone replacement therapy</td>
</tr>
<tr>
<td>IGF-IR</td>
<td>Type I Insulin-like growth factor receptor</td>
</tr>
<tr>
<td>IKZF1</td>
<td>Ikaros family zinc finger 1</td>
</tr>
<tr>
<td>MMTV</td>
<td>Mouse mammary tumor virus</td>
</tr>
<tr>
<td>MSI2</td>
<td>Musashi RNA binding protein 2</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian (mechanistic) target of rapamycin</td>
</tr>
<tr>
<td>mTORC1</td>
<td>Mammalian (mechanistic) target of rapamycin complex 1</td>
</tr>
<tr>
<td>NSCLC</td>
<td>Non-small cell lung carcinoma</td>
</tr>
<tr>
<td>OCR</td>
<td>Oxygen consumption rate</td>
</tr>
<tr>
<td>PDAC</td>
<td>Pancreatic ductal carcinoma</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Term</td>
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<tr>
<td>--------------</td>
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<tr>
<td>PI</td>
<td>Propidium Iodide</td>
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<tr>
<td>PLP</td>
<td>Pyridoxal-5-phosphate</td>
</tr>
<tr>
<td>PMP</td>
<td>Pyridoxamine-5-phosphate</td>
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<tr>
<td>PMT</td>
<td>Primary mammary tumor</td>
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<tr>
<td>PR</td>
<td>Progesterone receptor</td>
</tr>
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<td>PTEN</td>
<td>Phosphatase and tensin [gene]</td>
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<td>qRT-PCR</td>
<td>Quantitative reverse transcription polymerase chain reaction</td>
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<td>RST</td>
<td>Recurrent spindle tumor</td>
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<td>STK11</td>
<td>Serine/threonine kinase 11 [gene]</td>
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<tr>
<td>TCA</td>
<td>Tricarboxylic acid [cycle]</td>
</tr>
<tr>
<td>TIC</td>
<td>Tumor initiating cell</td>
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<tr>
<td>WT</td>
<td>Wild type</td>
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<tr>
<td>7-AAD</td>
<td>7 – Aminoactinomycin D</td>
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INTRODUCTION

Cancer is one of the leading causes of death around the world, with an unwavering incidence rate (National Cancer Institute 2018; Canadian Cancer Society’s Advisory Committee on Cancer Statistics 2017). In fact, it is projected that in the year 2030, 22 million new cancer cases will be diagnosed and 13 million patients will succumb to the disease (National Cancer Institute 2018). In response to the profound burden that cancer poses globally, investigators are tasked with uncovering the etiology and mechanisms of each cancer, and attempt to recognize more effective treatment options. In the year 2000, Hanahan and Weinberg proposed six hallmarks of cancer, capabilities acquired during, and necessary for, the development of neoplastic disease (Hanahan & Weinberg 2000). The six capabilities include evading growth suppressors, sustaining proliferative signaling, resisting cell death, enabling replicative immortality, inducing angiogenesis, and stimulating invasion and metastasis (Hanahan & Weinberg 2000). Almost a decade later, two imperative capabilities had been recognized and added to the list of hallmarks: evading immune destruction and the reprogramming of energy metabolism (Hanahan & Weinberg 2011). The focus of this thesis centers on one way that tumor cells have altered their metabolic profile. Specifically, this thesis will discuss a metabolic enzyme that has gained tremendous notoriety within only a few years of study – branched chain amino acid transaminase 1 (BCAT1). BCAT1 is the initiating enzyme for the metabolism of branched chain amino acids (BCAAs) – leucine, isoleucine, and valine (Suryawan et al. 1998). The metabolism of amino acids can play an important role in the process of oncogenesis (Vasquez et al. 2016). All amino acids can be used for protein synthesis or can be catabolized to produce metabolites for energy production; however, the BCAAs are the preferred source of amino acids for tumors (Vasquez et al. 2016; Anaieva 2015). When deregulated, BCAA
metabolism, and the enzymes involved in their metabolic pathways, have been linked not only to malignancy, in cases such as myeloid leukemia, nasopharyngeal carcinoma, glioblastoma, and triple negative breast cancer, but other disease states as well including heart failure and other cardiac pathologies and insulin resistance and type 2 diabetes (Tonjes et al. 2013; Hattori et al. 2017; Zhou et al. 2013; Huang et al. 2011; Ruiz-Canela et al. 2016; Lynch & Adams 2014).
LITERATURE REVIEW

Breast Cancer

Statistics

Breast cancer is one of the leading causes of cancer related deaths in women in developed countries, second only to lung cancer (Ferlay et al. 2015). In less developed countries, breast cancer remains the leading cause of cancer mortalities in women, and is the most commonly diagnosed cancer being attributed to 25% of all new cancer cases in women (Canadian Cancer Society’s Advisory Committee on Cancer Statistics 2017). In 2017, 26300 women and 230 men were diagnosed with breast cancer in Canada, of which 5000 women and 60 men died as a result (Canadian Cancer Society’s Advisory Committee on Cancer Statistics 2017). The incidence rate of breast cancer decreased through the early 2000s following a steady increase the previous decade (Canadian Cancer Society’s Advisory Committee on Cancer Statistics 2017). The increase has been attributed, at least in part, to increased surveillance using mammography, and the subsequent decrease attributed to a decrease in the use of hormone replacement therapy (HRT) in post-menopausal women (Canadian Cancer Society’s Advisory Committee on Cancer Statistics 2017). The incidence rate of breast cancer in Canada has since stabilized (Canadian Cancer Society’s Advisory Committee on Cancer Statistics 2017). The total death rates of breast cancer have steadily declined over the past three decades in thanks to early detection as well as improved therapeutic techniques (Canadian Cancer Society’s Advisory Committee on Cancer Statistics 2017). However, the Canadian Cancer Society has reported that, on average, 13 women died of breast cancer each day in 2017, stressing the continuing need for renewed clinical interventions (Canadian Cancer Society’s Advisory Committee on Cancer Statistics 2017).
Anatomy of the Human Breast

The human breast is a complex structure that undergoes a number of changes over the course of a female’s lifetime (National Breast Cancer Foundation 2019). The tissue of the breast spreads from the sternum and lower ribs to the collarbone and armpit (National Breast Cancer Foundation 2019). The human breast is made up of both parenchymal and stromal tissue (Javed & Lteif 2013). The parenchymal tissue is comprised of a network of lobes and ducts; within each breast are 15-20 lobes, where milk will be produced in lactating females, and 6-8 ducts that allow transfer of the milk from the lobes to the nipple during breast feeding (National Breast Cancer Foundation 2019; Javed & Lteif 2013). The breast also contains lymph nodes that will extend into the armpit and beyond (National Breast Cancer Foundation 2019). The rest of the breast is made up of fatty tissue, comprising the stromal tissue (National Breast Cancer Foundation 2019; Javed & Lteif 2013). The development of the parenchymal tissue network is known as branching morphogenesis and is initiated in fetal development (Javed & Lteif 2013). Following a halt in development at early childhood, the breast tissue will undergo further differentiation in response to hormonal stimuli accompanying puberty (Javed & Lteif 2013). Additional changes occur during pregnancy, lactation, and menopause (Javed & Lteif 2013). The modifications that the breast undergoes over the course of
a female’s lifetime is permitted through the breast tissue’s inherent cellular plasticity (Javed & Lteif 2013). This characteristic, however, makes the breast tissue susceptible to cancerous growth (Javed & Lteif 2013).

**Risk Factors**

A risk factor, as defined by the World Health Organization, is ‘any attribute, characteristic or exposure of an individual that increases the likelihood of developing a disease or injury (WHO 2019). However, an individual who displays one or more risk factors may never develop the disease in question, and an individual who presents no risk factors may still develop the disease (WHO 2019). The risk factors for developing breast cancer include: sex, reproductive history, family history and genetic mutations, and lifestyle or modifiable factors (Dietrich et al. 2014; Canadian Cancer Society 2017).

**Sex**

Biological sex is the most salient risk factor for developing breast cancer (Canadian Cancer Society’s Advisory Committee on Cancer Statistics 2017). According to the Canadian Cancer Society, in 2017, the incidence rate of breast cancer in males was 1.2 for every 100000 people whereas the incidence rate of breast cancer in females was 130.3 for every 100000 people (Canadian Cancer Society’s Advisory Committee on Cancer Statistics 2017).

**Reproductive History**

A woman’s reproductive history can influence her chances of developing breast cancer through associated fluctuations in estrogen and progesterone hormone levels (Canadian Cancer Society 2017; Ritte et al. 2013). Estrogen and progesterone play a role in the growth of breast cancer cells (Canadian Cancer Society 2017; Ritte et al. 2013). The age of menarche as well as
the age of menopause will determine the length of time that a woman is exposed to high levels of estrogen and progesterone (Canadian Cancer Society 2017; Ritte et al. 2013). Thus, when a woman has an early age at menarche and a late age of menopause, or one of the two, she is exposed to these hormones for an extended period of time, increasing the risk that she will develop breast cancer (Canadian Cancer Society 2017; Ritte et al. 2013). Similarly, women who have no pregnancies over their life time or who have their first pregnancy at a late age (>30 years of age) are at an increased risk of breast cancer as compared to women who have their first pregnancy early in life (<20 years of age) (Canadian Cancer Society 2017). During pregnancy, the exposure of breast cells to estrogen and progesterone is interrupted for an extended period (Canadian Cancer Society 2017). The menstrual cycle is also inhibited during pregnancy and that will remove the heightened monthly exposure of the breast cells to circulating hormones (Canadian Cancer Society 2017). Additionally, there is convincing evidence that lactation can reduce a woman’s risk of developing breast cancer as well as lessen the infant’s future risk of developing breast cancer through regulation of body fat and growth rates (Miles et al. 2008).

**Family History of Breast Cancer and Genetic Mutations**

Having a family history of breast cancer, that is, having one or more blood relatives that have or have had breast cancer, increases the possibility of an individual having breast cancer (Canadian Cancer Society 2017). This relationship may be due to different factors including shared lifestyle factors, genetic mutations, and similar exposures (Canadian Cancer Society 2017). Although only 5-10% of breast cancer cases are caused by an inherited gene mutation, some mutations and conditions have become well known in the medical and scientific community (Canadian Cancer Society 2017). One of the most commonly discussed genetic mutations is the BRCA (breast cancer) gene mutation that occur relatively frequently in women
of Ashkenazi Jewish ancestry (Canadian Cancer Society 2017). BRCA is a tumor suppressor gene meaning it is responsible for hindering the growth and proliferation of breast cells, and when mutated, is ineffective at controlling this growth, leading to increased risk of developing breast cancer (Canadian Cancer Society 2017). Other genetic conditions can contribute to an increased risk for developing breast cancer, these include: Li-Fraumeni syndrome which develops from a mutation in the tumor suppressor gene, p53, Ataxia telangiesctasia (AT) that is caused by a mutation to the ATM (ataxia-telangiesctasia mutated) gene which is responsible for repairing damaged DNA, Cowden syndrome, caused by a mutation in the PTEN (phosphatase and tensin homolog) gene, another tumor suppressor, and also, Peutz-Jeghers syndrome that is caused by a mutation to the suspected tumor suppressor gene, STK11 (serine/threonine kinase 11) (Canadian Cancer Society 2017).

**Lifestyle Factors**

Although many of the risk factors associated with breast cancer are unable to be controlled, lifestyle related risk factors, or modifiable risk factors, can be regulated by an individual (Dietrich et al. 2014). Modifiable risk factors include obesity and nutritional factors, physical inactivity, smoking, alcohol consumption, shift work, and hormone therapy and oral contraceptives (Dietrich et al. 2014).

The association between breast cancer and body weight is well established (AICR 2007). Factors including weight gain, body fat levels, and body fat distribution have all been linked to an increased risk of developing breast cancer following menopause (AICR 2007). In fact, when compared with females falling within a healthy weight range, the relative risk of developing breast cancer following menopause is 1.5 for females who are overweight and greater than 2 for females who are obese (La Vecchia et al. 2011). Although it is not certain what mechanisms
body weight is impacting causing the increased risk of breast cancer, it has been suggested that the association is due to increased inflammation and differing levels of circulating adiponectin, leptin, and estrogen, often seen in an overweight individual (McTiernan et al. 2005). Some studies have analyzed the influence of a healthy, well-balanced diet on breast cancer risk, however, due to the close relation between body weight and nutrition, these studies are limited in affect (Dietrich et al. 2014). It has been suggested that a Mediterranean style diet including vegetables, fruits, fish, and olive oil is associated with a lessened risk of developing breast cancer (Albuquerque et al. 2014). Other studies have simply recommended a diet high in fiber rich vegetables or low in red meats is sufficient to lessen the risk (AICR 2007; Aune et al. 2012).

Physical inactivity has been linked directly to a number of different cancers, and there is evidence that physical activity can have a dramatic protective effect against developing breast cancer following menopause (Dietrich et al. 2014; Friedenreich 2010). In fact, women who partake in regular moderate to intense exercise consistently throughout their lives are at a 25% reduced risk of developing breast cancer (Wu et al. 2013). Although it is not certain what mechanism is causing this reduced risk a number of positive benefits of regular physical activities have been suggested including: influence over estrogen and progesterone levels, reduction of body fat, improved immune system, and alterations in the free-radical levels in the body (Dietrich et al. 2014; Friedenreich 2010).

Both alcohol consumption and smoking tobacco are well known risk factors for many types of cancer (Dietrich et al. 2014). In terms of breast cancer, the link between smoking tobacco and the development of breast cancer has only been widely accepted within the last decade (Dietrich et al. 2014). It is now commonly agreed that individuals who are regular smokers or even past smokers are at an increased risk of developing breast cancer by
approximately 10% (Dietrich et al. 2014). The carcinogenic effects of tobacco can be potentiated by alcohol consumption (Anderson et al. 1995). Although ethanol itself is not carcinogenic, the downstream metabolites can be (Anderson et al. 1995). It has been recommended that the daily intake of alcohol be less than 10g, with every 10g more than the recommendation being associated to a 10% increased risk of developing breast cancer (AICR 2007; Thomson et al. 2014; Bagnardi et al. 2013; Giacosa et al. 2012).

There is strong evidence that shift work may increase the risk of developing breast cancer and this may in part be due to a reduction of melatonin release at night (Dietrich et al. 2014; Thomson et al. 2014; Giacosa et al. 2012; Gerber et al. 2003). Being in a lit environment will suppress the release of melatonin, and melatonin will subsequently not suppress the release of estrogen during the night (Gerber et al. 2003). These findings are controversial as there are a number of confounding variables as shift work has been found to alter other lifestyle factors such as poor nutrition, lack of physical activity, and an increased alcohol intake (Smith-Warner et al. 1998).

Hormone replacement therapies, used to treat the side effects of menopause, have been linked to an increased risk of breast cancer (Dietrich et al. 2014). This association is due to the increased levels of circulating estrogen and progesterone affecting the mammary tissue (Beral 2003). Although hormone replacement therapy is still in use, there was a stark reduction in its application following numerous studies indicating this relationship (Canadian Cancer Society’s Advisory Committee on Cancer Statistics 2017). For similar reasons, the use of oral contraceptives have been linked to an increased risk of developing breast cancer, especially in those who have used for over ten years (Canadian Cancer Society 2017). In both cases, cessation
of the treatment removes the increased risk of developing breast cancer (Canadian Cancer Society 2017; Beral 2003).

**Histological Classification**

Breast cancer can be generally categorized into two types of carcinoma, in situ or invasive (Malhotra et al. 2010). In situ breast carcinoma can be broken down into two types: ductal and lobular, depending on the cytological features and growth patterns of the tumor (Malhotra et al. 2010). Similarly, invasive breast carcinoma can be divided into seven distinct types including: ductal, lobular, ductal/lobular, mucinous, tubular, medullary, and papillary carcinomas (Malhotra et al. 2010; Li et al. 2003). Of both the in situ and invasive carcinomas, ductal is the most common subtype accounting for 70-80% of breast cancer cases and lobular is the second most common accounting for approximately 15% of breast cancer cases (Malhotra et al. 2010; Li et al. 2003; Li et al. 2005).

**Molecular Sub-classification**

In 2000, five distinct subtypes of breast cancer were identified based on the expression of 496 genes in breast cancers (Perou et al. 2000). Based on the gene expression patterns offered in this study breast cancers can be sub-classified into luminal subtype A, luminal subtype B, HER2-enriched, basal-like, and unclassified/normal breast-like (Perou et al. 2000). Since the formative study was published in 2000, the unclassified/normal breast-like subtype has ceased being considered and a new subtype was discovered, claudin-low breast cancer (Herschkowitz et al. 2007; Masood et al. 2016). Claudin-low breast cancer and basal-like breast cancer are often categorized together as triple-negative breast cancer (TNBC) as they have similar expression levels of hormone receptors (Masood et al. 2016). Each subtype varies broadly in their expression of potential therapeutic targets and in their clinical prognosis (Sorlie et al. 2003;
Nielsen et al. 2004; Prat et al. 2010). Subtype luminal A and B express the estrogen receptor (ER) and progesterone receptor (PR) and have a comparatively long overall survival and relapse free survival (Sorlie et al. 2003; Sorlie et al. 2001, van’t Veer et al. 2002). Subtype luminal A generally has a better clinical prognosis than subtype luminal B (Sorlie et al. 2003; Sorlie et al. 2001; van’t Veer et al. 2002). The HER2-enriched subtype, characterized by an overexpression of the human epidermal growth factor receptor 2, has a poor prognosis with a relatively short overall survival and relapse free survival (Sorlie et al. 2001; van’t Veer et al. 2002). Basal-like tumors, often characterized by their mesenchymal phenotype and gene expression profile, an absence of ER, PR, and HER2, as well as a tendency to have high levels of p53 mutations, has the lowest overall survival and relapse free survival along with the claudin-low subtype (Prat et al. 2010; Sorlie et al. 2001; van’t Veer et al. 2002).

Table 1: Molecular sub-classification of human breast cancer (Perou et al. 2000; Herschkowitz et al. 2007; Masood et al. 2016; Sorlie et al. 2003; Nielsen et al. 2004; Prat et al. 2010; Sorlie et al. 2001; van’t Veer et al. 2002)

<table>
<thead>
<tr>
<th>Subtype</th>
<th>Characteristics</th>
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<tr>
<td><strong>Luminal A</strong></td>
<td>ER+, PR+, HER2-</td>
</tr>
<tr>
<td></td>
<td>Appears as differentiated from luminal epithelial cells</td>
</tr>
<tr>
<td><strong>Luminal B</strong></td>
<td>ER+, PR+, potentially HER2+</td>
</tr>
<tr>
<td></td>
<td>Generally more aggressive than Luminal A</td>
</tr>
<tr>
<td><strong>HER2 Enriched</strong></td>
<td>ER-, PR-, HER2+</td>
</tr>
<tr>
<td></td>
<td>Aggressive with a high metastatic potential</td>
</tr>
<tr>
<td><strong>Triple Negative</strong></td>
<td>Basal-like</td>
</tr>
<tr>
<td>(TNBC)</td>
<td>ER-, PR- HER2-</td>
</tr>
<tr>
<td></td>
<td>Aggressive with high metastatic potential</td>
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<td></td>
<td>Claudin-low</td>
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<tr>
<td></td>
<td>ER-, PR-, HER2-</td>
</tr>
<tr>
<td></td>
<td>Aggressive with high metastatic potential</td>
</tr>
<tr>
<td></td>
<td>Low expression of claudins 3, 4, and 7</td>
</tr>
<tr>
<td></td>
<td>High expression of stem cell/ mesenchymal markers</td>
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</table>
Claudin-low tumors, that account for 7% of breast cancer cases, are classified as triple negative breast cancer along with basal-like tumors as they also do not express substantial levels of ER, PR, or HER2 (Prat et al. 2010). When compared with tumors of other subtypes claudin-low tumors showed high expression of immune response genes such as CD79b and CD14, cell-to-cell communication ligand genes, cell migration genes including integrin a5 and moesin, as well as genes involved in angiogenesis such as vascular endothelial growth factor C (VEGF-C) (Prat et al. 2010; Dennis et al. 2003). Claudin-low tumor cells have also been suggested to have stem cell like properties indicated by a high expression of genes linked with breast tissue stem cells and tumor initiating cells (TICs) as well as a low expression level of genes associated with luminal epithelial differentiation (Prat et al. 2010; Al-Hajj et al. 2003). Genes involved in cell-to-cell adhesion such as claudins 3, 4, and 7, E-cadherin and occludin were expressed at low and even absent levels (Herschkowitz et al. 2007; Prat et al. 2010). Additionally, genes associated with epithelial-to-mesenchymal transition (EMT) including Vim, Snai-1, Snai-2, Twist1, Twist2, Zeb1, and Zeb2 were highly expressed in the claudin-low tumors (Prat et al. 2010). EMT is one of the first steps in the metastatic process and is associated with enhanced invasiveness and migratory capabilities (Valastyan & Weinberg 2011). Within recent years, EMT has also been connected with a number of metabolic alterations, some of which account for increased aerobic glycolysis, a common feature of cancer cells (Kondaveeti et al. 2015). Using breast cancer cell lines, Kondaveeti et al. demonstrated that EMT induces the increased expression of transporters and enzymes related to glycolysis, such as transporters GLUT3 and GLUT12 (Kondaveeti et al. 2015). Additionally, Kondaveeti et al. showed a decreased expression of enzymes involved in anabolic pathways, such as glucose-6-phosphate dehydrogenase, the initiating enzyme of the pentose phosphate pathway (Kondaveeti et al. 2015). The mesenchymal phenotype and high
expression of EMT associated genes suggests that a similar metabolic shift could be found in claudin-low tumors.

**BCAT1 in Claudin-low Tumors**

Microarray analysis of human breast cancer tumor samples has shown that *Bcat1* is highly expressed in claudin-low tumors compared to the other breast cancer subtypes (Prat et al. 2010; University of North Carolina MicroArray Database). Additionally, RNA sequencing completed in the Moorehead lab has shown that *Bcat1* is significantly up regulated in a murine claudin-low cell line compared to a murine luminal epithelial cell line (GSE113612).

**Branched Chain Amino Acid Transaminase 1 (BCAT1)**

Branched chain amino acid transaminase (BCAT) is the initiating enzyme for the catabolism of the three branched chain amino acids (BCAAs) – leucine, isoleucine, and valine (Suryawan et al. 1998). There are two isoforms of the BCAT enzyme: mitochondrial BCAT (BCATm) and cytosolic BCAT (BCATc) (Suryawan et al. 1998). BCATm is found in majority of the body tissues whereas BCATc is often confined to the brain, ovaries, and placenta (Hutson et al. 1992). Neither isoform of the BCAT enzyme is found in the liver, defining the unique process of BCAA catabolism compared to the other essential amino acids that are primarily catabolized hepatically (Hutson et al. 1992; Hall et al. 1993). The cytosolic isoform is also known as branched chain amino acid transaminase 1 (BCAT1) or ECA39, and controls the only catabolic step in the metabolism of the BCAAs occurring outside of the mitochondria (Xu et al. 2016; Rajendram et al. 2015). BCAT1 acts by reversibly transferring an α-amino group from a BCAA to α-ketoglutarate, producing a branched chain α-keto acid and glutamate, respectively (Ichihara et al. 1966). Both isoforms of the enzyme utilize Vitamin B6 cofactors to complete the transamination reaction (Islam et al. 2007). The α-amino group being donated from the BCAA is
transferred temporarily to pyridoxal-5-phosphate (PLP) complexed with BCAT, which thereby becomes pyridoxamine-5-phosphate (PMP) (Islam et al. 2007). The BCAT-PMP complex then transfers the amino group to α-ketoglutarate forming glutamate and returning PMP to PLP (Islam et al. 2007). As stated, BCAT is capable of catalyzing the reverse reaction, again utilizing the Vitamin B6 cofactors, producing a BCAA and α-ketoglutarate (Ichihara et al. 1966).

**Direction of the BCAT1 Reaction**

The function that Bcat1 plays in cancer appears to be tissue and malignancy specific. BCAT1 catalyzes the reversible reaction taking an α-amino group from a BCAA and adding it to α-ketoglutarate thereby producing glutamine and a branched chain α-keto acid (Ichihara et al. 1966). In healthy tissues, the breakdown of the BCAAs is predominant as opposed to the reverse reaction converting glutamate and a branched chain α-keto acid into α-ketoglutarate and a BCAA, respectively (Hattori et al. 2017). By tracking valine and α-keto-isovalerate levels using a stable-isotope tracer, it was determined that the production of BCAAs from branched chain α-keto acids is the principal reaction in myeloid leukemia (Hattori et al. 2017). Similarly, BCAA level in the serum of breast cancer patients was significantly higher than that of healthy equivalents, indicating that the production of BCAAs is also favored in breast cancer (Zhang et al. 2017). Conversely, glioblastomas appear to maintain the forward reaction catalyzing the breakdown of BCAAs (Tonjes et al. 2013). Inhibition of BCAT1 using gabapentin in glioblastoma cell lines resulted in a substantial accumulation of BCAAs and reduction of glutamate release (Tonjes et al. 2013).

**Regulation of BCAT1**

The upstream regulation of Bcat1 has been a point of inquiry for a number of different cancers. One signaling molecule that has been implicated in malignancy, c-Myc, has been
suggested to regulate the expression levels of Bcat1 (Zhou et al. 2013; Xu et al. 2016; Wang et al. 2015). Commonly considered to be an oncogene, c-Myc induces tumorigenesis through its actions as a transcription factor (Xu et al. 2016). Genes downstream of c-Myc containing the c-Myc binding element (CACGTG) will be targeted by the transcription factor, which can thereby achieve its oncogenic role when up regulated (Zhou et al. 2013). The binding element for c-Myc was found in the 5’ regulatory region of the Bcat1 gene in a study analyzing the enzyme’s role in nasopharyngeal carcinoma (Zhou et al. 2013). A subsequent study investigated the role of BCAT1 in hepatocellular carcinomas and exposed a correlation between the protein expression of BCAT1 and c-Myc protein using immunohistochemical staining (Xu et al. 2016). Furthermore, when c-Myc was knocked down in hepatocellular carcinoma cell lines using siRNA, the expression level of Bcat1 also dropped (Xu et al. 2016). Conversely, when Bcat1 was knocked down in the same cell lines, again using siRNA, the expression level of c-Myc was unaffected, indicating a unidirectional relationship between Bcat1 and c-Myc (Xu et al. 2016). The up-regulation of Bcat1 by c-Myc was also recognized in epithelial ovarian cancers (Wang et al. 2015).

Another protein shown to regulate expression levels of Bcat1 was identified in chronic myeloid leukemia (Hattori et al. 2017). Based on gene correlation analyses, BCAT1 and MSI2 are associated in a number of malignancies, including leukemias, breast and colorectal cancers (Hattori et al. 2017). MSI2 is a member of the Musashi RNA binding protein family that is important in determining cell fates during development (Hattori et al. 2017). Notably, this family of genes is abnormally regulated in different cancers, including human breast cancer (Wang et al. 2010). The direct link between Bcat1 and MSI2 in myeloid leukemia cells was established using immunoprecipitation and flagged MSI2 protein. Bcat1 mRNA transcripts co-precipitated with
the flagged MSI2 1500-fold greater than with the vector control (Wang et al. 2010). Additionally, when a mutant, RNA binding defective form of MSI2 was used, the co-precipitation with Beclin1 was significantly lowered (Hattori et al. 2017). Furthermore, MSI2 knockdown resulted in a stark decrease of BCAT1 protein levels (Hattori et al. 2017).

MSI2 and c-Myc have both been linked to the development of breast cancer (Hattori et al. 2017; Zhou et al. 2013; Xu et al. 2016; Wang et al. 2015; Wang et al. 2010). It has also been shown that the expression of Beclin1 in breast cancer may be regulated by DOT1L histone methyltransferases (Oktyabri et al. 2016). Specifically, DOT1L histone methyltransferases have been shown to regulate Beclin1 expression in triple negative breast cancer (Oktyabri et al. 2016). First determined to have oncogenic effects in leukemia, DOT1L methyltransferases catalyze the methylation of H3K79, which is important in the regulation of normal differentiation and proliferation (McLean et al. 2014). Induction of DOT1L hypermethylation of H3K79 on a gene will ultimately result in aberrant expression of that gene (Oktyabri et al. 2016). In human breast cancer cell lines that overexpress DOT1L, subsequent up-regulation of Beclin1 was determined by qRT-PCR and confirmed by Western blotting (Oktyabri et al. 2016). Regardless of the underlying mechanism, Beclin1 expression is up-regulated in a number of malignancies including nasopharyngeal carcinomas, hepatocellular carcinomas, epithelial ovarian cancer, triple negative breast cancer, urothelial carcinomas, glioblastomas carrying wild type isocitrate dehydrogenase 1, and ERα-negative breast cancers (Tonjes et al. 2013; Zhou et al. 2013, Xu et al. 2016; Wang et al. 2015; Oktyabri et al. 2016; Chang et al. 2016; Thewes et al. 2017).

**BCAT1 and BCAA Metabolism in Cancer**

The three BCAAs are among the nine essential amino acids required in the human diet (Hutson et al. 2005). In a healthy individual and a healthy tissue the BCAAs are required for
normal growth and proliferation of cells (Hutson et al. 2005). In addition to being nitrogen donors for alanine and glutamine and promoters of protein synthesis, the BCAAs are important sensors for different physiological processes such as activation of the mTOR pathway and stimulating the release of insulin from the pancreas (Zhou et al. 2010; Garber et al. 1976). Dependent upon the tissue of origin of the cancer and cancer type, the processes that the BCAAs influence can be coopted in different ways to benefit tumor growth (Mayers et al. 2016).

BCAAs and the mTOR Pathway

The effect of BCAT1 and BCAA metabolism on mitochondrial activity has been specifically studied in breast cancer cells lines (Zhang et al. 2017). When BCAT1 was knocked down in breast cancer cell lines, the mitochondrial DNA content was reduced, and when BCAT1 was overexpressed, the mitochondrial DNA content was increased, suggesting that BCAT1 controls mitochondrial number (Zhang et al. 2017). It appears that BCAT1 does this by controlling the expression of genes required for mitochondrial biogenesis (Zhang et al. 2017). Along with the reduced DNA content, knockdown of BCAT1 resulted in lowered mRNA levels of mitochondrial transcription factor A, nuclear respiratory factor 1, and peroxisome proliferator-activated receptor gamma coactivator 1α; overexpression led to the opposite effects (Zhang et al. 2017). Furthermore, in the BCAT1 overexpression breast cancer cell lines, an increase in the activity of citrate synthase and an amplified cellular ATP level was seen, indicating that BCAT1 also regulates the activity of the mitochondria (Zhang et al. 2017). Finally, BCAT1 exerts these effects through its influence over the mTOR signaling pathway (Zhang et al. 2017). BCAT1, working in the reverse reaction, will produce BCAAs that are known activators of mTOR signaling (Lynch & Adams 2014). Recently, it was discovered that leucine is a direct regulator of the mTORC1 pathway through its influence over Sestrin2
(Wolfson et al. 2016). Sestrin2, when unbound, inhibits the mTORC1 pathway, however once bound by leucine, the mTORC1 pathway is left unimpeded (Wolfson et al. 2016). The mTOR pathway is responsible for enabling cell survival and proliferation, cell growth and protein, lipid, and nucleotide synthesis (Kim et al. 2013). BCAT1 knockdown decreased mTOR signaling, whereas BCAT1 overexpression activated the pathway (Zhang et al. 2017). When the mTOR signaling pathway was inhibited using rapamycin, BCAT1 overexpression was unable to produce the increase in mitochondrial biogenesis regulators or mitochondrial activity discussed above (Zhang et al. 2017).

**BCAAs as a Nitrogen Source**

In 2016, Mayers and colleagues analyzed the fate of BCAA derived nitrogen in non-small cell lung carcinoma (NSCLC) and pancreatic ductal carcinoma (PDAC), both of which are Kras mutation driven cancers (Mayers et al. 2016). Despite having an identical genetic component, the tumors of these two cancers appear to utilize different mechanisms to fulfill their metabolic requirements (Mayers et al. 2016). To determine this effect, mice were fed a modified diet with 50% of the leucine labeled with $^{15}$N (Mayers et al. 2016). Compared to the control, mice with PDAC presented no difference in the amount of $^{15}$N incorporated into the tumor tissue and less $^{15}$N incorporated into other amino acids (Mayers et al. 2016). In contrast, when compared with normal lung tissue, mice with NSCLC showed increased levels of $^{15}$N in tumor tissue as well as in free and protein-bound amino acids (Mayers et al. 2016). NSCLC exhibits a high expression of the BCAT enzyme compared to healthy lung tissue, whereas PDAC tumors exhibit a lower expression of the BCAT enzyme than control tissue, indicating that NSCLC, but not PDAC, is allowing for increased nitrogen utilization for protein and nucleotide biosynthesis by altering the expression of BCAA metabolic enzymes (Mayers et al. 2016).
**BCAAs and the TCA Cycle**

BCAT1 acts by reversibly transferring an α-amino group from a BCAA to α-ketoglutarate, producing glutamate and a branched chain α-keto acid, respectively (Ichihara et al. 1966). The resulting branched chain α-keto acids, α-ketoisovaleric acid, α-ketoisocapric acid, and α-keto-β-methylvaleric acid, are further catabolized through a series of steps into acetyl-CoA, propionyl-CoA, and succinyl-CoA similar to the other amino acids (Rajendram et al. 2015; Ichihara et al. 1966). It is worth noting that at many of the catabolic steps the products can be diverted into other pathways and utilized for processes such as cholesterol synthesis (Rajendram et al. 2015). However, the final products – acetyl-CoA, propionyl-CoA, and succinyl-CoA – feed into the tricarboxylic acid cycle providing substrates for energy production (Tonjes et al. 2013). In addition to the downstream metabolites of the BCAAs feeding into the TCA cycle, the reverse reaction – in which an α-ketoglutarate and a BCAA are produced – α-ketoglutarate is itself an intermediate of the TCA cycle, allowing it to move forward (Ananieva 2015).
Figure 2: Catabolic pathway of leucine, isoleucine, and valine. Taken and adapted from “Branched Chain Amino Acids in Clinical Nutrition: Volume 1” (Rajendram et al. 2015)

**BCAT1 and Cell Cycle Inhibitors**

BCAT1 was shown to have pro-proliferative effects in ER-negative, anti-estrogen-responsive and anti-estrogen-resistant, MCF-7 breast cancer cell lines (Thewes et al. 2017). Thewes and colleagues (2017) determined that this might in part be reflective of BCAT1 indirectly regulating the expression of cell cycle inhibitor, $p27^{\text{Kip1}}$ (Thewes et al. 2017). Knockdown of BCAT1 in the MCF-7 cell lines was accompanied by an increased expression of the cell cycle inhibitor $p27^{\text{Kip1}}$ and subsequent phosphorylation of the tumor suppressor Retina.
blastoma protein thereby allowing the tumor cells to transition from S phase (Thewes et al. 2017; Knudsen et al. 2008).

**Clinical Trials**

A number of studies have investigated the value of supplementing BCAAs into the diet in combination with established cancer treatments on disease progression (Nojiri et al. 2016; Iwasa et al. 2015; Park et al. 2017). In hepatocellular carcinoma, when supplemented following radiative ablation of the tumor, BCAAs led to an increased event-free survival and fewer overall complications (Nojiri et al. 2016). These findings are corroborated by another study analyzing BCAA supplementation in hepatocellular carcinoma as well as in advanced liver disease; both trials demonstrated that supplementation was beneficial for the patient group (Park et al. 2017; Shiozawa et al. 2016). Additionally, the level of circulating methylated DNA for Bcat1 and ikaros family zinc finger 1 (IKZF1) in a patient’s blood has been suggested as a prognostic marker for recurrence of colorectal cancer (Symonds et al. 2016; Young et al. 2016). In this comparative study, 75% of the blood tests were positive for methylated Bcat1 and Ikzf1 in patients who had relapsing colorectal cancer (Symonds et al. 2016). Although further studies are necessary to determine the diagnostic value of Bcat1, this does indicate a relationship between BCAT1 and colorectal cancer recurrence (Symonds et al. 2016; Young et al. 2016).
HYPOTHESIS AND OBJECTIVES

Hypothesis

The up-regulation of BCAT1, and consequently BCAA metabolism, is driving the aggressive nature of claudin-low breast cancer tumors. Furthermore, disruption of BCAT1 and BCAA metabolism in claudin-low cell lines will result in impaired cell metabolism and thereby inhibit growth and proliferation.

Objectives

1. Confirm the up-regulation of BCAT1 in the claudin-low cell line, RJ423, recurrent spindle tumor tissue, and human cell line, MDA-MB-231.
2. Knockdown expression of BCAT1 in the claudin-low cell line, RJ423
3. Investigate the effects of BCAT1 knockdown on the proliferation, cell survival, migration, and metabolism of the claudin-low cell line, RJ423
**METHODOLOGY**

**Cell Lines**

The mammary cell lines RJ423 and RJ345 were derived from a transgenic mouse model expressing human IGF-IR under a MMTV promoter system inducible by doxycycline, previously created in the Moorehead lab (Jones et al. 2006). Cells were cultured in 1X DMEM media (Sigma Aldrich) supplemented with 10% FBS (Clontech), 2% L-glutamine (Thermo Fisher Scientific), 1% AB/AM (Wisent Bioproducts), 1% sodium pyruvate (Sigma Aldrich), 1% hepes solution (Sigma Aldrich) as well as 0.001% estrogen (Sigma Aldrich), 0.0005% EGF (Sigma Aldrich), 0.001% insulin (Roche), and 0.0002% hydrocortisone (Sigma Aldrich).

Table 2: Characteristics of cell lines (Jones et al. 2006; Campbell et al. 2011; Jones et al. 2009; Franks et al. 2011)

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>RJ423</th>
<th>RJ345</th>
</tr>
</thead>
<tbody>
<tr>
<td>Derived from</td>
<td>Recurrent Spindle Tumor</td>
<td>Primary Mammary Tumor</td>
</tr>
<tr>
<td>Genotype</td>
<td>High Expression: <em>Zeb1, Zeb2, Twist1, Twist2, Snai1, Snai2,</em> and <em>Vim</em></td>
<td>High Expression: E-cadherin, Cytokeratin 8 and 18, Claudins 3, 4, 7</td>
</tr>
<tr>
<td></td>
<td>Low Expression: E-cadherin, Claudins 3, 4, and 7</td>
<td>Low Expression: <em>Zeb1, Zeb2, Twist1, Twist2, Snai1, Snai2,</em> and <em>Vim</em></td>
</tr>
<tr>
<td>Morphology</td>
<td>Spindle/ Mesenchymal</td>
<td>Luminal Epithelial</td>
</tr>
<tr>
<td>Representative of</td>
<td>Claudin-low breast cancer</td>
<td>Luminal breast cancer</td>
</tr>
</tbody>
</table>
**Cell Counting**

Cells in culture were washed with sterile PBS and trypsinized using 10x trypsin (Wisent Bioproducts) for 2-5 minutes. The cells and trypsin were then diluted in 5mL of media. 50µL of the solution was then mixed thoroughly with 50µL of Trypan Blue (Thermo Fisher Scientific). Live cells were manually counted using a haemocytometer. Cell density was calculated by dividing total cell count by ten (the number of chambers counted), multiplying the product by 2 (the dilution factor), and multiplied by $10^4$.

**siRNA Transfection – Bcat1 Knockdown**

Bcat1 Silencer Select siRNA s62890 and s62889, and Silencer Select Negative Control #2 siRNA were purchased from Thermo Fisher Scientific. siRNAs were resuspended in nuclease free water to a working concentration of 200µM. RJ423 cells were plated at a density of 30000 cells/well in a 6 well dish and incubated overnight. For each well 10µL of Lipofectamine 2000 (Thermo Fisher Scientific) was combined with 240µL of Opti-mem media (Thermo Fischer Scientific) and 1µL of siRNA suspension (200pmole) was combined with 249µL of Opti-mem media, each was incubated at room temperature for 5 minutes and then combined together. The combined solution was incubated at room temperature for 20 minutes, during which time media was removed from the cells and replaced with 500µL of antibiotic-free media. 500µL of the siRNA combination was added to each respective well and then incubated at 37°C for 5 hours, after which the media was replaced with culture media. All tests were run 48 hours following the start of the incubation period. For optimization process, see Appendix 2.

**RNA Isolation and Quantitative Reverse Transcription Polymerase Chain Reaction**

Cells were grown to 70% confluency in 10cm cell culture plates or as per the siRNA transfection protocol. RNA was extracted using the RNeasy Mini Kit from Qiagen. RNA
concentration and quality was determined using a Nanodrop 200c Spectrophotometer (Thermo Fisher Scientific) and stored at -80°C. 1µg of RNA was combined with 4µL of qScript cDNA mix (Quanta Biosciences) and nuclease-free water (Life Technologies) to a total of 20µL. The RNA was reverse transcribed in a thermo-cycler for 5 min at 25°C, 30 min at 42°C, 5 min at 85°C, left at 4°C, and the resulting cDNA was stored at -20°C.

mRNA expression level was determined by quantitative real-time PCR reactions with a SYBR Green qPCR Mastermix from Bioline utilizing a CFX Connect Real-time PCR Detection System from Bio-Rad. The cycling parameters include initial denaturation at 94°C for 2 min, and then 40 cycles of denaturation at 94°C for 15 sec and annealing and extension at 60°C for 1 min. Each sample was analyzed using three biological replicates and three technical replicates and normalized to Hprt using CFX-Manager 3.1 (Bio-Rad). Primers for Bcat1 (cat#10025635), Bcat2 (cat#10025636), and Hprt (cat#10025636) were purchased from Bio-Rad.

**Protein Isolation and Western Blotting**

Cells were grown to 70% confluency in 10cm cell culture plates or as per the siRNA transfection protocol. Cells were washed with ice-cold PBS and lysed with 300µL (10cm cell culture plates) or 150µL (siRNA transfection) of RIPA lysis buffer (Appendix I). Immediately following application of lysis buffer the cells were scraped and transferred to microcentrifuge tubes and incubated on ice for 15 minutes. The cell lysates were centrifuged at 13000 x g for 20 minutes at 6°C. Supernatant was collected and stored at -80°C. Protein concentration was determined using a Bradford protein assay (Bio-Rad). 30µg of protein was combined with reducing buffer (Appendix I) and RO water to a total of 30µL. Samples were separated in 10% sodium dodecyl sulphate (SDS) polyacrylamide gels for 1 hour and 45 minutes at 125V in a XCell SureLock™ Mini-Cell Electrophoresis System (Thermo Fisher Scientific). Protein was
transferred for 2 hours at 25V onto Hybond ECL nitrocellulose membranes (Amersham Pharmacia Biotech). Membranes were left in blocking solution (Appendix 1) for 1 hour at room temperature. Membranes were incubated with primary mouse monoclonal BCAT1 antibody (Origene; #TA504360) diluted in antibody diluting solution (Appendix I) at a concentration of 1:2000 and agitated overnight at 4°C. Membranes were then washed with TBST for 10 minutes three times and incubated in secondary anti-mouse IgG, HRP-linked antibody (Cell Signaling Technology; #7076S) in antibody diluting solution at a concentration of 1:2000 for 1 hour at room temperature. The membranes were then washed with TBST for 10 minutes 3 times. Membranes were placed in a plastic covering with 1mL of Clarity Western ECL substrate (Bio-Rad) for 1 minute. The protein was then imaged using the ChemiDoc™XRS+ System (Bio-Rad) and analyzed using Image Lab software (Bio-Rad). Membranes were washed in TBST for 10 minutes 3 times and then reprocessed and imaged using primary goat polyclonal α-TUBULIN antibody (Santa Cruz Biotechnology; sc-31779) and secondary rabbit-anti-goat IgG, HRP-linked antibody (Santa Cruz Biotechnology; sc-2922) both at a concentration of 1:5000 for normalization.

**Flow Cytometry**

**Bromodeoxyuridine (BrdU)**

Cells were plated and processed as per the siRNA transfection protocol. At 48 hours following the start of the incubation period, the FITC BrdU Flow Kit from BD Biosciences was used to determine the cell cycle profile of each cell line, following the prescribed protocol. Fluorescence was measured on a BD Accuri C6 flow cytometer (BD Biosciences) and analysis of data was done using Accuri C6 software (BD Biosciences).
Annexin V

Cells were plated and processed as per the siRNA transfection protocol and Flow Cytometry Annexin V-FITC Apoptosis detection kit from eBiosciences was used to measure apoptosis. At 24 hours following the start of the incubation period positive control cells were treated with 12.5µM of cisplatin. At 48 hours following the start of the incubation period media was removed from the cells and retained. The cells were then washed in PBS, which was also retained. The cells were then trypsinized with 10x trypsin and added to the initial media and PBS. The combination was centrifuged at 150 x g for 10 minutes. The cells were resuspended in PBS and centrifuged again at 300 x g for 5 minutes. The cells were then resuspended in binding buffer and centrifuged. The cells were resuspended in 195µL of binding buffer and 5µL of Annexin V-FITC and incubated for 10 minutes at room temperature. Cells were washed with 200µL of binding buffer and centrifuged. Cells were then resuspended in 190µL of binding buffer and 10µL of Propidium Iodide. Fluorescence was measured on a BD Accuri C6 flow cytometer (BD Biosciences) and analysis of data was done using Accuri C6 software (BD Biosciences).

Immunofluorescence

Phospho-Histone H3

Cells were plated and processed as per the siRNA transfection protocol, with the addition of sterile coverslips in the wells. At 48 hours following the start of the siRNA transfection incubation period the cells were washed 2 times in PBS and then fixed for 1 hour in 10% buffered formalin phosphate. The cells were again washed 2 times with PBS and permeabilized for 5 minutes in permeabilizing solution (Appendix I). Cells were again washed 2 times in PBS then blocked for 10 minutes with blocking solution (Appendix I). Cells were incubated overnight
at 4°C in primary mouse monoclonal phospho-histone H3 antibody (Abcam; Ab-14955) in antibody diluting fluid at a concentration of 1:1500. The following day cells were washed 2 times in PBS and incubated at room temperature in the dark in secondary AlexaFluor 594 donkey anti-rabbit IgG antibody (Thermo Fisher Scientific; A-11032) in antibody diluting fluid at a concentration of 1:500. Cells were then washed 3 times in PBS and then the coverslips were mounted on slides with Prolong Gold Antifade Mountant with Dapi (Life Technologies) and left at room temperature overnight and then at -20°C until analysis. Images were captured using an Olympus BX961 fluorescent microscope (Olympus) with Metamorph Imaging software (Molecular Devices). Images were analyzed and cells manually counted using Image J software (National Institute of Health).

**Population Doubling Time**

Cells were plated and processed as per the siRNA transfection protocol. At the end of the incubation period of the transfection, the siRNA cells were counted as per the cell counting protocol. 20000 cells of each siRNA cell line were then plated in a new 6 well culture dish. At the same time, RJ423 and RJ345 cells were counted and plated at 20000 cells/well in the 6 well culture dish. 48 hours following the start of the siRNA transfection period all cell lines were washed in PBS, trypsinized with 250µL of 10x trypsin, diluted with 1mL of media and then counted. Cell counts obtained in the form of cells per mL were multiplied by 1.25mL for the total cell count. Population growth rate was determined using the following equation. 

\[ P(t) = P(\theta)e^{rt}, \]

where \( P(t) \) is the final amount, \( P(\theta) \) is the initial amount, \( r \) is the exponential growth rate, and \( t \) is the total time. Doubling time was calculated by dividing 70 by the exponential growth rate (r).
Scratch Wound Assay

Cells were plated and processed as per the siRNA transfection protocol and at the end of the 5 hour incubation period the cells were counted as per the cell counting protocol. 200000 cells of each Bcat1 siRNA cell line and RJ423 and RJ345 were seeded in a 24 well dish and incubated overnight. A scratch was created using a P1000 micropipette tip at which time the wells were washed with PBS and media replaced with DMEM media containing 5% FBS as opposed to the regular culture media that contains 10% FBS. Cells were imaged at times 0, 12, 18, and 24. At 24 hours post scratch the cells were washed twice with ice-cold PBS and fixed using ice-cold methanol for 10 minutes. The cells were then stained with crystal violet for 10 minutes. Images were analyzed by comparing the scratch at T0 and T24 with ImageJ software (National Institute of Health).

Seahorse Analyzer XFe24

Cell Energy Phenotype Kit

Cells were plated and processed as per the siRNA transfection protocol and incubated for 24 hours. At this time the cells were counted as per the cell counting protocol and 40000 RJ423 and RJ345 cells, and 80000 cells of the Bcat1 siRNA transfected cell lines, were plated per well in the Seahorse XF Cell Culture Microplate (Agilent Technologies) and incubated overnight at 37°C in a 5% CO₂ incubator. The sensor cartridge (Agilent Technologies) was hydrated in Agilent Seahorse XF Calibrant (500µL/well) (Agilent Technologies) and incubated overnight at 37°C in a 0% CO₂ incubator. The following day the assay media was prepared using 50mL of Seahorse XF Base Medium (Agilent Technologies). 1500uL of the base medium was removed and 500uL of dextrose (10mM), glutamine (2mM), and pyruvate (1mM) were added. The cell energy phenotype kit compounds were re-suspended using the prepared assay medium;
oligomycin was re-suspended in 630uL of medium (100uM) and FCCP was resuspended in 720uL (100uM) and then vortexed. The prepared compounds were then combined to create the stressor mix using 2400uL of prepared assay medium, 300uL oligomycin, and 300uL FCCP. 56uL of the stressor mix was then added to port A of the sensor cartridge (1uM of oligomycin and FCCP/well). The cells plated in the Seahorse XF Cell Culture Microplate were washed 2x with 500uL prepared assay medium. 500uL of prepared assay medium was then added to the microplate wells and incubated for 1 hour at 37°C in a 0% CO₂ incubator. Using the Seahorse Analyzer XF instruction prompts the sensor cartridge was loaded and calibrated. Following the instruction prompts, the microplate was then loaded into the Seahorse Analyzer XFe24 (Agilent Technologies) and analyzed using Wave software (Agilent Technologies).

*Mito Fuel Flex Kit – Energy Pathway Dependence*

Cells were plated and processed as per the siRNA transfection protocol and incubated for 24 hours. At this time the cells were counted as per the cell counting protocol and 40000 RJ423 and RJ345 cells, and 80000 cells of the Bcat1 siRNA transfected cell lines, were plated per well in the Seahorse XF Cell Culture Microplate (Agilent Technologies) and incubated overnight at 37°C in a 5% CO₂ incubator. The sensor cartridge (Agilent Technologies) was hydrated in Agilent Seahorse XF Calibrant (500µL/well) (Agilent Technologies) and incubated overnight at 37°C in a 0% CO₂ incubator. The following day the assay media was prepared using 50mL of Seahorse XF Base Medium (Agilent Technologies). 1500uL of the base medium was removed and 500uL of dextrose (10mM), glutamine (2mM), and pyruvate (1mM) were added. The cell energy phenotype kit compounds were re-suspended using the prepared assay medium; BPTES, Etomoxir, and UK5099 in 700uL and then vortexed. The re-suspended compounds were then combined into six mixes and added to the cartridge as prescribed in Tables 4 and 6 of the Agilent
Seahorse XF Mito Fuel Flex Test Kit User Manual. The cells plated in the Seahorse XF Cell Culture Microplate were washed 2x with 500uL prepared assay medium. 500uL of prepared assay medium was then added to the microplate wells and incubated for 1 hour at 37°C in a 0% CO₂ incubator. Using the Seahorse Analyzer XFe24 instruction prompts the sensor cartridge was loaded and calibrated. Following the instruction prompts, the microplate was then loaded into the Seahorse Analyzer XF and analyzed using Wave software (Agilent Technologies).

**Conditioned Media Metabolite Analysis**

Cells were plated and processed as per the siRNA transfection protocol and RJ423 and RJ345 cells were also seeded in the 6 well dish at this time such that 70-80% confluence would be reached the following day. The cells were incubated overnight and then the media was removed and the cells were washed with PBS. 1mL of new regular culture media was added to the wells. The cells were incubated for 24 hours at which time the media was collected. The collected media was centrifuged at 3000g for 5 minutes after which the top layer was transferred to a new tube. Samples were stored at -20°C until shipment to The Metabolomics Innovation Centre at the University of Alberta for analysis by liquid chromatography mass spectrometry.
RESULTS

_Bcat1_ is up-regulated in RJ423 cells, RST tissue, and a Claudin-low human cell line

To confirm the up-regulation of _Bcat1_ observed in the RNA sequencing data previously conducted (GSE113612), qRT-PCR was performed on cell lines RJ423 and RJ345, normal mammary tissue sample (WT), and mammary tumor tissue samples (PMT and RST), as well as human cell lines MCF7 and MDA-MB-231. At the mRNA level, _Bcat1_ was significantly higher in the RJ423 cells compared to the RJ345 cells. (Figure 3A). A significant difference was found between the _Bcat1_ mRNA levels in the mammary tissue samples with WT tissue having the lowest expression, PMT tissue expressing a higher level, and RST tissue expressing the highest level (Figure 3B). In the human cell lines, _Bcat1_ expression was significantly higher in the claudin-low MDA-MB-231 cells than the luminal MCF7 cells (Figure 3C). At the protein level, BCAT1 is expressed highly in the RJ423, and is expressed at an indistinguishably low level in RJ345 cells (Figure 4). Western blotting was not performed on the mammary tissue samples or human breast cancer cell lines.

Additionally, the expression of _Bcat2_ mRNA was determined in the cell lines RJ423 and RJ345 (Figure 5A), and in the mammary tissue samples WT, PMT, and RST (Figure 5B) using qRT-PCR. No significant difference was seen in _Bcat2_ mRNA levels between RJ423 and RJ345 cells or between the mammary tissue samples.
Figure 3: Relative mRNA expression of *Bcat1* in A) murine cell lines RJ345 and RJ423 (n=3), B) mammary tissue wild type (WT, n=5), primary mammary tumor (PMT, n=11), and recurrent spindle tumor (RST, n=8), and C) human luminal cell line MCF7 (n=3) and claudin-low cell line MDA-MB-231 (n=3). Bars represent relative mRNA expression ± SEM normalized to *Hprt* expression. Statistical significance was determined using one-way Anova and post-hoc Tukey test (p<0.05). Annotations: Figure 1A: a – significantly different than RJ423, b – significantly different than RJ345. 1B: a – significantly different than WT, b – significantly different than PMT, c – significantly different than RST. Figure 1C: a – significantly different than MCF7, b – significantly different than MDA-MB-231.
Figure 4: Western blot of BCAT1 (43kDa) in RJ423 and RJ345 cells. β-ACTIN (42kDa) was used as a loading control.
Figure 5: Relative mRNA expression of Bcat2 in A) murine cell lines RJ345 and RJ423 (n=3) and B) mammary tissue wild type (WT, n=5), primary mammary tumor (PMT, n=11), and recurrent spindle tumor (RST, n=8). Bars represent relative mRNA expression ± SEM normalized to Hprt expression. Statistical significance was determined using one-way Anova and post-hoc Tukey test (p<0.05). No significant difference was observed.
siRNA Knockdown

Bcat1 was transiently knocked down in RJ423 using Bcat1 siRNA. The knockdown was confirmed at the mRNA level using qRT-PCR (Figure 6A) and protein level using Western Blotting (Figure 6B). The level of Bcat1 in the Bcat1 siRNA A and Bcat1 siRNA B cell was significantly lower than that of the parental RJ423 cell line and Bcat1 siRNA Control cell line.
Figure 6: A) Relative mRNA expression of $Bcat1$ in RJ423, $Bcat1$ siRNA A, $Bcat1$ siRNA B, and $Bcat1$ siRNA Control. Bars represent relative mRNA expression ± SEM (n=6) normalized to $Hprt$ expression. Statistical significance was determined using one-way Anova and post-hoc Tukey test (p<0.05). Annotations: a – significantly different than RJ423, b – significantly different than $Bcat1$ siRNA A, c – significantly different than $Bcat1$ siRNA B, d – significantly different than $Bcat1$ siRNA Control. B) Western blot of BCAT1 (43kDa) in RJ423, $Bcat1$ siRNA A, $Bcat1$ siRNA B, and $Bcat1$ siRNA Control. $\alpha$-TUBULIN (50kDa) was used as a loading control.
Effects of *Bcat1* siRNA Knockdown on Proliferation

*Population Doubling Time*

To determine the effect of *Bcat1* siRNA knockdown of *Bcat1* in the RJ423 cell line, the population doubling times were determined (Figure 7A). The population doubling time of *Bcat1* siRNA A, *Bcat1* siRNA B, and *Bcat1* siRNA Control was significantly increased over the population doubling time of RJ423 cell line. The population doubling time of *Bcat1* siRNA B was significantly increased over the *Bcat1* siRNA Control cell line, however, no significant difference was seen between *Bcat1* siRNA A and *Bcat1* siRNA Control cells.

*Phospho-histone H3 Immunofluorescence*

To further elucidate the effects of *Bcat1* siRNA knockdown of *Bcat1* in the RJ423 cell line, immunofluorescence of mitotic cell cycle marker phospho-histone H3 was conducted (Figure 7B). No significant difference was seen in the percent of cells staining for phospho-histone H3 between cells lines *Bcat1* siRNA A, *Bcat1* siRNA B, *Bcat1* siRNA Control, and RJ423.

*Bromodeoxyuridine (BrdU)*

To further clarify the effects of *Bcat1* siRNA knockdown of *Bcat1* in the RJ423 cell line, analysis of the cell cycle phases was conducted using BrdU and 7-AAD. No significant difference was observed between the respective cell cycle phases of each cell line and *Bcat1* siRNA cell line (Figure 7C). Additionally, no significant difference was observed between the percent of cells staining positive for BrdU in each cell line and *Bcat1* siRNA cell line (Figure 7D).
Figure 7: A) Cell population doubling time of RJ423 (n=7) Bcat1 siRNA A (n=5), Bcat1 siRNA B (n=5), Bcat1 siRNA Control (n=5). Bars represent cell population doubling time in hours ± SEM. Statistical significance was determined using one-way Anova and post-hoc Tukey test (p<0.05). Annotations: a – significantly difference than RJ423, b – significantly different than Bcat1 siRNA A, c – significantly different than Bcat1 siRNA B, d – significantly different than Bcat1 siRNA Control. B) Percent of the cell population staining positive for phospho-histone H3 in RJ423, Bcat1 siRNA A, Bcat1 siRNA B, Bcat1 siRNA Control. Bars represent percent of cells staining positive for phospho-histone H3 ± SEM (n=3). Statistical significance was determined using one-way Anova and post-hoc Tukey test (p<0.05). No statistical significance seen. C) Cell cycle distribution of RJ423, Bcat1 siRNA A, Bcat1 siRNA B, Bcat1 siRNA Control. Bars represent percent of cells in each cell cycle ± SEM (n=4). Statistical significance determined using one-way Anova and post-hoc Tukey test (p<0.05). D) Percent of cells staining positive for BrdU in RJ423, Bcat1 siRNA A, Bcat1 siRNA B, Bcat1 siRNA Control. Bars represent percent of cells staining for BrdU ± SEM (n=4). Statistical significance determined using one-way Anova and post-hoc Tukey test (p<0.05)
Effects of Bcat1 siRNA Knockdown on Cell Survival

In an attempt to explain the difference observed in cell population doubling time, Annexin V and Propidium Iodine staining was conducted and measured using flow cytometry. Cells in early apoptosis were determined by percent of cells staining positive for Annexin V and negative for Propidium Iodide (Figure 8A). Cells in late apoptosis were determined by percent of cells staining positive for both Annexin V and Propidium Iodide (Figure 8B). No significant difference was seen between cell lines RJ423, Bcat1 siRNA A, Bcat1 siRNA B, and Bcat1 siRNA Control in either condition.
Figure 8: A) Percent of the cell population staining positive for Annexin V and negative for Propidium Iodide in RJ423, Bcat1 siRNA A, Bcat1 siRNA B, Bcat1 siRNA Control. Bars represent percent of cells staining positive for Annexin V and negative for Propidium Iodide ± SEM (n=4). B) Percent of the cell population staining positive for Annexin V and Propidium Iodide in RJ423, Bcat1 siRNA A, Bcat1 siRNA B, Bcat1 siRNA Control. Bars represent percent of cells staining positive for Annexin V and Propidium Iodide ± SEM (n=4). Statistical significance was determined using one-way Anova and post-hoc Tukey test (p<0.05). No significant difference observed.
Effects of \textit{Bcat1} siRNA Knockdown on Migration

To determine the effects of \textit{Bcat1} siRNA knockdown on migration, a scratch wound assay was performed (Figure 9A). The rate of migration was determined by subtracting the area covered by cells at time of initial scratch by the area covered by cells at 24 hours post scratch. No significant difference was observed between the RJ423 and \textit{Bcat1} siRNA cells.
Figure 9: A) difference in area covered by RJ423, Bcat1 siRNA A, Bcat1 siRNA B, and Bcat1 siRNA Control between time of initial scratch and 24 hours post scratch. Bars represent the change in area covered by cells in pixel units ± SEM (n=3). Significance determined by One-way Anova and post-hoc Tukey test (p<0.05). No significant difference observed.
**Effects of Bcat1 siRNA Knockdown on Oxidative Phosphorylation and Glycolysis**

In order to determine the effects of *Bcat1* knockdown on metabolism, a Cell Energy Phenotype test was performed using the Seahorse XFe24 Analyzer, in which the oxygen consumption rate (OCR; Figure 10A) and extracellular acidification rate (ECAR; Figure 10B) was measured both prior to and after an injection of stressor compounds. At the representative time point of 18 minutes there was a significant difference in basal OCR between the RJ423 cells and the RJ345, *Bcat1* siRNA B, and *Bcat1* siRNA Control cells (Figure 10C). At the representative time point of 44 minutes there was a significant difference in stressed OCR between *Bcat1* siRNA A and *Bcat1* siRNA Control (Figure 10D). At the representative time point of 18 minutes there was a significant difference in basal ECAR between the RJ423 cells and the RJ345, *Bcat1* siRNA B, and *Bcat1* siRNA Control cells (Figure 10E). At the representative time point of 44 minutes there was no significant differences observed in stressed ECAR (Figure 10F).
Figure 10: A) oxygen consumption rate (OCR) of RJ423, Bcat1 siRNA A, Bcat1 siRNA B, and Bcat1 siRNA control at each time point. Points represent the OCR (pmol/min) ± SEM (n=3). B) extracellular acidification rate (ECAR) of RJ423, Bcat1 siRNA A, Bcat1 siRNA B, and Bcat1 siRNA control at each time point. Points represent the ECAR (mpH/min) ± SEM (n=3). C) basal OCR observed at time point of 18 minutes in RJ423, Bcat1 siRNA A, Bcat1 siRNA B, and Bcat1 siRNA control. Bars represent the OCR (pmol/min) ± SEM (n=3). Annotations: a – significantly different than RJ423; b – significantly different than RJ345; d – significantly different than Bcat1 siRNA B; e – significantly different than Bcat1 siRNA control (p<0.05). D) stressed OCR observed at time point of 44 minutes in RJ423, Bcat1 siRNA A, Bcat1 siRNA B, and Bcat1 siRNA control. Bars represent the OCR (pmol/min) ± SEM (n=3). Annotations: c – significantly different than Bcat1 siRNA A; e – significantly different than Bcat1 siRNA control (p<0.05). E) basal ECAR observed at time point of 18 minutes in RJ423, Bcat1 siRNA A, Bcat1 siRNA B, and Bcat1 siRNA control. Bars represent the ECAR (mpH/min) ± SEM (n=3). Annotations: a – significantly different than RJ423; b – significantly different than RJ345; d – significantly different than Bcat1 siRNA B; e – significantly different than Bcat1 siRNA control (p<0.05). F) stressed ECAR observed at time point of 44 minutes in RJ423, Bcat1 siRNA A, Bcat1 siRNA B, and Bcat1 siRNA control. Bars represent the ECAR (mpH/min) ± SEM (n=3). No significant differences observed. Significance calculated using one-way Anova and post-hoc tukey analysis.
**Effect of Bcat1 siRNA Knockdown on Mitochondrial Fuel Dependency**

In order to further elucidate the effects of Bcat1 knockdown on metabolism, a Mito Fuel Flex test was performed using the Seahorse XFe24 Analyzer, in which the oxygen consumption rate (OCR) is measured before and after the addition of a specific inhibitor – inhibitors of glutamine, fatty acid, and glucose oxidation - and after the addition of all inhibitors. Under these conditions the OCR was measured over a period of time in RJ423 cells (Figure 11A), RJ345 cells (Figure 11B), Bcat1 siRNA A cells (Figure 11C), Bcat1 siRNA B cells (Figure 11D), and Bcat1 siRNA Control cells (Figure 11E). The dependency of the RJ345 cells (Figure 12A) and Bcat1 siRNA Control cells (Figure 12B) to the three mitochondrial fuels was then calculated using the formula,

\[
\frac{\text{basal } OCR - \text{target inhibitor } OCR}{\text{basal } OCR - \text{all inhibitors } OCR} \times 100
\]

RJ345 cells are highly dependent on glucose with a significant difference seen between glucose dependence and glutamine and fatty acid dependence. siRNA Control cells are also significantly dependent on glucose over the other the fuel oxidation options. As the OCR of the RJ423, Bcat1 siRNA A, and Bcat1 siRNA B did not follow the anticipated pattern of subsequently dropping after addition of one and then all of the inhibitors, the calculation does not yield valid dependency values and thus are not shown.
Figure 11: oxygen consumption rate (OCR) over a span of approximately two hours with the addition of a BPTES, a glutamine (GLN) oxidation inhibitor, Etoxomir, a fatty acid (FA) oxidation inhibitor, or UK5099, a glucose (GLC) oxidation inhibitor at approximately 18 minutes, and the addition of the outstanding two inhibitors at approximately 70 minutes in A) RJ423 cells, B) RJ345 cells, C) Bcat1 siRNA A cells, D) Bcat1 siRNA B cells, and E) Bcat1 siRNA Control cells. Points represent OCR ± SEM (n=3).
Figure 12: A) Percent dependency on mitochondrial fuels glutamine (GLN), fatty acids (FA), and glucose (GLC), in RJ345 cells. Bars represent percent dependency on each mitochondrial fuel oxidation pathway ± SEM (n=3). Annotations: a – significantly different than glutamine dependency; b – significantly different than fatty acid dependency; c – significantly different than glucose dependency. B) Percent dependency on mitochondrial fuels glutamine (GLN), fatty acids (FA), and glucose (GLC), in Bcat1 siRNA Control cells. Bars represent percent dependency on each mitochondrial fuel oxidation pathway ± SEM (n=3). Annotations: a – significantly different than glutamine dependency; b – significantly different than fatty acid dependency; c – significantly different than glucose dependency. Significance calculated using one-way Anova and post hoc tukey analysis.
**Metabolite utilization and production in the RJ423 and RJ345 cell lines**

To clarify the metabolic differences in RJ423 and RJ345 cells, cells were incubated for 24 hours in our regular cell culture media (with 10% FBS). The conditioned media samples were collected and tested by liquid chromatography mass spectroscopy (Figure 13) by The Metabolomics Innovation Centre at the University of Alberta. Significant differences were seen in 29 of the 116 metabolites analyzed with serine, taurine, leucine, glutamine, methionine, α-amino adipate, arginine, tryptophan, kynurenine, creatine, pyruvic acid, glucose, lysoPhosphatydylecholine acyl C16.1, C16.0, C18.2, C18.1, C18.0, C20.4, C24.0, and sphingomyeline C16.0 and C18.1, being utilized significantly more in the RJ423 cells than the RJ345 cells, with putrescine, α-ketoglutaric acid, citric acid, butyric acid, fumaric acid, and methylmalonic acid being produced significantly more in the RJ423 cells than the RJ345 cells, and with proline, glutamate, and lysoPhosphatydylecholine acyl C17.0 being produced more in the RJ345 cells than the RJ423 cells. Significance was determined using two-tailed T tests.
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<th>RJ345</th>
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| LYSOC17:0      | 0.13  | 0.16  | 0.13       |
| LYSOC18:2      | 0.11  | 0.17  | 0.27       |
| LYSOC18:1      | 0.89  | 1.55  | 1.50       |
| LYSOC18:0      | 1.09  | 1.40  | 1.25       |
| LYSOC20:4      | 0.12  | 0.32  | 0.56       |
| LYSOC24:0      | 0.22  | 0.31  | 0.32       |
| LYSOC26:1      | 0.02  | 0.02  | 0.02       |
| LYSOC26:0      | 0.05  | 0.05  | 0.05       |
| LYSOC28:1      | 0.02  | 0.02  | 0.02       |
| LYSOC28:0      | 0.04  | 0.05  | 0.04       |
| 14:1SMOH       | 0.06  | 0.07  | 0.07       |
| 16:1SM         | 0.14  | 0.17  | 0.17       |
| **16:0SM**     | 1.03  | 1.27  | 1.28       |
| 16:1SMOH       | 0.08  | 0.09  | 0.09       |
| **18:1SM**     | 0.09  | 0.10  | 0.10       |
| PC32:2AA       | 1.09  | 1.11  | 1.06       |
| 18:0SM         | 0.44  | 0.49  | 0.48       |
| 20:2SM         | 0.01  | 0.01  | 0.01       |
| PC36:0AE       | 0.05  | 0.05  | 0.05       |
| PC36:6AA       | 0.02  | 0.02  | 0.02       |
| PC36:0AA       | 0.17  | 0.19  | 0.21       |
| 22:2SMOH       | 0.07  | 0.07  | 0.07       |
| 22:1SMOH       | 0.08  | 0.10  | 0.10       |
| PC38:6AA       | 0.22  | 0.24  | 0.24       |
| PC38:0AA       | 0.06  | 0.07  | 0.08       |
| PC40:6AE       | 0.05  | 0.06  | 0.06       |
| 24:1SMOH       | 0.03  | 0.03  | 0.03       |
| PC40:6AA       | 0.36  | 0.39  | 0.41       |
| PC40:2AA       | 0.02  | 0.02  | 0.01       |
| PC40:1AA       | 0.02  | 0.02  | 0.02       |
Figure 13: heat map of metabolite concentration in µM in 10% FBS conditioned media samples of RJ423 cells and RJ345 cells (n=3), and background media samples (n=2).

|       | C0   | C2    | C3    | C4    | C3OH | C5:1  | C5    | C4OH  | C6:1  | C6    | C5OH  | C8    | C5MDC | C9    | C10:2 | C10   | C12:1 | C12   | C14:2 | C14:1 | C14   | C12DC | C14:2OH | C14:1OH | C16:2 | C16:1 | C16   | C16:2OH | C16:1OH | C16:OH | C18:2 | C18:1 | C18    | C18:1OH |
|-------|------|-------|-------|-------|------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|--------|--------|-------|-------|-------|---------|---------|-------|-------|-------|--------|---------|-------|-------|-------|--------|
| RJ423 | 2.15 | 0.73  | 0.09  | 0.49  | 0.19 | 0.28  | 0.17  | 0.08  | 0.47  | 0.87  | 0.29  | 0.66  | 0.16  | 0.10  | 0.10  | 0.16  | 0.19  | 0.32  | 0.09  | 0.08  | 0.27  | 0.62   | 0.17    | 0.13  | 0.19  | 0.15  | 0.19   | 0.14    | 0.26   | 0.21  | 0.26  | 0.11   | 0.16    |
| RJ345 | 2.34 | 0.75  | 0.10  | 0.51  | 0.20 | 0.30  | 0.16  | 0.09  | 0.49  | 1.11  | 0.30  | 0.67  | 0.18  | 0.10  | 0.10  | 0.20  | 0.18  | 0.30  | 0.09  | 0.08  | 0.26  | 0.57   | 0.18    | 0.13  | 0.17  | 0.16  | 0.18   | 0.15    | 0.26   | 0.23  | 0.24  | 0.10   | 0.18    |
| Background | 2.38 | 0.71  | 0.09  | 0.52  | 0.21 | 0.26  | 0.15  | 0.09  | 0.47  | 1.25  | 0.30  | 0.69  | 0.19  | 0.10  | 0.10  | 0.22  | 0.18  | 0.29  | 0.08  | 0.08  | 0.25  | 0.60   | 0.19    | 0.13  | 0.18  | 0.16  | 0.19   | 0.14    | 0.26   | 0.22  | 0.23  | 0.11   | 0.18    |
DISCUSSION

The metabolic enzyme, branched chain amino acid transaminase 1, or BCAT1, is responsible for initiating the reversible catabolism of the branched chain amino acids (BCAAs) (Suryawan et al. 1998). The BCAAs – leucine, isoleucine, and valine – play a number of roles in a healthy individual including regulating growth and proliferation, acting as a nitrogen source for alanine and glutamine, and acting as components and regulators of protein synthesis (Hutson et al. 2005; Zhou et al. 2010; Garber et al. 1976). As a result of their physiologic importance, the BCAAs and their metabolism can be coopted by tumors to support a high metabolic demand (Mayers et al. 2016). Deregulation of BCAT1 expression and the subsequent deregulation of BCAA metabolism has been linked to nasopharyngeal carcinomas, hepatocellular carcinomas, epithelial ovarian cancer, triple negative breast cancer, urothelial carcinomas, glioblastomas carrying wild type isocitrate dehydrogenase 1, and ERα-negative breast cancers (Tonjes et al. 2013; Zhou et al. 2013; Xu, et al. 2016; Zhang et al. 2017; Wang et al. 2015; Oktyabri et al. 2016; Chang et al. 2016; Thewes et al. 2017). As Bcat1 RNA level was found to be up-regulated in the murine claudin-low cell line, RJ423, created in the Moorehead lab, and in human claudin-low breast cancer tumors as determined by a team at the University of North Carolina, it was the goal of this project to confirm the up-regulation of Bcat1 in the murine claudin-low cell line and determine what effects the enzyme has on the aggressive and metastatic nature of the claudin-low subtype (University of North Carolina MicroArray Database).

To first determine the viability of an investigation determining the effects of BCAT1 utilizing our lab’s murine claudin-low cell line, RJ423, the expression of BCAT1 was confirmed to be up-regulated in claudin-low breast cancer subtype compared to luminal breast cancer subtype. Specifically, the mRNA level of Bcat1 was confirmed to be up-regulated in the human
claudin-low cell line, MDA-MB-231, claudin-low murine tumor tissue, and claudin-low murine cell line, RJ423, compared to human luminal cell line, MCF-7, luminal murine tumor tissue, and luminal murine cell line, RJ345, respectively. The expression of BCAT1 was confirmed to be up-regulated at the protein level in the cell line RJ423 compared to the cell line RJ345. Furthermore, Bcat2, the second and more physiologically common isotype of the BCAT enzyme, was determined to not be expressed at significantly different levels in the RJ423 cell line or the claudin-low murine tumor tissue compared to the RJ345 cell line and luminal murine tumor tissue, respectively. Therefore, we can trust that alterations in Bcat1 expression, and not Bcat2, is responsible for results collected from experiments discussed below.

Utilizing a siRNA mediated knockdown of BCAT1, we first sought to determine the influence of the enzyme on cellular proliferation. Based on the evidence put forth in the literature demonstrating that BCAT1 has a driving influence on cellular proliferation and growth rate in gliomas and breast cancer, we expected to also see significant differences in our own cell lines (Tonjes et al. 2013; Zhang et al. 2017; Thewes et al. 2017). However, the level of cellular proliferation in RJ423 was not impacted by a siRNA BCAT1 knockdown based on phospho-histone H3 staining or BrdU and 7-AAD staining. Although no significant difference was observed in these cell cycle markers, the manual cell count indicating population doubling time was significantly longer in the Bcat1 siRNA knockdown cell lines compared to the RJ423 cell line. However, the Bcat1 siRNA Control cell line also had a significantly longer population doubling time over the RJ423 cell line, with Bcat1 siRNA Control cell line only having a significant difference between the Bcat1 siRNA B cell line and not the Bcat1 siRNA A cell line. This inconsistency lead us to believe that the difference seen may not in fact be caused by a change in proliferation, but instead by a change in the level of cell death thereby decreasing cell
number during a manual count. Upon further research, increased BCAT1 expression has been linked to apoptotic features in murine cells when grown under serum deprived conditions (Eden & Benvenisty 1999). Although citing an opposite effect, that increased BCAT1 expression will increase apoptotic events, this study lends the idea that BCAT1 may impact cell death in different ways depending on the direction of the BCAT1 reaction in the cells in question (Eden & Benvenisty 1999). Again, contrary to our expectation, siRNA knockdown of BCAT1 had no significant impact on cell death as determined by Annexin V and Propidium Iodide staining. The results of our proliferation and apoptosis studies, demonstrating that the Bcat1 siRNA Control cell line trended closely with the Bcat1 siRNA knockdown cell lines and away from the RJ423 cell line, suggest that some of the effects seen may be side effect of the siRNA transfection protocol, and not BCAT1 itself. We next analyzed the impact of siRNA mediated BCAT1 knockdown on cellular migration. BCAT1 influence on migration was determined through scratch wound assays comparing the difference in area covered by cells at time of initial scratch and 24 hours post scratch. Although contradicting with results cited in studies looking at hepatocellular carcinoma, nasopharyngeal carcinoma, and a human claudin-low breast cancer cell line demonstrating that BCAT1 is linked to migration, BCAT1 knockdown did not significantly impact the migration of RJ423 cells (Zhou et al. 2013; Xu et al. 2016; Oktyabri et al. 2016). It is worth noting that although a significant difference was not observed, the results are trending towards a decreased rate of migration in the Bcat1 siRNA knockdown cell lines. However, again, the difference was seen in the Bcat1 siRNA Control cell line as well. Although BCAT1 was analyzed in a claudin-low human cell line in respects to migration by another group, the knockdown method was different in that Oktyabri and colleagues utilized a stable knockdown through shRNA or cDNA expressing retroviruses (Oktyabri et al. 2016). This
method would likely avoid the transfection protocol side effects believed to be impacting the current study. Additionally, as the knockdown in the claudin-low human cell line was stable, the level of BCAT1 would be consistent over the course of the migration period. In our study utilizing a transient knockdown of BCAT1, it was determined that the protein level showed the best knockdown at 48 hours following the transfection [see Appendix 2]. However, in order to allow for a 24 hour incubation period the scratch wound assay spanned the time of approximately 36 to 60 hours following the transfection period. This variability in BCAT1 expression levels may be affecting the migration results discussed.

As BCAT1 is a metabolic enzyme, the next step was to analyze what impact it has on the overall metabolic phenotype of the claudin-low cell line, RJ423, compared to our luminal cell line, RJ345. This was accomplished using the Seahorse metabolic analyzer technology along with the Cell Energy Phenotype kit from Agilent. This kit allows the user to distinguish whether a cell line is relying more on oxidative phosphorylation or on glycolysis (Agilent Technologies Inc. 2017). Additionally, the kit will determine the maximum capacity of these metabolic pathways to sustain the cells under stressed conditions (Agilent Technologies Inc. 2017). Specifically, measuring the change in oxygen consumption rate, or OCR, over a period of time, attains the basal rate of oxidative phosphorylation (Agilent Technologies Inc. 2017). The maximum potential of the oxidative phosphorylation pathway is determined by measuring the change in OCR after the addition of a mitochondrial membrane proton gradient uncoupling agent, carbonylcyanide p-trifluoromethoxyphenylhydrazone, or FCCP (Agilent Technologies Inc. 2017). FCCP drives an increase in the OCR as the cells attempt to restore the proton gradient. Measuring the extracellular acidification rate, or ECAR, over a period of time, will attain the basal rate of glycolysis (Agilent Technologies Inc. 2017). When the ATPase inhibitor
oligomycin is added, it provides the maximum potential of the glycolytic pathway as the cells attempt to restore ATP levels through glycolytic production (Agilent Technologies Inc. 2017). The claudin-low cell line, RJ423, had both a higher basal rate of oxygen consumption and extracellular acidification. This is a logical finding for our cell line, as RJ423 cells appear more energetic, with a faster proliferation rate, and more metabolically active, based on a faster colour change in the culture media indicating a pH change, in culture. Additionally, the literature suggests that the claudin-low phenotype has greater glycolytic usage compared to oxidative phosphorylation, with multiple studies analyzing the human breast cancer cell line, MDA-MB-231, demonstrating a greater basal ECAR than other breast cancer subtypes (Pelicano et al. 2014; Lanning et al. 2017). When stressed however, our RJ423 cells and the RJ345 cells did not have a significant difference in maximum capacity. This suggests that the RJ345 cells have greater metabolic potential – a greater difference between their basal and maximum metabolic rate – than the RJ423 cells, although this is likely due to the fact that the RJ423 cells are functioning at a high rate of metabolic activity to start with. To determine whether BCAT1 was responsible for the higher rate of basal metabolic activity the cell energy phenotype test was conducted on the Bcat1 siRNA cell lines. Although the Bcat1 siRNA knockdown cell lines showed a significantly decreased basal OCR and basal ECAR, this difference was also observed in the Bcat1 siRNA Control cell line. As the Bcat1 siRNA Control cell line did not show similar results to the RJ423 cell line, it is possible that the difference seen in the Bcat1 siRNA knockdown cell lines was, again, a side effect of the siRNA transfection, not BCAT1 itself. There was no consistent effect of BCAT1 observed on the stressed OCR or ECAR based on the Bcat1 siRNA knockdown cell line results of this assay. However, this data will be impacted by the fact that we are unable to normalize the data to cell number due to the cells lifting during the assay. Agilent recommends
normalizing the data to cell number by one, or a combination of, three methods (Kam et al. 2018). These methods include measuring total cellular protein by such means as Bradford or BCA protein detection agents, measuring nuclear DNA content via a variety of fluorescence or colorimetric stains that incorporate into double stranded DNA, and cell counting through stain and automated imaging technologies (Kam et al. 2018). Each method of normalization has benefits and limitations depending on the experiment parameters being conducted and the method used should be decided on by such (Kam et al. 2018). However, each method shares the limitation that the cells need to be adherent following the metabolic assay in order to be normalized (Kam et al. 2018). This fact is especially concerning in the case of the current study as, although there was no significant difference observed in proliferation or cell survival between our parental cell lines and the Bcat1 siRNA cell lines, there was a trending difference in both considerations, high standard error margins, as well as a significant difference seen in population doubling time by manual cell counting, discussed previously. Although the cells are seeded such that they reach 100% confluence by the time of the assay, this is measured only qualitatively, and subject to variability.

To further tease out the effect of BCAT1 on metabolic activity in the RJ423 cell line compared to the RJ345 cell line, the Mitochondrial Fuel Flex kit from Agilent was conducted. Specifically, this kit allows users to compare the cells dependency on three different fuels to sustain mitochondrial respiration: glucose, fatty acids, and glutamine (Agilent Technologies Inc. 2019). The basal OCR is measured and then compared to the OCR when a specific inhibitor is added; the basal OCR is then compared to the OCR when all three fuel pathways are inhibited (Agilent Technologies Inc. 2019). The difference in these two conditions gives the dependence on the one fuel compared to dependence on all three fuels together (Agilent Technologies Inc.
To measure the dependency of the cells on glucose, the compound UK5099 is added (Agilent Technologies Inc. 2019). UK5099 is an inhibitor of glucose oxidation by blocking the mitochondrial pyruvate carrier (Agilent Technologies Inc. 2019). The compound etoxomir is an inhibitor of fatty acid oxidation by inhibiting carnitine palmitoyl-transferase 1A, which is important for transporting the long chain fatty acids into the mitochondria (Agilent Technologies Inc. 2019). Finally, to measure the dependency of the cells on glutamine, the inhibitor BPTES is added, which will inhibit glutaminase (Agilent Technologies Inc. 2019). Glutaminase is responsible for converting glutamine to glutamate, which will then be converted into alpha-ketoglutarate that will be oxidized in the tricarboxylic acid (TCA) cycle (Agilent Technologies Inc. 2019). A search of the literature for previous studies analyzing mitochondrial fuel dependency in claudin-low breast cancer yields relatively few but very informative studies. Based on these works analyzing the claudin-low human breast cancer cell line, MDA-MB-231, and luminal human breast cancer cell line, MCF-7, our claudin-low cell line, RJ423, was anticipated to present with an increased dependence on fatty acid and glucose oxidation compared to our luminal cell line, RJ345 (Lopez et al. 2017). Additionally, the literature would suggest that our luminal cell line be significantly more dependent on glucose oxidation compared to the other mitochondrial fuel oxidation pathways (Lopez et al. 2017; Kam et al. 2017). Although flexibility of these fuel oxidation pathways was not analyzed in the current study, it is worth noting that in these human cell line studies the MDA-MB-231 cell line demonstrated a limited flexibility in response to inhibition of one or multiple of the fuel oxidation pathways, with only a slight increase in fatty acid and glutamine oxidation, under the relative trial conditions (Lopez et al. 2017). Consistent with what has been demonstrated in the literature, our luminal cell line, RJ345, demonstrated a significant dependence on glucose oxidation compared
to the other two mitochondrial fuel sources analyzed by Agilent’s Mito Fuel Flex test. Unfortunately, the data obtained for our claudin-low cell line, as well as our Bcat1 siRNA knockdown cell lines, did not produce the anticipated result based on the literature, and more unexpectedly, failed to even follow the anticipated trend in the raw data such that the OCR was not decreased after addition of one and subsequently all of the mitochondrial fuel pathway inhibitors. This unexpected and difficult to explain outcome would indicate that our claudin-low cell line, RJ423, and our Bcat1 siRNA knockdown cell lines, are not dependent on any of the major mitochondrial fuel oxidation pathways, however, a dependency calculation using the relative basal OCR, target inhibitor OCR, and all inhibitors OCR data, yields an invalid result and so this is only an anecdotal and not quantitative answer. To further confuse the matter, the Bcat1 siRNA Control cell line did in fact follow the anticipated trend in the raw data producing calculations indicating a significant dependency on glucose oxidation over the other two mitochondrial fuels, similar to the RJ345 cell line. With the information that we have obtained through this study, at best we can speculate why the data from this assay has deviated so far from what was expected. Of course, the inability to normalize the data to cell number as discussed previously is the first consideration worth noting. This limitation makes it difficult to confidently compare the different condition groups within the same cell line as well as compare the same condition group across the different cell lines. Additionally, the lack of normalization will increase the likelihood of variability between technical replicates within each condition group and cell line leading to increased standard error margins. The lack of normalization, however, does not account for the raw data deviating from the expected drop in OCR following each subsequent addition of inhibitors. When the individual technical replicate well results were analyzed, in the majority of cases, the results also did not follow the anticipated trend. The lack
of normalization also cannot logically explain why the RJ345 cell line and Bcat1 siRNA Control cell line responded to the assay as intended when the other cell lines did not. Another limitation to consider is the range within which the OCR values fall. The seeding density for our cell lines was optimized such that the cells would be at 100% confluency at the time of the assay and that the OCR values would be measured between 50 and 400 pmols/minute, and ECAR values, in the case of our Cell Energy Phenotype experiment, would be measured between 20 and 120 mpH/minute, as recommended by Agilent Technical Overview (Agilent Technologies Inc. 2017). Despite maintaining the same seeding number and protocol throughout both Seahorse Metabolic experiments, the OCR values of the RJ423 cell line glucose dependency condition hover around the 50 pmols/minute mark with individual technical replicate values reading as low as 35.41427 pmols/minute even in the basal OCR results. Similarly, some individual technical replicates within the RJ345 and Bcat1 siRNA B cell lines come close to, and fall below, 50 pmols/minute. Below this level the standard error margin of the readings become exacerbated and will lower confidence in the results. Although this limitation may explain the lack of adherence to the anticipated trend in OCR in the RJ423 cell line glucose oxidation condition, as well as the relatively high standard error margins in other conditions, it does not account for the lack of adherence to the anticipated trend in OCR for the rest of the cell lines and conditions as the values for those conditions fell comfortably within the range of 50 to 400 pmols/minute at each step of the assay. Finally, the efficiency of the inhibitors themselves must be taken into consideration. The concentration of inhibitor added to each well during the assay was a set value laid out in the Mito Fuel Flex Kit User Manual and was not indicated to need optimization (Agilent Technologies Inc. 2019). An example OCR readout and calculation are provided within the manual, analyzing specifically the glutamine oxidation pathway in HepG2 cells, a human
hepatocellular carcinoma cell line. Although the exact values are not indicated in this example, the figure presented indicates a drop in OCR to approximately half when all inhibitors are added and only a decrease of approximately 20 pmols/minute from when the basal OCR was measured and when the target inhibitor OCR, in this case BPTES, was measured. This is an extremely low magnitude of change and, when combined with the high variability reported in the current study, may explain why significant differences are not appearing in our calculations and why it appears that the RJ423, Bcat1 siRNA A, and Bcat1 siRNA B cell lines are not affected by any or all of the inhibitors, especially when taken into account that the RJ423 cell line is highly energetic as determined by our Cell Energy Phenotype experiment. As the basal OCR is high in the RJ423 cells, compared to the RJ345 cells, again as determined in the current study, it is possible that the percent inhibition by each inhibitor used compared to the total oxidative phosphorylation activity of this cell line is too low to be quantified by the Mito Fuel Flex assay.

In order to clarify the results of the Seahorse metabolic assays, conditioned media samples were collected and sent to The Metabolomic Innovations Center at the University of Alberta for analysis by liquid chromatography mass spectroscopy. A total of 116 common metabolites were analyzed as per the protocol of the Prime Metabolome Profiling Assay. Seahorse metabolic technologies offer many benefits in that researchers can obtain a real-time view of the metabolic activities of the cell population in question, allowing us to not only see metabolic differences between cell lines, but the rate at which these differences are changing, whereas technologies such as mass spectroscopy only allow for a snapshot of the metabolic activity in the cells in question (Agilent Technologies Inc. 2019). However, mass spectroscopy can analyze a large number of metabolites, such as the 116 analyzed in the current study, whereas Seahorse metabolic analyzer tests Cell Energy Phenotype and Mito Fuel Flex are
limited to looking at glycolysis and oxidative phosphorylation on a large scale and the mitochondrial fuels glutamine, fatty acids, and glucose, respectively (Agilent Technologies Inc. 2017; 2019). One key benefit that Seahorse metabolic assays offer is the ability to interfere with the target pathways and thereby glean more information – such as dependency on a particular fuel pathway – than what mass spectroscopy can provide (Agilent Technologies Inc. 2019). Of course, the Seahorse metabolic assays are not without their challenges, as discussed previously. For this reason, it was our hope that analysis of common metabolites by liquid chromatography mass spectroscopy would provide explanations for the unexpected metabolic results thus far. Of the 116 metabolites measured in the Prime Metabolome Profiling Assay, 29 metabolites were significantly different between the RJ423 and RJ345 cells. Of these 29, some metabolites stood out as potentially being key indicators into the metabolic activities of our cells. First, our RJ423 cells appear to be producing less glutamate and more α-ketoglutaric acid than our RJ345 cells, as well as utilizing more glutamine than the RJ345 cells, which may indicate that, in our claudin-low cell line, the reverse BCAT1 reaction is favoured, creating a BCAA and α-ketoglutarate from an BCKA and glutamate, respectively. This finding is consistent with the literature, which demonstrates that the reverse reaction is favoured in both myeloid leukemia and breast cancer (Hattori et al. 2017; Zhang et al. 2017). This idea is contradicted, however, by the lower concentration of the BCAA, leucine, in our RJ423 cells compared to the RJ345 cells as well as no significant difference observed in the other BCAAs. However, it is possible that leucine is being rapidly utilized by other pathways and thus appears to have a low concentration. The BCAAs, namely leucine, are often the preferred source of nitrogen to be used in protein anabolism and it has been shown that, in some cases, relatively few BCAA derivatives end up cycling through the TCA cycle (Mayers, et al. 2016; Green et al. 2015). Additionally, glucose
utilization was significantly higher in RJ423 cells compared to the RJ345 cells. As our RJ423 cell line, as well as human claudin-low cell line, MBA-MD-231, has a higher basal glycolytic rate as per our Cell Energy Phenotype data and findings from the literature, respectively, this is a logical finding (Pelicano et al. 2014; Lanning et al. 2017). Based on this we would also expect to see a greater production of lactic acid in the RJ423 cells over the RJ345 cells, however, the difference was not significant. Both glucose and glutamine are utilized more, and components of the TCA cycle, butyric acid and citric acid, are produced more, in the RJ423 than in the RJ345 cells. Aside from its involvement in the TCA cycle, butyric acid has been shown to have variable effects as at times it has shown to be oncogenic and other anti-oncogenic through its influence over proliferation and apoptosis (Human Metabolome Database 2019). Further, pyruvic acid, which will feed into the TCA cycle is utilized more by the RJ423 cells. These findings are consistent with the Cell Energy Phenotype data that shows a greater basal rate of oxidative phosphorylation compared to the RJ345 cells. However, fumaric acid, a precursor to the TCA metabolite malate, and methylmalonic acid, whose derivatives will feed into the TCA cycle, are produced more in the RJ423 cells (Human Metabolome Database 2019). Fumaric acid has been identified as an oncometabolite as it appears to regulate HIF1-α (Human Metabolome Database 2019). It is difficult to compare the results of our Mito Fuel Flex assay with our metabolite concentration data as, in addition to most cell lines not adhering to anticipated trends, dependency does not necessarily equate to utilization. Interestingly, proline production was significantly higher in the RJ345 cells compared to the RJ423 cells. Proline metabolism has previously been linked to the aggressive nature of esophageal squamous cell cancer and has been shown to regulate stress responses modulating either the production of energy for survival or reactive oxygen species for apoptosis (Phang et al. 2008; Togashi et al. 2014). The metabolism
of proline has relevance to the metabolic analyses of our BCAT1 study as it can be converted into a number of other substrates, notably, glutamine, as well as act as a source for carbon exchange in the TCA cycle (Phang et al. 2008). Although it appears that proline is being produced more by the RJ345 cells, this may simply indicate that the RJ423 cells are utilizing what proline they are producing endogenously in the metabolic pathways discussed. Another amino acid that has been linked with cancer, and is utilized significantly more in our RJ423 cells, is serine (Mattaini et al. 2016). Serine can be attained by a cell by intake from the extracellular environment or through endogenous synthesis from glucose (Mattaini et al. 2016). Increased serine in the extracellular media alone has been shown to be sufficient to drive cancer cell proliferation (Mattaini et al. 2016). Although the metabolite concentration analysis has provided more confident data to interpret than the Seahorse metabolic assays, it is important to note that the transient nature of our BCAT1 knockdown will be limiting the accuracy of the results, similar to our migration assay, as the knockdown is shown to be most effective at 48 hours post transfection, but the conditioned media was incubated for 24 hours spanning that time and so it is possible that the BCAT1 expression levels varied greatly during this time.
LIMITATIONS AND FUTURE DIRECTIONS

Although the current study did not show BCAT1 to be a regulator of proliferation, apoptosis, migration, or metabolism in our murine claudin-low breast cancer cell line, it may be worth further investigating the effects of the metabolic enzyme, BCAT1, on claudin-low breast cancer and its aggressive nature, as the literature strongly indicates a causal relationship in other cancer types. One of the major limitations of this study was the method of knockdown and reliability of that knockdown. Transient knockdown of a target protein can be a valuable tool when studying pathologies in vitro, but in the current study the Bcat1 siRNA knockdown was highly variable between transfections requiring six biological replicates before we were confident in the results and significance was seen. It is possible that this variability compounded into high standard error margins in the experiments run. In most cases, including the cell proliferation and cell survival analyses, the results showed no significance and yet were trending towards a change in the Bcat1 siRNA cell lines. It is possible that due to the high standard error margins the results were not statistically significant even though appearing so. Additionally, the transient nature of the knockdown limited the reliability of the migration study as the level of BCAT1 expression was variable of the 24 hour period, as well as the conditioned media samples that were also incubated over a 24 hour period. Although stable knockdowns were attempted in the RJ423 cell line though shRNA constructs in lentiviral GFP vectors, and stable up-regulation of Bcat1 attempted in the RJ345 cell line through cDNA ORF Clone with GFPSpark® tagged N-terminal [Appendix 2], both proved unsuccessful despite numerous optimization attempts. However, if a stable Bcat1 knockdown or up-regulated cell line could be created, it would lessen the variability of BCAT1 expression between biological replicates, ideally allowing for significance to be seen in future experiments. Further, it would be ideal that if a stable cell line
could be created, it would be a RJ345 cell line with BCAT1 stably up-regulated. Another of the major limitations of this study was the inability to normalize the metabolic data obtained from the Seahorse Metabolic Analyzer. Although the RJ423 cells consistently lifted from the microplate, the RJ345 cells remained adherent. Working with the assumption that BCAT1 does not affect adherence of the cells, a stable up-regulation of BCAT1 in the RJ345 cell line would allow for more reliable analysis of the effects of BCAT1 on metabolic phenotype and mitochondrial fuel dependency using the Seahorse Metabolic Analyzer. A stable BCAT1 cell line, either a knockdown or up-regulated, would also allow for in vivo studies analyzing the effect of BCAT1 on tumor growth and metastasis.
SUMMARY AND CONCLUSION

In this study we analyzed the influence of the metabolic enzyme, branched chain amino acid transaminase 1, or BCAT1, on the aggressive nature of claudin-low breast cancer though investigation with a murine claudin-low cell line, RJ423, and murine luminal cell line, RJ345. BCAT1 is significantly up-regulated in our claudin-low cell line, its originating tumor, as well as human claudin-low breast cancer cell lines. However, based on the results of this study utilizing a transient Bcat1 knockdown by siRNA, BCAT1 does not exert influence over claudin-low breast cancer cell proliferation, cell survival, or migration, and does not have consistent effects on the overall metabolism of claudin-low phenotype. Based on metabolite concentration analysis, BCAT1 may be catabolizing the reverse reaction, creating a BCAA and α-ketoglutarate, in claudin-low breast cancer. Due to numerous factors potentially compounding into extremely high variability in these experiments, this study is not decisive to contradict the abundant literature stating the importance of BCAT1 in other cancers or to disprove its relevance in claudin-low breast cancer.
APPENDIX 1: SOLUTION RECIPES

Phosphate Buffered Saline (PBS) (2L)

Sodium Chloride 16.0 g  
Potassium Chloride 0.4 g  
Sodium Potassium Dibasic Anhydrous 2.3 g  
Potassium Phosphate Monobasic 0.4 g  
P pH to 7.4 with HCL

Western Blot Reagents

RIPA Lysis Buffer

50mM Tris HCL, pH 7.5 3.0285 g  
150mM NaCl 4.383 g  
1% Triton X-100 5mL  
0.1% sodium dodecyl sulfate (SDS) 0.5mL  
10mM EDTA 1.4612 g  
1% Sodium Deoxycholate 5mL

For every 1mL buffer was supplemented with 10uL of Phosphate Inhibitor Cocktail A, B, and C purchased from Biotool

1.0M Tris

Tris Base 12.12 g  
H2O 100mL  
P pH to 6.8 with HCL

1.5M Tris

70
Tris Base 18.16 g
H2O 100mL
pH to 8.8 with HCL

10% Ammonium Persulfate
Ammonium Persulfate 1 g
H2O 1mL

10% SDS
SDS 10 g
H2O 100mL

Resolving Gel (10% Polyacrylamide)
H2O 4.0mL
30% Acrylamide Mix 3.3mL
1.5M Tris (pH 8.8) 2.5mL
10% Ammonium Persulfate 0.1mL
10% SDS 0.1mL
TEMED 0.004mL

Stacking Gel
H2O 2.1mL
30% Acrylamide 0.5mL
1.0M Tris (pH 6.8) 0.38mL
10% SDS 0.03mL
10% Ammonium Persulfate 0.03mL
TEMED 0.003mL
3x Sample Buffer

187.5mM Tris HCL 1.47 g
6% SDS 3.0 g
0.03% Bromophenol Blue 0.015 g
30% Glycerol 15mL
PBS 35mL

Reducing Buffer

3x Sample Buffer 350uL
1M DTT 50uL

5x Running Buffer

Tris Base 15.1 g
Glycine 72.1 g
10% SDS 10mL
H₂O to 1L

25x Transfer Buffer

Tris Base 18.2 g
Glycine 90 g
H₂O to 500mL

Transfer Buffer

25x Transfer Buffer 40mL
Methanol 200mL
H₂O to 1L

10x Tris Buffered Saline (TBS)
20mM Tris Base  24.2 g
NaCl  80 g
pH to 7.6
H₂O to 1L

**Tris Buffered Saline with Tween (TBST)**
TBS  100mL
Tween 20  1mL
H₂O to 1L

**Blocking Solution and Antibody Diluting Solution**
5% Bovine Serum Albumin (BSA)  2.5 g
TBST  50mL

**Immunofluorescence Reagents**

**Permeabilizing Solution (0.2% Triton X-100 in PBS)**
Triton X-100  100uL
PBS  50mL

**Blocking Solution (5% BSA in 0.1% Triton X-100/PBS)**
BSA  2.5 g
Triton X-100  50uL
PBS  50mL

**Antibody Diluting Solution (1% BSA in 0.1% Triton X-100/PBS)**
BSA  0.5 g
Triton X-100  50uL
PBS 50mL
APPENDIX 2: ATTEMPTED PROCEDURES AND OPTIMIZATIONS

siRNA Transfection Optimization

Transfection Efficacy

Bcat1 Silencer Select siRNA s62890 and s62889, and Silencer Select Negative Control #2 siRNA were purchased from Thermo Fisher Scientific. siRNAs were resuspended in nuclease free water to a working concentration of 200µM as per the recommendation in the manufacturer’s protocol. Following the Lipofectamine 2000 (Thermo Fisher Scientific) protocol, siRNA transfections were attempted at concentrations outlined in the table below. Trial 4 showed the greatest knockdown of Bcat1 mRNA by qRT-PCR.

Table 3: Optimizing siRNA and Lipofectamine 2000 concentrations

<table>
<thead>
<tr>
<th>Trial 1</th>
<th>Trial 2</th>
<th>Trial 3</th>
<th>Trial 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>siRNA Amount</td>
<td>100pmole</td>
<td>200pmole</td>
<td>300pmole</td>
</tr>
<tr>
<td>Lipofectamine 2000 Amount</td>
<td>10µL</td>
<td>5µL</td>
<td>5µL</td>
</tr>
<tr>
<td>Time Point</td>
<td>24 hours</td>
<td>24 hours</td>
<td>24 hours</td>
</tr>
<tr>
<td>Following</td>
<td>transfection</td>
<td>following</td>
<td>following</td>
</tr>
</tbody>
</table>

Timing for mRNA

Using the concentration determined to be most effective from the above table, siRNA transfections were performed to determine at what time point following the transfection the cells showed the greatest knockdown of Bcat1 mRNA by qRT-PCR. Bcat1 mRNA levels were decreased most at the 48 hour time point with a sharp rise over the 72 and 96 hour time points.
Table 4: Optimizing siRNA transfection timing for mRNA level

<table>
<thead>
<tr>
<th>Trial</th>
<th>Trial 2</th>
<th>Trial 3</th>
<th>Trial 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>siRNA Amount</td>
<td>200pmole</td>
<td>200pmole</td>
<td>200pmole</td>
</tr>
<tr>
<td>Lipofectamine 2000 Amount</td>
<td>10µL</td>
<td>10µL</td>
<td>10µL</td>
</tr>
<tr>
<td>Time Point</td>
<td>24 hours following transfection</td>
<td>48 hours following transfection</td>
<td>72 hours following transfection</td>
</tr>
</tbody>
</table>

**Timing for Protein**

Using the concentration determined to be most effective from the above table, siRNA transfections were performed to determine at what time point following the transfection the cells showed the greatest knockdown of BCAT1 by Western Blotting. BCAT1 levels were decreased most at the 48 hour time point.

Table 5: Optimizing siRNA transfection timing for protein level

<table>
<thead>
<tr>
<th>Trial 1</th>
<th>Trial 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>siRNA Amount</td>
<td>200pmole</td>
</tr>
<tr>
<td>Lipofectamine 2000 Amount</td>
<td>10µL</td>
</tr>
<tr>
<td>Time Point</td>
<td>24 hours following transfection</td>
</tr>
</tbody>
</table>

**Gabapentin Treatment**

Gabapentin (20mg) was purchased from ApexBio and diluted to a 10mM stock. Three technical replicates of RJ423 cells were seeded in a 96 well dish at a density of 750 cells/well and incubated overnight. Each day for three days the media was removed from each well and new media containing a 1, 2, 5, 10, 15, 25, 50, 75, or 100µM concentration of Gabapentin was added back to the well. On the day following the third treatment a WST-1 Cell Viability assay was performed. Media was removed from each well and 90µL of media and 10µL of WST-1
reagent (Roche) were added back to the well. The optical density at 450nm was recorded at 10, 20, and 30 minutes of incubation in a Bio-Tek EL800 Universal Microplate Reader from Bio-tek Instruments Inc.

**Bcatc Inhibitor 2 Treatment:**

Bcatc Inhibitor 2 (5mg) was purchased from ApexBio and diluted in ethanol to a working stock of 10mM. Three technical replicates of RJ423 cells were seeded in a 96 well dish at a density of 750 cells/well and incubated overnight. Each day for three days the media was removed from each well and new media containing a 0.05, 0.1, 0.2, 0.4, 0.6, 0.8, or 1.0µM concentration of Bcatc Inhibitor 2 was added back to the well. On the day following the third treatment a WST-1 Cell Viability assay was performed. Media was removed from each well and 90µL of media and 10µL of WST-1 reagent (Roche) were added back to the well. The optical density at 450nm was recorded at 10, 20, and 30 minutes of incubation in a Bio-Tek EL800 Universal Microplate Reader from Bio-tek Instruments Inc.

**BCAA Assay**

A Branched Chain Amino Acid (BCAA) Kit and a Deproteinizing Sample Preparation Kit – TCA were purchased from Abcam. RJ345 and RJ423 cells were grown to 80% confluency in 6cm culture plates. Following the manufacturer’s protocols, the cell samples were deproteinized and then processed using the BCAA Kit. Samples were loaded into a 96 well dish at amounts ranging from 5-50µL and the well topped up to a total of 50µL with the prescribed BCAA Assay Buffer. The plate was agitated for 30 minutes at room temperature. The optical density at 450nm was recorded after this time in a Bio-Tek EL800 Universal Microplate Reader from Bio-tek Instruments Inc.
**Bcat1 shRNA Transfection – Bcat1 Knockdown**

Four Bcat1 mouse shRNA constructs in lentiviral GFP vectors (TL500193A, TL500193B, TL500193C, TL500193D) and one scrambled negative control shRNA cassette were purchased from Origene and resuspended in 50µL of nuclease free water. RJ423 cells were seeded in 6 well dishes at a density allowing for 40% confluence at the time of transfection. 10µL of Lipofectamine 2000 (Thermo Fisher Scientific) was combined with 240µL of Opti-mem media (Thermo Fischer Scientific) and 10µL of lentiviral plasmid was combined with 240µL of Opti-mem media, each was incubated at room temperature for 5 minutes and then combined together. The combined solution was incubated at room temperature for 20 minutes, during which time media was removed from the cells and replaced with 500µL of antibiotic-free media. 500µL of the lentiviral plasmid combination was added to each respective well and then incubated at 37°C for 5 hours, after which the media was replaced. Knockdown was tested using qRT-PCR and Western blotting, for mRNA and protein level of Bcat1, respectively.

**Bcat1 Plasmid Transfection – Bcat1 Overexpression**

**Transfection Protocol**

A murine Bcat1 cDNA ORF Clone with GFPSpark® tagged N-terminal and a Control Vector (CV) with GFPSpark® tagged N-terminal, both with hygromycin resistance, were purchased from Sino Biological. Both Bcat1 and Control Vector were resuspended in 100µL of nuclease free water as per the manufacturer’s protocol. As per the Lipofectamine 3000 protocol (Thermo Fisher Scientific) the cells were plated in a 6 well dish such as to reach 80% confluence at the time of transfection. 15µL of Lipofectamine 3000 was combined with 250µL of Opti-mem media while 20µL of each plasmid was combined with 110µL of Opti-mem media and 4µL of P3000 reagent. Both mixtures were incubated at room temperature for 5 minutes. To both the
Bcat1 and CV mixtures, 125μL of the Lipofectamine 3000-Opti-mem media mixture was added and then incubated at room temperature for 10 minutes. The 250μL Bcat1 and CV mixtures were then added to their respective wells on top of the existing media (750μL).

**Hygromycin Kill Curve**

RJ345 cells were seeded in a 24 well culture dish in duplicate at a density of 20,000 cells/well and incubated overnight. The following day the media from each well was removed and replaced with media containing hygromycin (Thermo Fisher Scientific) at concentration increments from 100μg/mL to 600μg/mL. The cells were monitored for cell death each day for the three following days. A second kill curve was seeded in the same manner and treated with hygromycin at concentration increments from 600μg/mL to 2000μg/mL and monitored for three days for cell death. A working concentration was determined when all RJ345 cells had died as a result of the treatment.
APPENDIX 3: MATERIAL SOURCES

Abcam, Cambridge, UK
Agilent Technologies Inc., Santa Clara, California, USA
Amersham Pharmacia Biotech, Buckinghamshire, UK
ApexBio, Houston, Texas, USA
BD Biosciences, San Jose, California, USA
Bio-Rad, Mississauga, Ontario, CA
Bio-tek Instruments Inc., Winooski, Vermont, USA
Bioline, London, UK
Cell Signaling Technology, Beverly, Massachusetts, USA
Clontech, Mountain View, California, USA
eBioscience, San Diego, California, USA
Graphpad Software, Inc., La Jolla, CA
Life Technologies, Burlington, Ontario, CA
Molecular Devices, Sunnyvale, California, USA
National Institute of Health, Maryland, USA
Olympus, Tokyo, JPN
Origene, Rockville, Maryland, USA
Quanta Biosciences, Beverly, Massachusetts, USA
Roche, Mississauga, Ontario, CA
Santa Cruz Biotechnology, Dallas, Texas, USA
Sigma Aldrich, Oakville, Ontario, CA
Sino Biological, Burlington, Ontario, CA

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The Metabolomics Innovation Centre, Edmonton, Alberta, CA

Thermo Fisher Scientific, Waltham, Massachusetts, USA

Wisent Bioproducts, St. Bruno, Quebec, CA
REFERENCES


https://www.cancer.gov/about-cancer/understanding/statistics


University of North Carolina. MicroArray Database.


