INVESTIGATING THE MECHANISM OF OBESITY-ASSOCIATED ESTROGEN RECEPTOR NEGATIVE BREAST CANCER TO IDENTIFY NOVEL AGENTS FOR CHEMOPREVENTION

By

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ABSTRACT

INVESTIGATING THE MECHANISM OF OBESITY-ASSOCIATED ESTROGEN RECEPTOR NEGATIVE BREAST CANCER TO IDENTIFY NOVEL AGENTS FOR CHEMOPREVENTION

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The United States is undergoing an obesity epidemic as nearly 40% of adults are obese. This number is only expected to increase. Evidence over the last two decades demonstrates the obesity public health crisis has devastating impacts on cancer incidence, specifically breast cancer. As a breast cancer risk factor, elevated body mass index (BMI) is associated primarily with postmenopausal, estrogen receptor (ER) positive (ER+) breast cancer. However, BMI does not describe adipose tissue distribution. Evidence suggests not all adipose tissue is "unhealthy", nor is adipose tissue universally associated with breast cancer risk. Now we are learning that central obesity reflects accumulation of a type of adipose tissue that is particularly harmful because it is a strong predictor of pre-menopausal ER negative (ER–) breast cancers: triple negative breast cancer (TNBC) and HER2+ER–. Currently, there are limited pharmacological means that target ER– breast cancer including PARP inhibitors and anti-PD-L 1 inhibitors. Furthermore, TNBC, a subtype lacks the ER, is an aggressive form of breast cancer that affects 10-20% of all patients. These patients would greatly benefit from preventative agents. Selective estrogen receptor modulators (SERMs), like tamoxifen, effectively reduce the risk of ER+ breast cancers by 50%. Unfortunately, SERMS are ineffective against ER– breast cancers. Because the mechanisms of how obesity promotes ER– breast cancer are unknown, thus developing targeted prevention strategies is difficult. Therefore, the objective of this dissertation is to determine the mechanisms of visceral obesity driven ER– breast cancer and use this mechanism to identify chemopreventive compounds.

Literature suggests fibroblast growth factor 2 (FGF2) and its main receptor fibroblast growth factor receptor 1 (FGFR1) could be a potential mechanism of obesity-promoted pre-menopausal ER– breast cancer. I previously demonstrated that one ER– mammary epithelial cell line undergoes malignant transformation when treated with factors from visceral adipose tissue (VAT). In addition to the Bernard lab's studies on FGF2-FGFR1 mediated malignant transformation, another group recently identified FGFR1 activation as a primary pathway for obesity-associated progression of ER+ breast cancer after estrogen deprivation. This exciting discovery also implicates FGFR1 signaling as a primary node of breast cancer

progression independent of estrogen signaling. Together, this suggests FGFR1 activation in the context of obesity may contribute to both malignant transformation and progression. Therefore, I hypothesize FGF2/FGFR1 activation is a critical mechanism in VAT-associated breast epithelial cell transformation and is a potential target for chemoprevention.

Herein, I have demonstrated that VAT and FGF2 transforms ER– breast epithelial cells and this is prevented/attenuated by a selective FGFR1 inhibitor. This revealed FGF2/FGFR1 as a critical signaling mechanism that I utilized to develop a target-based, phenotypic transformation high throughput screen (HTS) to identify chemopreventive compounds that prevent/attenuate FGF2-stimulated transformation. With this assay, fluvastatin was identified as the lead candidate for chemoprevention. Fluvastatin inhibits HMG-CoA reductase, the rate-limiting enzyme in the mevalonate pathway. Interestingly, there is no known mechanistic connection between FGF2/FGFR1 signaling and the mevalonate pathway. My data revealed that factors from VAT upregulated protein expression of mevalonate pathway enzymes such as HMG-CoA synthase 1 (HMGCS1), farnesyltransferase (FNTA), squalene synthesis (FDFT1), and HRas. Furthermore, a selective FGFR1 inhibitor effectively prevented this VAT-induced upregulation of these enzymes. This suggests that FGF2/FGFR1 influences the mevalonate pathway. Products of the mevalonate pathway include cholesterol and isoprenoids like farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP). These isoprenoids could be important in transformation as GTP binding proteins (GTPase) like oncogenic Ras require FPP to undergo prenylation, a process that is necessary for oncogenic Ras activation. While previous literature has demonstrated HRas activation stimulates transformation of ER– breast epithelial cells, my data did not show that HRas activation as a critical step in FGF2/VAT stimulated transformation. Further studies are needed to identify exactly how FGF2/FGFR1 and the mevalonate pathways are interconnected. Overall, these studies implicate the mevalonate pathway in FGF2/FGFR1 signaling and suggest that components of the pathway may serve as targets for prevention.

This dissertation is dedicated to my family,
Thank you for your love and support.

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KEY TO ABBREVIATIONS

ADDRC	Assay Drug and Development Repurposing Core
ACAT1	Acetyl-CoA C-acetyltransferase
ASC	Adipocyte-derived stem cell
Atorv	Atorvastatin
BaP	Benzo(a)pyrene
BMD	Bone mineral density
BMI	Body mass index
BRCA1	Breast cancer 1
BSA	Bovine serum albumin
CDC	Center for Disease Control and Prevention
cDNA	Complementary deoxyribonucleic acid
CHX	Cycloheximide
CRP	C-reactive protein
CSC	Cancer stem cell
CT	Computerized tomography
DCIS	Ductal carcinoma *in situ*
DGAC	Dietary Guidelines Advisory Committee
DMSO	Dimethyl sulfoxide
DTP	Developmental Therapeutics Program
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EndoR	Endoplasmic Reticulum
ER	Estrogen receptor
ER–	Estrogen receptor negative
ER+	Estrogen receptor positive

ERE	Estrogen response elements
ERK	Extracellular-signal-regulated kinase
EtOH	Ethanol
FBS	Fetal bovine serum
FDPS	Farnesyl pyrophosphate synthase
FDFT1	Squalene synthesis
FFA	Free fatty acid
FGFa	Fibroblast growth factor acidic
FGFb	Fibroblast growth factor basic
FGF1	Fibroblast growth factor 1
FGF2	Fibroblast growth factor 2
FGFR	Fibroblast growth factor
FGFR1	Fibroblast growth factor receptor 1
FGFR1 Ab	Fibroblast growth factor receptor 1 antibody
Fluv	Fluvastatin
FNTA	Farnesyl transferase
FPP	Farnesyl pyrophosphate
FTF	Fat tissue filtrate
FTO	Fat mass and obesity-associated protein
GAP	GTPase-accelerating proteins
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GGPP	Geranylgeranyl pyrophosphate
GPP	Geranyl pyrophosphate
GGPS1	Geranylgeranyl pyrophosphate synthase 1
GH	Growth hormone
GTP	Guanosine triphosphate

GTPase	GTP binding protein
NCHS	National Center for Health Statistics
HDL	High-density lipoproteins
HER2	Human epidermal growth factor receptor 2
HFD	High-fat diet
HMG-CoA	3-hydroxy-3-methyl-glutaryl-coenzyme A
HMGCR	HMG-CoA reductase
HMGCS1	HMG-CoA synthase 1
HMW	High molecular weight
HPG axis	Hypothalamic-pituitary-gonadal axis
HRas	Harvey rat sarcoma viral oncogene homolog\
HS	Horse serum
HSPG	Heparan-sulfate proteoglycans
HTS	High-throughput screen
HuFTF	Human fat tissue filtrate
IARC	International Agency for Research on Cancer
ICAM-1	Intracellular adhesion molecule
IBET	I-BET-762
IDT	Integrated developmental technologies
iFGFR1	Inducible fibroblast growth factor receptor 1
IGF	Insulin-like growth factor
IGF1	Insulin-like growth factor 1
IGF1R	Insulin-like growth factor receptor 1
IGF2R	Insulin-like growth factor receptor 2
IGF3R	Insulin-like growth factor receptor 3
IGFBP	Insulin-like binding protein
IgI-III	Ig-like domains I-III
IHC	Immunohistochemistry

IL6	Interleukin 6
IPP	Isopentenyl pyrophosphate
IR	Insulin receptor
JAK/STAT	Janus kinase/Signal transducer and activators of transcription
JNK	c-Jun N-terminal kinase
KRas	Kirsten rat sarcoma 2 viral oncogene homolog
LDL	Low-density lipoprotein
LFD	Low-fat diet
LMW	Low molecular weight
MAPK	Mitogen-activated protein kinase
MAT	Mammary adipose tissue
MCP	Monocyte chemoattractant
MC4R	Melanocortin-4-receptor gene
MEK	Mitogen-activated protein kinase
MEM	Minimum essential media
MeOH	Methanol
MetS	Metabolic syndrome
MFI	Median fluorescence intensity
MFTF	Mouse fat tissue filtrate
MM	Multiple myeloma
MMP9	Matrix metalloproteinase 9
MmC	Mitomycin C
MSU	Michigan State University
MVL	Mevalonate
NCD	Non-communicable disease
NCI	National Cancer Institute
NFDM	Non-fat dairy milk
NFkB	Nuclear kappa-light chain enhancer of activated B cells

NMU	N-methyl-N-nitrosourea
NRas	Neuroblastoma RAS viral (v-ras) oncogene homolog
NVP	NVP-AWD742
Ob-R	Leptin Receptor
P/s	Penicillin/streptomycin
PAI-1	Plasminogen activator inhibitor-1
PD	PD166866
PI3K	Phosphoinositide 3-kinase
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PIP_3	Phosphatidylinositol (3,4,5)-trisphosphate
POMC	Proopiomelanocortin
PPP	Picropodophyllin
PR	Progesterone receptor
Prav	Pravastatin
PR+	Progesterone receptor positive
PR–	Progesterone receptor negative
PTEN	Phosphatase and tensin homolog
RNA	Ribonucleic acid
Rosu	Rosuvastatin
RTK	Receptor tyrosine kinase
RT-PCR	Reverse transcription polymerase chain reaction
SAT	Subcutaneous adipose tissue
SERM	Selective estrogen receptor modulator
SHP2	Src homology region 2-containing protein kinas phosphate 2
SHUB	Spectrum Health Universal Biorepository
Sim	Simvastatin
SNP	Single nucleotide polymorphism
SREBP	Sterol regulatory element binding proteins

SREBF2	Sterol regulatory element binding protein 2
TBST	1X tris-buffered saline, 0.1% triton X
TLR4	Toll-like receptor 4
TNBC	Triple negative breast cancer
UVB	Ultra-violet radiation B
VAT	Visceral adipose tissue
WHO	World Health Organization
WHR	Waist-to-hip ratio

CHAPTER 1

The complex relationship between obesity and estrogen receptor negative breast cancer risk and the immediate need for novel prevention strategies

Overview

In this chapter, I will discuss the epidemiology evaluating the complex relationship between obesity and cancer with a focus on pre-menopausal estrogen receptor (ER) negative (ER–) breast cancer. Next, I will address why the molecular mechanisms of obesity-associated cancers continue to elude modern science. Furthermore, I will explain how understanding the driving molecular mechanisms of obesity associated ER– breast cancer will reveal biomarkers for at-risk individuals and identify targets for prevention. I will discuss preliminary data and current literature that suggests FGF2/FGFR1 signaling is critical in transformation of ER– breast epithelial cells. Next, I will explain how the drug discovery process using a target based (FGF2/FGFR1) phenotypic screening can help elucidate the complex mechanisms of obesity associated ER– breast cancer. Because the developed HTS identified fluvastatin as the lead candidate, I will discuss the current literature and what is known of the relationship between statins and cancer.

The Obesity Cancer Association

What is Obesity?

Obesity is an emerging disease of global epidemic proportions as it coincides with surges in heart-disease, cancer, and diabetes creating a heavy burden for the health care system. The Center for Disease Control and Prevention (CDC) defines being over overweight or obese as having a weight that is higher than what is considered healthy for a given height[1], defined by body mass index (BMI). BMI is used as a screening tool that determines if a person is underweight, normal, overweight, or obese (Table 1.1)[1,2]. Using

Table 1.1. BMI and weight class

Class	**BMI** (kg/m^2)
Underweight	<18.5
Normal	18.5 – 24.9
Overweight	25.0 – 29.9
Obese	
Class 1	30.0 - 34.9
Class 2	35.0 - 39.9
Class 3	>40.0

BMI to determine health is controversial because it does not consider body stature, muscle mass, or adiposity distribution, making BMI a crude measure of fat mass. While BMI is a reliable screening tool in evaluating an individual's health, it is not diagnostic of fat mass or health. Measures of abdominal fat mass are better indicators for determining health risks.

Obesity: a public health concern

The obesity epidemic emerged within the first two decades of the 21st century. First, the early 20th century saw people in developed countries gaining proportionally more weight than height (increasing the average BMI). While this did not capture the attention of the media or general public for a few decades, it was the start of a burgeoning obesity epidemic. Obesity progressively increased in industrialized counties so much so that the year 2000 was a historical landmark, as for the first time in human evolution, the number of adults with excess weight surpassed those who were underweight[3].

Obesity is a major public health concern since it contributes to many chronic diseases including metabolic syndrome, insulin resistance, type 2 diabetes mellitus[4], cardiovascular disease[4,5], and cancer[4-11]. Rising obesity rates coincide with increases in obesity-related diseases that take an expensive toll on the health care system. From 2005 to 2010 the health care costs of obesity in adults rose from $212.4 billion to $315.8 billion in the United States, a 48.7% increase[12]. According the National Center for Health Statistics (NCHS) brief reports, United States obesity reports rose ~1.5% from 2005-2010[13]. Therefore, a 1.5% increase in obesity rates translated to a $100 billion dollar increase in health care costs. This cost increase during a five year period is a culmination of increase in population, increase in cost per individual, and the increase in obesity prevalence[12]. Therefore, obesity has drastic negative health consequences on society and places a large economic burden on the health care system.

Excess adiposity is considered a global non-communicable disease (NCD) target, and the World Health Organization (WHO) Global Action Plan has a defined goal to halt rising obesity rates by 2025[14,15]. Obesity rates are continuing to increase and obesity is now a pandemic[16]. In 2016, the NCD Risk Factor Collaboration performed the longest, most complete analysis of body weight trends from 1975 to 2014[16]. They accessed 1698 population-based data sources that included more than 19.2 million participants. In this study they showed rising obesity rates have slowed in high-income countries, while rates have

increased in other parts of the world, meaning the global obesity rate has not slowed down[16]. Since 1980, the prevalence of global obesity has more than doubled and is at 12% in adults[17]. Furthermore, if present trends continue, by 2025, global obesity prevalence will reach 18% in men and surpass 21% in women[16].

Obesity in the United States is even higher as 42.4% of adults were considered obese (2017-2018)[13]. Interestingly, obesity rates vary among different ethnicities. Collectively, African Americans have the highest obesity rates at 49.6%, followed by Hispanics at 44.8%, then Caucasians at 42.7%. Obesity rates are similar among men of different ethnicities but vary among women of different ethnicities. Hispanic men have the highest obesity rates at 45.7%, followed by Caucasian men at 44.7%, then African American men at 41.4%. African American women experience the highest obesity rates at 56.9%, followed by Hispanic women at 43.7%, then Caucasian women at 39.8%. However, age has less of an impact on obesity in women. The prevalence of obesity in women aged 20-39, 40-59, and 60+ is 39.7%, 43.3%, and 43.3% respectively[13]. Overall, obesity is serious health risk with pandemic proportions and needs swift effective strategies to prevent or reduce obesity related diseases.

Obesity-associated adipose tissue alterations

Adipose tissue, previously thought to be a passive energy store, is a complex and highly active endocrine organ. Adipose produces hormones and an array of cytokines called adipokines[18,19]. For example, adipose tissue produces growth factors, pro- and anti-inflammatory cytokines, and estrogen. Adipose tissue is composed of adipocytes, pre-adipocytes, immune cells, stromovascular cells, connective tissue matrix, and nerve tissue which forms an interactive network. This network is capable of releasing factors in a paracrine and/or endocrine manner that communicates/acts with local and distant sites[18]. Cohesively, this array of cell types and their adipokine production and secretion are central to understanding the metabolic abnormalities associated with the development of obesity[20].

Adipose tissue depots exhibit unique adipokine expression, secretion profiles, and receptor expression densities[18,21,22]. Adipose tissue is distributed into two main depots, subcutaneous adipose tissue (SAT) and visceral adipose tissue (VAT). SAT is found below the skin, whereas VAT surrounds the intra-abdominal organs (e.g. omental, mesenteric, retroperitoneal, gonadal, and pericardial)[22]. For example, VAT adipocytes are more sensitive to catecholamine induced lipolysis and less sensitive to lipolysis inhibition

than SAT adipocytes[23-25]. Triacylglycerol lipolysis in VAT adipocytes produce and release more glycerol and free fatty acids into the body than SAT adipocytes[23,24]. Additionally, VAT and SAT differentially produce and secrete adipokines[21] and exhibit regional differences in receptor densities. Insulin upregulates adiponectin secretion from visceral adipocytes and has no effect on subcutaneous adipocytes[26]. Whereas leptin is primarily secreted from SAT[23]. This demonstrates that VAT and SAT are functionally different. Quantitatively, VAT accounts for 6% of total adipose tissue, but is biologically very active. Over the BMI range of 22 to 36, VAT increased by ~400% and SAT increased by ~100% [(fat mass at 36 BMI – Fat mass at 22 BMI / fat mass at 22) x 100][23,27]. Individuals can gain weight in either or both depots but increases in VAT is associated with obesity-related diseases.

Obesity altered adipokine profile

As the percentage of VAT increases, VAT undergoes more functional and structural changes than SAT, including more adipocyte hypertrophy. When adipocytes become hypertrophic, they become dysfunctional that can cause changes that have whole body effects as the adipokine profile can affect processes at distant sites. Firstly, circulating levels of leptin is directly related to adipose tissue mass as adipose tissue in obese individuals as they produce and secrete higher leptin concentrations levels compared with normal-weight individuals[28]. Whereas, the adipokine adiponectin is downregulated in obese individuals. In addition, BMI is positively associated with increased aromatase expression and estrogen levels[29,30]. Furthermore, increases in VAT was positively associated with increased plasma levels of c-reactive protein (CRP), intracellular adhesion molecule 1 (ICAM-1), monocyte chemoattractant protein (MCP), matrix metalloproteinase 9 (MMP9), and plasminogen activator inhibitor-1 (PAI-1), whereas increases in SAT was only positively associated with increased plasma levels of CRP[21]. Additionally, adipocytes contain aromatase, a key enzyme in estrogen biosynthesis, but in normal, pre-menopausal women, ovarian granulosa cells are the main estrogen source with a small fraction of estrogen being produced from other tissues. In obese premenopausal women, estrogens are predominantly produced in adipose tissue[31]. Additionally, excess VAT also exhibits low-grade chronic inflammation and produces increased inflammatory cytokines like interleukin 6 (IL6). These obesity related changes are theorized to be central to the underlying molecular mechanisms of obesity-related diseases.

Obesity-associated inflammation

Obesity results in low-grade, chronic inflammation in adipose tissue[32,33]. When adipocytes enlarge and increase in number, this can create a hypoxic environment and not all adipocytes have access to nutrients and/or oxygen. Cell death then causes the number of immune cells to increase within the tissue to phagocytize the debris, which results in low-grade chronic inflammation. In excess, VAT experiences changes in immune cell infiltration, macrophages being the major innate recruited immune cells and are the major source of inflammatory cytokines[34]. Functionally, macrophages phagocytize debris from adipocytes that underwent hypoxia-induced adipocyte cell death. Adipocyte cell death releases free fatty acids (FFAs) that act as toll-like receptor 4 (TLR4) agonists and this further induces an inflammatory response and enhances macrophage recruitment[35]. Furthermore, the macrophage phenotype ratio switches. M2-like phenotype is an anti-inflammatory form and the M1-like phenotype is a more pro-inflammatory form and in obese adipose tissue, the macrophage phenotype shifts to be predominantly M1. Additionally, the low-low grade inflammation is promoted by Leptin. Leptin is pro-inflammatory whereas its counterpart adiponectin is anti-inflammatory, and increased leptin production leads to increased macrophage production by monocyte differentiation[33]. Macrophages are phagocytic leukocytes that are part of the innate immunological response, the body's nonspecific defense mechanism, and obesity also impacts the adaptive immunological response[33]. This is a small snapshot of the long-recognized relationship between obesity, cancer and inflammation and definitively identifying inflammation as the causal mechanism has yet to be determined. Overall, these obesity-associated VAT changes are suspected to promote the etiology of obesity-related diseases.

Combating Obesity

Reducing obesity rates would prevent the occurrence of obesity related diseases, however, this is an ineffective strategy as combating obesity is more complicated then limiting caloric intake. Obesity can be caused by an interplay between behavior, environment, and genetics. An unhealthy diet was recognized as a risk factor for colon and breast cancer by the Dietary Guidelines Advisory Committee (DGAC) in 2015[36,37]. Diet and cancer risk is still a relatively young field and current studies have significant inter-study heterogeneity[37,38]. It is difficult to holistically investigate dietary patterns. Additionally, for some people, a

healthy lifestyle is an unattainable privilege as low socio-economic status is disproportionately associated with obesity[39]. This includes physical activities such as going for a run outside or a walk in the park as these communities are not safe environments. Additionally, healthy eating is notoriously expensive compared to 'junk food' and certain people have limited access to affordable nutritious food and only have corner stores, stocked with predominantly high-calorie food, as their main food source[40]. Furthermore, genetics can predispose people to obesity. For example, the fat mass and obesity-associated gene (FTO) has variants associated with increased obesity rates and is found in up to 43% of the population[41,42]. Despite this complicated interplay, proper diet and exercise reverse the risk for obesity-associated diseases regardless of weight loss. However, poor compliance makes this an ineffective strategy to combat obesity, leaving this disease with a dire need for effective prevention strategies.

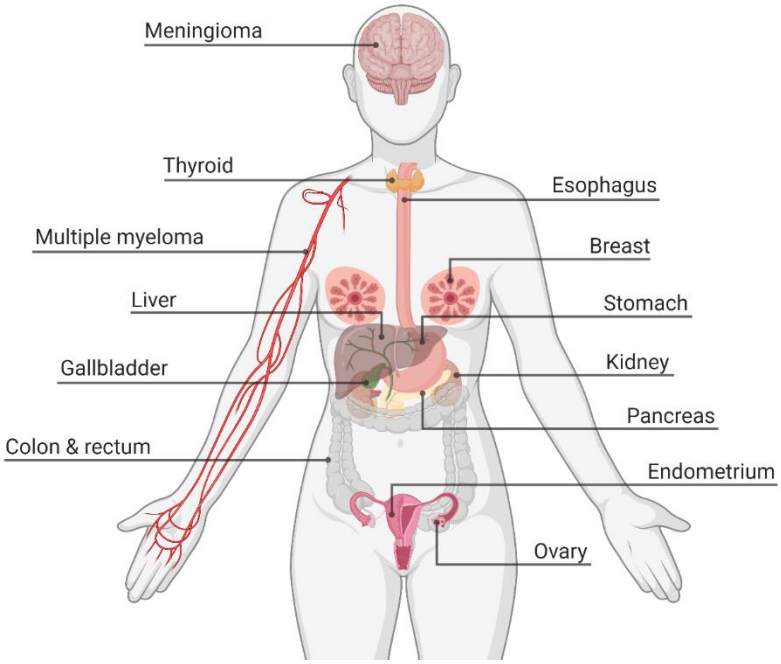

Figure 1.1. Obesity is linked to 13 different types of cancer. Obesity increases the risk of meningioma (cancer in the tissue covering the brain and spinal cord), thyroid cancer, multiple myeloma (cancer of the blood), liver cancer, gallbladder cancer, colon cancer, esophageal cancer (adenocarcinoma), breast cancer (post-menopausal), stomach cancer, kidney cancer, pancreatic cancer, endometrial cancer, and ovarian cancer. Image was adapted from Centers for Disease Control & Prevention. Image made in BioRender.

Obesity and Cancer

From 1959-1972, the American Cancer Society performed a long-term prospective study, the first large-scale study of mortality according to weight[43]. Here, they evaluated 750,000 men and women and found obese individuals had distinctly elevated mortality for colon and rectum cancer and breast, uterus, and endometrium cancer[43]. The magnitude of the obesity/cancer relationship was revealed in 2003 when the New England Journal of Medicine conducted a study evaluating almost a million U.S. adults demonstrating significant trends of increasing risk with eight cancer types[44]. Today, the epidemiological studies have linked excess weight to the increased risk of 13 different cancer types including breast cancer (Figure 1.2)[2,45,46]. Interestingly, obesity is differently associated to each cancer type. These 13 cancer types account for 40% of all U.S. cancers diagnosed[47]. These statistics highlight the drastic effects obesity has on society's cancer burden.

Breast cancer: defining triple negative breast cancer

Breast cancer is the most frequently diagnosed cancer in women, affecting 1 in 8 women, after skin cancer, with around 1.3 million cases worldwide. Breast cancer is categorized based on differential expression of ER, progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2). Breast cancers are classified as either ER-positive (ER+) or PR-positive (PR+), HER2 overexpressing, or triple negative. Triple negative breast cancer (TNBC) is characterized as ER–, PR-negative (PR–) with no HER2 overexpression. Approximately 10% to 20% of all breast cancers are triple negative[48]. TNBC prognosis is very poor compared to non-TNBC types because it is a more aggressive breast cancer subtype with no effective targeted therapy. Furthermore, the oncogenic drivers, the mutations responsible for cancer initiation and maintenance of TNBC are not understood[48]. Identifying the molecular mechanisms of TNBC tumorigenesis will inform as to the aberrant signaling mechanisms that arise in TNBC and can be used to develop prevention strategies and targeted therapies. This illustrates several critical deficits in the prevention and treatment of TNBC because of its aggressive nature and poor prognosis.

TNBC is hard to treat and does not have any clinically approved prevention strategies. While, selective ER modulators (SERMs), like tamoxifen effectively prevent ER+ breast cancer regardless of menopausal status[46], there are no clinically approved chemopreventive compounds for ER– breast cancer. ER– breast

cancer is often slated under TNBC[48,49]. TNBC is a heterogenous breast cancer subtype, therefore a one-strategy fits all is highly unlikely, further emphasizing an unmet need of ER-independent chemopreventive strategies[50]. Preventing ER– tumorigenesis has the potential to significantly reduce breast cancer burden[51].

Obesity and estrogen receptor negative breast cancer

The relationship between obesity and breast cancer is complicated and controversial. Initial studies demonstrated obesity increased the risk of post-menopausal breast cancer and had an inverse relationship for premenopausal breast cancer, meaning obesity has a protective effect against premenopausal breast cancer. Unsurprisingly, obesity is officially recognized as a risk factor for post-menopausal breast cancer, but not premenopausal breast cancer. However, more current studies undermine the previously generalized protective effect of pre-menopausal obesity. Pre- and post-menopausal women exhibit significant biological differences in breast cancer clinical expression. Stratifying breast cancer according to hormone receptor statuses revealed that obesity increases the risk of postmenopausal ER+ breast cancer and premenopausal ER– breast cancer. In postmenopausal women, obesity increases the risk of ER+ breast cancer and an inverse/no relationship with ER– breast cancer[52]. Conversely, in premenopausal women, obesity increases the risk of ER– breast cancer and has no association with ER+ breast cancer[52]. In 2013, a meta-analysis demonstrated premenopausal obese women (BMI > 30 kg/m^2) have a 42% higher risk of developing TNBC compared with premenopausal non-obese women. This study also revealed no significant association between obesity and TNBC for postmenopausal women[53]. Since 2013, at least seven studies have further supported BMI (≥30 kg/m^2) is associated with increased incidence of ER– breast cancer in premenopausal patients[54-60]. Another pooled analysis from 2011 found obesity (BMI ≥ 30 kg/m^2) was more frequent in case patients with ER–/PR– tumors compared with other hormone receptor expression combinations[61]. Overall, menopausal status is a significant factor in breast cancers clinical expression, and epidemiology stratification by breast cancer subtype supports obesity as a risk factor for premenopausal ER– breast cancer[53-61].

Studies that focus on VAT mass instead of BMI demonstrate stronger associations with pre-menopausal ER– breast cancer and not ER+ breast cancer. Computed tomography (CT) and waist-to-hip ratio (WHR), a measure of VAT, found generally higher abdominal VAT distributions was associated with

ER– breast cancer, but not with ER+ breast cancer[62,63]. This suggests that VAT and not SAT promote ER– breast cancer in pre-menopausal women.

Despite ample evidence supporting visceral obesity as a risk factor for ER– breast cancer in pre-menopausal women, the underlying molecular mechanisms have not been clearly elucidated. Therefore, the objective of this thesis is to determine the mechanism of visceral obesity driven ER– breast cancer in order to find new targets for prevention.

Molecular mechanisms of obesity-associated cancer

Linking the epidemiology of obesity and cancer risk with a molecular, biological basis has proved difficult. The etiology of obesity associated cancer is hypothesized to involve growth factors, inflammation, and/or hormones. However, obesity is a complex multifactorial pathophysiology and these candidate systems often overlap making it unlikely that the obesity and cancer risk etiology is attributed to a "one system fits all" mechanism which makes elucidating the exact biological mechanisms difficult. While, obesity-mediated alterations in adipose endocrine functions are hypothesized to be mechanistically involved in obesity associated tumorigenesis, the exact molecular mechanisms remain unclear.

Modeling transformation

In vitro assays are necessary for experimental analysis of cancer development on cellular and molecular levels because they offer insights that are often unattainable in whole animal studies[64]. Cancer development is a multistep process that begins with initiation[50]. One model of carcinogenesis involves initiation, promotion, and progression. Initiation is caused by an initiating agent such as a carcinogen that induces an irreversible mutation in gene(s) that control proliferation, survival, or apoptosis that leads to abnormal cell growth. The next step is promotion by which the initiated cell proliferates. The last step is progression that results from continued proliferation and accumulation of further mutations that have invasive and metastatic potential[50]. To model cancer development, researchers turn to *in vitro* assays that detect cell transformation, a process that describes the conversion of normal (non-tumorigenic) cells to transformed (tumorigenic) cells in cell culture. *In vitro* transformation assays are easier to quantitate and control than tumor induction in an *in vivo* model[64].

Transformation, the steps a normal cell undergoes to become malignant, is modeled *in vitro* by the soft agar assay that measures anchorage-independent proliferation. For normal cells, proliferation is inhibited by cell-cell contact or deprivation of a solid substrate for cells to anchor to. When cells transform and become tumorigenic, they lose cell-cell contact inhibition and no longer require anchorage-dependence to proliferate, a hallmark of transformed cells. Therefore, suspending cells in agar selects for cells that can only undergo anchorage-independent growth. Normal cells will remain as single cells suspended in agar as they are not anchored to a solid substrate and transformed cells will proliferate forming colonies. These colonies can be counted and used as a measure for transformation. Soft agar is considered the gold standard of transformation because the ability of cells to form colonies in agar directly correlates to the tumorigenicity of these cells in a mouse xenograft model. This signifies that *in vitro* cell transformation is a valid indicator of cancer development *in vivo*[65].

Overall, current literature lacks a fundamental understanding of the exact molecular mechanisms responsible for breast cancer development. It is difficult to define the sequential processes that cause a normal cell to lose anchorage-dependence because this can be achieved by different systems. Anchorage-dependent growth is regulated by dynamic cellular interactions between cells and extracellular matrix (ECM). When normal cells are not attached to a supportive matrix, cells undergo a specific type of apoptosis called anoikis[66]. Anoikis is important for physiological function that removes displaced cells, maintains tissue homeostasis, and prevents disease. Cells achieve matrix anchorage through mechanisms including ECM components and cell adhesion molecules. Conversely, changes in these mechanisms can be hijacked inducing changes that lead a cell to lose anchorage dependence. It is unknown if or how ECM-cell interactions contribute to or promote transformation.

Receptor tyrosine kinase dysregulation in triple negative breast cancer

Dysregulation of several receptor activated signaling pathways can lead to breast cancer development. One such family of receptors is Receptor Tyrosine Kinases (RTKs) and they bind growth factors, making RTKs a potential target for obesity associated ER– breast cancer prevention[67]. RTKs are cell surface receptors that mediate a range of biological functions, including cell proliferation, migration, survival, and differentiation. Cancer initiation is the first step in carcinogenesis, which is a multistep process. Cancer

initiation involves an irreversible mutation that causes alterations in cell proliferation, survival, and differentiation that allows the cells to evade apoptosis, expand within normal tissue without constrains on cell-cell contact. Not surprisingly, mutations that affect RTK signaling often lead to cell transformation, which is observed in a wide variety of malignancies[68,69]. Therefore, as RTKs regulate these relevant biological processes, many RTKs are known oncogenes and suspected to be involved in tumorigenesis and progression.

RTK signaling pathways are frequently dysregulated in TNBC patients[67]. RTKs activates multiple signal transduction pathways: the phosphoinositide 3-kinase (PI3K)/Akt, Ras/mitogen-activated protein kinase (MAPK) pathway, and janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway and other pathways (Figure 1.2). Targeting the Ras/MAPK pathway with selumetinib, a MEK inhibitor, *in vitro* inhibits the invasiveness and motility of TNBC cell lines. Selumetinib also prevented lung metastasis in TNBC-bearing mouse xenograft model[67]. Regarding the PI3K/Akt pathway, RTKs activate PI3K which phosphorylates phosphatidylinositol-4,5-diphosphate (PIP_2) to phosphatidylinositol-4,5-triphosphate (PIP_3), which enables Akt phosphorylation that results in protein synthesis and cell growth[67]. The PI3K/Akt pathways is frequently dysregulated in TNBC. Approximately 24% of TNBC tumors have activating PI3K mutations, and 25-30% of TNBC cases have loss of function mutations in Phosphatase and tensin homolog (PTEN), a tumor suppressor that dephosphorylates PIP_3 to PIP_2[67]. PTEN also negatively regulates the Ras/MAPK pathway, making PTEN one example of the high level of crosstalk occurring between Ras/MAPK and PI3K/Akt pathways that has been detected in basal-like breast cancer models. This biochemical multiplicity limits the efficacy and applicability of RTK inhibitors for TNBC[67].

RTKs consist of several subfamilies of receptors including but not limited to epidermal growth factor receptors (EGFRs), fibroblast growth factor receptors (FGFRs), and insulin and insulin-like growth factor receptors (IR and IGFR respectively)[70]. RTK activity is tightly regulated under normal physiologic conditions. However, RTKs can undergo potential mutations/alterations that induces the constitutive RTK activation seen in cancer: amplification, gain-of-function mutations, chromosomal rearrangements, and/or autocrine/constitutive activation[68]. For example, overexpression (a result of gene amplification) of RTKs, such as FGFRs, are found in breast cancer[68,70].

FGF2/FGFR1 in triple negative breast cancer

FGFRs are a subclass of RTKs that when bound to their endogenous ligands, serve multiple functions and dysregulation of FGF/FGFR signaling can lead to cancer formation[70]. The FGF family consists of 22 FGF ligands. The first two FGF ligands were isolated according to pH and received the names FGF acidic (FGFa) and FGF basic (FGFb). When more FGF ligands were discovered FGFa and FGFb were renamed FGF1 and FGF2 respectively. The FGFR family has four FGFR receptors (FGFR1-4). RTKS, including FGFR1-4, typically have an extracellular ligand binding domain with three Ig-like domains (IgI-III), a transmembrane domain, and an intracellular tyrosine kinase domain. However, due to alternative splicing, FGFR1-3 has multiple isoforms with varying FGF ligand specificity[71]. Receptor activation requires two compulsory events, ligand binding then receptor dimerization. Without cognate ligands, RTKs remain inactive by autoinhibitory mechanisms[70]. Ligands, like those from the FGF family, bind to FGFRs, leading to dimerization and subsequent activation of the intracellular kinase domain by phosphorylation and activation of the aforementioned downstream signaling pathways[70].

FGF2/FGFR1 signaling is associated with TNBC progression and may be involved in TNBC tumorigenesis. First, FGF signaling is essential for mammary gland formation; specifically FGF2 regulates ductal elongation in the mammary gland, through processes like cell proliferation and epithelial expansion[72,73]. In a breast cancer mouse model, tumors of obese mice had increased expression of FGF2 compared with tumors from lean mice[74,75]. In addition, 62% of basal-like breast cancers expressed FGF2 compared with only 5% of non-basal-like breast cancers and basal-like breast cancer cell lines also express FGF2[71]. While these cases are not exclusively triple negative, approximately 80% of TNBC are basal-like, therefore, these two terms describe similar cancer forms[48]. Another study demonstrated FGFR1 expression was upregulated in TNBC cells, and TNBC cell lines were highly sensitive to FGFR inhibition in anchorage-independent conditions[48,76]. As FGF2 and FGFR1 is induced in breast cancer, FGF2/FGFR1 signaling could promote tumorigenesis.

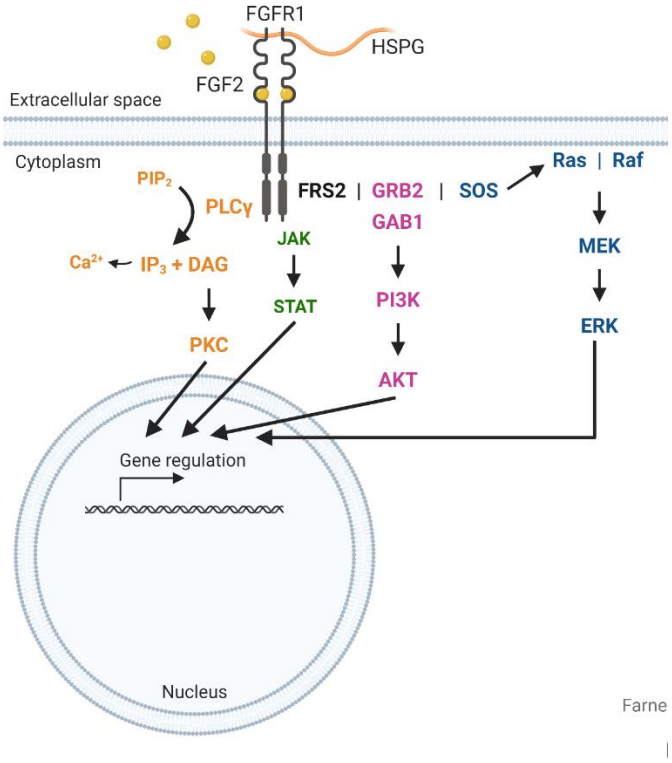

Figure 1.2. Signaling pathways activated by RTKs. FGF2 is an endogenous ligand for FGFR1, an RTK. RTK's activates the PLCγ/PKC (orange), the JAK/STAT pathway (green), the PI3K/Akt pathway (fuchsia), and the Ras/MAPK pathway (blue). Image made in BioRender.
Abbreviations: FRS2- fibroblast growth factor receptor substrate 2; GRB2- growth factor receptor-bound protein 2; GAB1- GRB2 associated binding protein; SOS- son of sevenless; DAG- diacylglycerol; CA^{2+}- calcium; IP_3- inositol triphosphate.

FGF2 in obesity

VAT produces FGF2 and in obesity, FGF2 production and secretion is significantly increased. Epidemiology studies have demonstrated FGF2 plasma and serum levels increase with high BMI and increased fat mass respectively[77,78]. *In vivo*, high-fat diet (HFD)-fed mice had elevated FGF2 levels in VAT and serum compared to low-fat diet (LFD)-fed mice. Additionally, when HFD-fed mice underwent surgical lipectomy of the parametrial fat pad (the largest VAT depository in mice), serum FGF2 levels decreased to the FGF2 serum levels seen in LFD-fed mice[79]. This suggests that excess VAT is a main source for elevated circulating FGF2 levels. Physiologically, FGF2 exhibits a concentration dependent biphasic effect on adipogenesis. Low concentrations (2 ng/mL or lower) of FGF2 enhanced adipogenic expression whereas high concentrations (10 ng/mL or higher) suppressed adipogenesis[80]. As FGF2 has a physiological role in VAT and mammary gland development, FGF2 could link obesity with cancer.

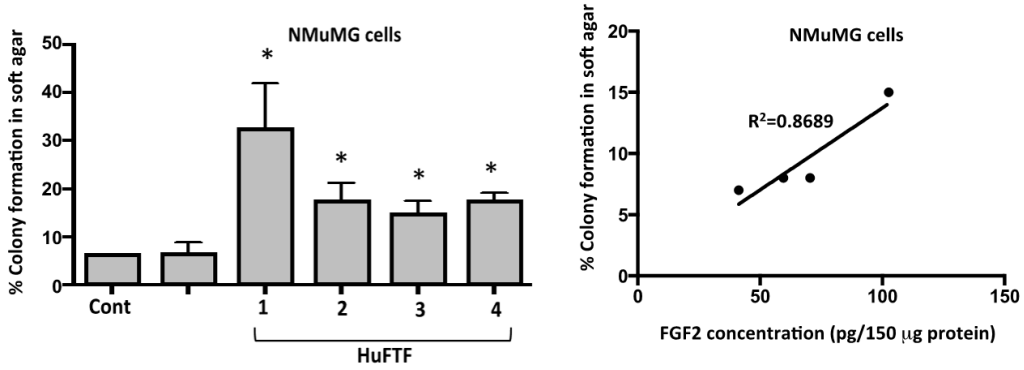

Figure 1.3. Transforming activity of HuFTF is associated with FGF2 levels. Fat tissue filtrates were made from human visceral adipose tissue (HuFTF) from four donors undergoing hysterectomy. (a) Percentage of clones growing in soft agar (% colony formation) significantly increases in JB6 P+ and NMuMG cells cultured with HuFTF from donors 1, 2 and 4 compared no treatment (control;Cont). (b) HuFTF with higher concentrations of FGF2 are more potent at stimulating cell transformation[79].

FGF2/FGFR1 in Transformation

FGF2/FGFR1 signaling is potentially a critical signaling mechanism between obesity and TNBC. Animal models have shown that VAT promotes tumorigenesis. One study demonstrated that surgical removal of intra-abdominal VAT (lipectomy) in a rat model of HFD-promoted intestinal cancer significantly reduced obesity-associated tumorigenesis[81]. Additionally, another study used this concept with HFD-fed mice, the parametrial fat pads were either lipectomized or the mice underwent a sham-operation, and both groups were irradiated with ultra-violet radiation (UVB). Compared with the sham operated mice, the lipectomized mice had significantly reduced squamous cell carcinomas by 75-80%, suggesting VAT in HFD-fed mice promoted skin tumorigenesis[82]. Our previous data investigated how HFD-VAT promoted skin tumorigenesis. First, we showed FGF2 was elevated in the VAT and serum of HFD-fed mice compared to LFD fed mice. Next, they demonstrated factors from VAT (made into a fat tissue filtrate (FTF)) stimulated transformation of mouse mammary epithelial (NMuMG) cells and skin epidermal (JB6 P^+) cells as determined by the soft agar assay[79]. The ability of FTF to transform NMuMG and JB6 P^+ cells was associated with the concentration of FGF2 in the FTF. Furthermore, knocking out FGFR1 in JB6 P^+ cells prevented FTF from stimulating transformation[79]. Additionally, exogenous FGF2, subcutaneously injected, stimulated JB6 P^+ cells to form tumors in an immunocompromised mouse xenograft model[79]. These studies identified FGF2/FGFR1 as a critical signaling pathway in VAT stimulated transformation of skin epidermal cells. Because FGF2/FGFR1 is characterized in ER– breast cancer, we hypothesize that FGF2/FGFR1 is a critical mechanism in VAT-associated ER– breast epithelial cell transformation and is a potential target for chemoprevention strategies (Figure 1.4).

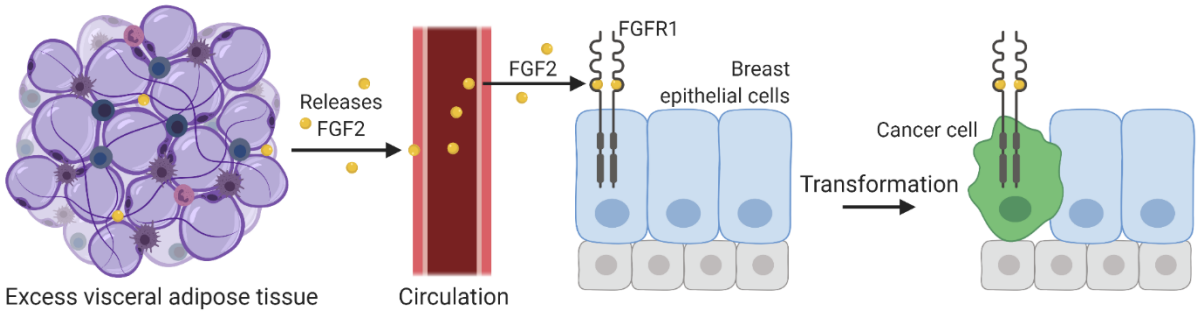

Figure 1.4. FGF2/FGFR1 is a critical mechanism in VAT-associated, ER independent, epithelial cell transformation. Excess visceral adipose tissue produces and secretes FGF2 into the circulation. FGF2 activates FGFR1 on breast epithelial cells to stimulate transformation. Image made in BioRender.

Prevention of obesity driven cancer

Chemoprevention: success and failures

Cancer chemoprevention uses agents that prevent cancer development and can significantly reduce cancer burden. For chemopreventive compounds that target ER– breast cancer to be successful, potential compounds must uphold high standards in order to successfully 1) reduce cancer cases that 2) fosters consumer compliance in 3) high-risk individuals 4) that has a tolerable safety profile. First, to reduce cancer cases, effective chemopreventive agents prevent cancer formation by either eliminating premalignant cells or by protecting normal cells from undergoing malignant transformation[83,84]. Second, chemopreventive compounds must be well tolerated with minor to no side effects. Many chemotherapeutics have significant adverse side effects. Chemoprevention has different standards because it entails women taking a drug for a prolonged period of time to reduce the possibility cancer might develop. Therefore, the presence of side effects can severely impact consumer compliance and safety. Tamoxifen is backed by strong data showing it does prevent ER+ breast cancer, but has significant adverse side effects including stroke, blood clots, and endometrial cancer and a long list of common side effects[46]. As a result, tamoxifen compliance is less than optimal. Third, target populations should consist of high-risk individuals in order for cancer-preventive drugs to have the highest benefit[85]. A targeted population shifts the risk/benefit ratio that further encourages compliance. Lastly, chemopreventive compounds need minimal to no side effects because they are being administered in a healthy population of people. Side effects can negatively effect consumer compliance, lowering the effectiveness of the prevention strategy. Overall, pre-menopausal obese women with elevated circulating levels of FGF2 represent a high-risk population that would benefit from chemoprevention. In this scenario, FGF2 not only functions as a biomarker, but as a potential new target for obesity associated TNBC.

Chemoprevention drug discovery

The fundamental goal of chemoprevention drug discovery is to identify compounds that eliminate or prevent the normal cells from transforming while minimizing or preventing negative side effects on normal cells. This is most readily accomplished with high-throughput screens (HTS) that enables testing of compounds in a high-throughput efficient manner. Challenges faced with drug discovery culminate to the

paucity of novel targets and attrition of drugs in the pipeline due to insufficient clinical efficacy. This has spurred debates on the most effective drug discovery approaches which center on target-based or phenotypic based approaches. Target-based is self-descriptive and focuses on the target. In the context of FGF2/FGFR1, it would be identifying compounds that specifically target FGF2 or FGFR1. Phenotypic screens focus on a function or process and in this context would be targeting the transformation process. Both approaches have advantages and disadvantages. Target-based is mechanism informed, however, target inhibition can be negated by RTK compensatory crosstalk and redundancy that often prevents clinical efficacy. Phenotypic approaches are at risk of identifying non-selective compounds and promiscuous compounds are more likely to have off-target effects and in turn side effects. This is problematic for chemoprevention as these compounds need to be efficacious with minimal to no side effects[86].

Chemoprevention HTS

Utilizing high-throughput screening methods to identify chemopreventive compounds is an underdeveloped area and currently faces many challenges. Current chemoprevention assays predominantly use cancer cell lines in a 2D format, methodology that is not representative of chemoprevention. Cancer cells are morphologically and functionally dissimilar to non-tumorigenic cells and inducing cancer cell death is different than preventing a normal cell from transforming. Additionally, HTSs in a 2D format (cells that attach to the cell cultured treated plates that proliferate in a monolayer) does not inform on the process of transformation. Both transformed and non-transformed can proliferate in 2D culture conditions, giving no way to distinguish between the two cell varieties, and cell death does not mean the process of transformation is being inhibited or prevented. In contrast, 3D culture conditions (anchorage-independent growth) like those employed in the soft agar assay and ultra-low attachment conditions do distinguish between transformed and non-transformed cells. Furthermore, cell proliferation in 2D and 3D utilize different factors and signaling pathways. This is demonstrated by a study that demonstrated Erbβ4 (HER4) overexpression (by lentivirus transduction) transformed MCF-10A cells but did not induce proliferation of TNBC cell lines whereas Erbβ2 (HER2) did not transform MCF-10A cells but did stimulate proliferation of TNBC cell lines[87]. MCF-10A cells are non-tumorigenic human breast epithelial cells. Consequently, mechanisms of cancer cell proliferation are not reflective of mechanisms of transformation.

The limitations and challenges that come from using cancer cell lines in a 2D format have yet to be overcome with a novel HTS that models the process of transformation starting with non-transformed cells. Currently, no HTS assays models the transformation process. The soft agar assay is not suitable for HTS as scaling down the assay is not feasible, nor would that method be easily quantified. One study demonstrated that growth of transformed or non-transformed cells in ultra-low attachment conditions, correlated to growth in soft agar, in a high-throughput format (384-well). Therefore, this platform using 3D growth in ultra-low attachment conditions could be utilized to develop the first HTS to model the transformation process and enable screening of compound libraries to identify chemopreventive compounds against obesity-associated transformation of breast epithelial cells. This methodology enables the second part of this thesis's objective of identifying chemopreventive compounds against obesity associated ER– breast cancer.

Fluvastatin as a chemopreventive agent

The HTS methodology identified fluvastatin as the lead candidate for chemoprevention. Fluvastatin is a part of a large class of statins that inhibit 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase (HMGCR), the rate limiting step in the mevalonate pathway[88]. The mevalonate pathway begins with acetyl-CoA, the product of glycolysis. Two molecules of acetyl-CoA are combined by acetyl-CoA C-acetyltransferase (ACAT1) to make acetoacetyl-CoA. Here, acetoacetyl-CoA is condensed to form HMGCR by HMGCS1 and HMG-CoA is converted into mevalonate by HMGCR. Mevalonate is metabolized to isopentenyl pyrophosphate (IPP), an isoprenoid precursor to FPP and then GGPP by FDPS and GGPS1, respectively. FPP can also be converted to squalene by squalene synthesis (FDFT1) and squalene is the precursor of cholesterol (Figure 1.5)[89]. Additionally, FPP and GGPP are essential for prenylation, a series of posttranslational modifications that enable protein function through plasma membrane localization and anchoring molecules (Figure 1.6)[90,91]. Prenylation is a three-step process that leads to the addition of a prenyl moiety to a small GTP binding (GTPase) protein. There are two types of prenylation: farnesylation and geranylgeranylation. Farnesylated proteins include the Ras superfamily (K/N/HRas) and geranygeranylated proteins include RhoA/B/C, Rap1A, Rac-1, and Cdc42. The Ras GTPase superfamily is a well-recognized class of prenylated proteins. Protein prenylation is required for protein activity. When

H/NRas has been farnesylated and palmitoylated (attachment of fatty acids), Ras has a 100-fold higher affinity for the plasma membrane than farnesylated Ras. Plasma membrane localization places Ras within proximity of cell surface effector proteins where they can activate HRas and subsequent signaling (Figure 1.6). While this is a small snapshot of the mevalonate pathway, it plays a key role in cellular metabolism and homeostasis.

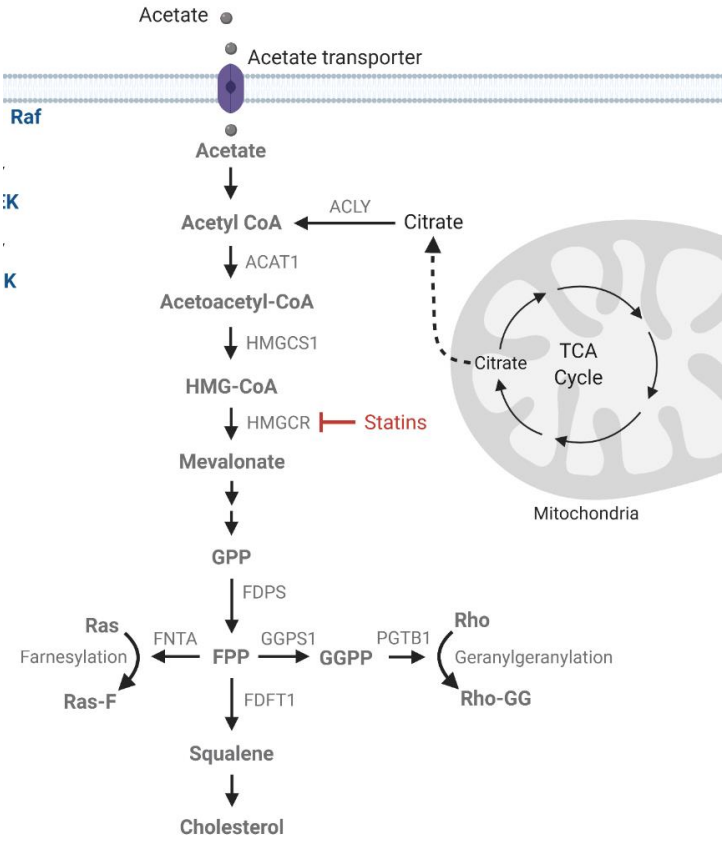

Figure 1.5. The mevalonate pathway. The mevalonate pathway begins with Acetyl CoA. Acetyl CoA can be derived from Acetate or can be absorbed through the cell or derived from citrate produced in the TCA cycle. Acetyl CoA and is converted to Acetoacetyl CoA by ACAT1. HMGCS1 converts Acetoacetyl CoA to HMG-CoA. Here, HMGCR produces mevalonate. Mevalonate produces intermediates FPP and then GGPP, isoprenoids that can undergo farnesylation and geranylgeranylation respectively. The FGF2 ligand binding to FGFR1 stimulated multiple signaling pathways including the Ras/MAPK pathway which regulates gene transcription.
Abbreviations: ACAT1- Acetyl-CoA Acetyltransferase 1; PGTB1- geranylgeranyl transferase type 1[89].

Statins are efficacious cholesterol lowering agents that are essential medical therapy in preventing heart attack and stroke. Statins are classified based on lipophilicity which reflects tissue selectivity. Lipophilic statins like fluvastatin, simvastatin, and atorvastatin penetrate hepatic and extrahepatic cells whereas hydrophilic statins like pravastatin and rosuvastatin have less absorption with non-hepatic cells. Regarding cholesterol-lowering and cardiovascular disease, different statin types have slightly different efficacies and there is currently no clinical standard for selecting statin type. Studies have shown similar effects and safety in patients with hydrophilic and lipophilic statins in coronary artery disease and acute coronary syndrome[92]. Statins also have a relatively good safety profile, making them ideal candidates for disease prevention.

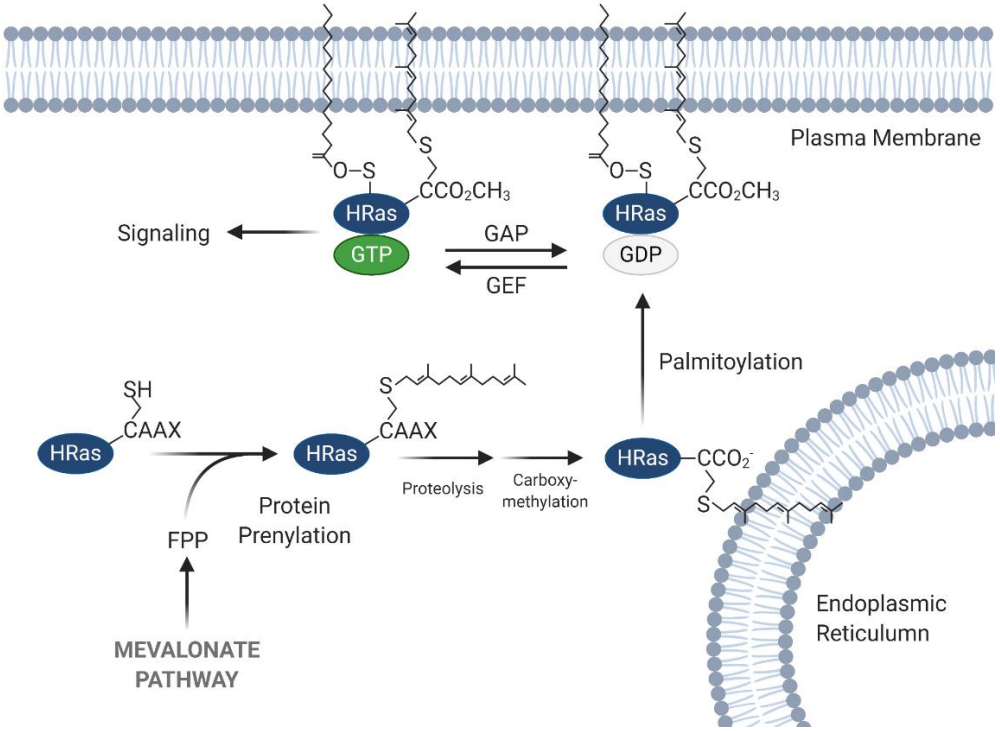

Figure 1.6. Prenylation of HRas enables plasma membrane localization and in turn activation. Prenylation is a three-step process that attaches the farnesyl moiety, proteolysis of the aaX residue, and carboxymethylation. Prenylated proteins like HRas and NRas then undergo palmitoylation then membrane localization. This puts HRas in proximity to cell surface receptors and adaptor proteins where they can be activated and participate is cell signaling pathways. Ras bound to GDP is inactive. GEF exchanges GDP for GTP and GAPs exchanges GTP for GDP. Active Ras with appropriate adaptor proteins.
Abbreviations: GEFs- guanine nucleotide exchange factor; GAPs- GTPase activating proteins; GTP- guanosine triphosphate; GDP- guanosine diphosphate.

The transformation HTS is not the first to associate the mevalonate pathway with tumorigenesis. The mevalonate pathway is often dysregulated in tumor cells in order to produce more cholesterol and accommodate higher energy demands[93]. Mevalonate pathway dysregulation can also promote tumorigenesis through protein prenylation. As Rho and Ras proteins are known oncogenes, the tumor suppressive effects of statins are likely from reduced prenylation of Ras and Rho, thus reduced oncogene activity[88]. However, the exact mechanisms of how mevalonate pathway dysregulation promotes tumorigenesis is unknown.

Statins and cancer

Investigating fluvastatin as a potential chemopreventive agent in ER– breast cancer poses many challenges as the epidemiological literature surrounding statins and cancer is highly heterogenous and inconsistent. Initial retrospective analyses investigating statins and cancer prevention demonstrated statins did not increase cancer risk nor decrease cancer risk. However, these initial studies were not designed to evaluate cancer risk but to evaluate cardiovascular outcomes[94]. When subsequent *in vitro* experiments indicated statins slow the growth of cancer cell growth[94], it revealed a need for further investigations to evaluate statins for chemoprevention.

Statins are investigated for chemopreventive properties as well as adjuvant chemotherapeutics. Today, meta-analyses show that adjuvant statin use is associated with modest reductions in cancer-related mortality[95]. There is still inter-study heterogeneity, but cumulative analyses show a reduction. For instance, a meta-analysis of 95 studies including, 18+ cancer types, found consistent evidence that statin use was associated with reduced cancer-specific mortality[96]. While the majority of these studies investigated prostate, breast, and colorectal cancer, this association was up held when stratified by cancer type[96]. Another more recent meta-analysis of 60 observational studies found similar findings regarding statin use and reduced cancer-specific mortality[95]. As a result, studies are assessing statins as adjuvant chemotherapeutics. Overall, this is still a burgeoning area of research and needs a better understanding of what tumor molecular characteristics equate to statin sensitivity for different cancer types.

In contrast, epidemiological studies investigating statin use with cancer prevention are overall inconsistent. This is a result of highly heterogeneous literature. Meta-analyses that evaluated the effect of

statin use and prostate cancer risk identified five studies where statin increased this risk, 10 studies that decreased the risk, and 18 studies that demonstrated there was no effect[97]. Literature on statin use and colorectal cancer predominantly describes no effect of statin use on cancer risk[98] whereas statin use was strongly associated with a lower risk of liver cancer[99]. Breast cancer literature is more challenging to investigate as statin chemoprevention is affected by statin type, tumor subtype and age. More research is needed to determine which cancers could benefit from statin chemoprevention including stratification by statin type (lipophilicity). Furthermore, a better understanding of how statins prevent tumorigenesis of different cancer types and would further inform on populations that could benefit from statin use.

Statins and breast cancer prevention

Statin chemoprevention efficacy on breast cancer risk is influenced by lipophilicity, cancer site, and hormone receptor expression. This presents many challenges as this requires study designs to have enough statistical power to evaluate these endpoints. Studies demonstrate that stratifying by breast cancer subtype and statin type suggest lipophilic statins have a protective against ER– breast cancer and not ER+ breast cancer. For instance, Langballe *et al*. demonstrated that statin use reduced the risk of contralateral breast cancer among patients with ER– breast tumors and not with patients with ER+ tumors[100]. Interestingly, Kumar *et al*. had a striking observation in that lipophilic statin use (for more than one year) appeared to significantly reduce ER– breast cancers by 37% relative to the proportion of ER+ breast cancers[101]. Another study revealed younger patients experienced more favorable outcomes with statin use reducing risk of breast cancer reoccurrence[102]. Moreover, two meta-analysis found lipophilic statins were associated with decreased breast cancer risk[103,104], supporting the results of two previously published randomized control trials[105,106]. The studies analyzed by Manthravadi *et al*. described the 'paucity of data' pertaining to breast cancer subtype, a limitation that might have influenced their conflicting results[104]. These data demonstrate that statin type, breast cancer subtype, and menopausal status all have the potential to influence or alter associations between statins and obesity-associated breast cancer risk. It is difficult to address all of these variables and maintain enough statistical power to determine associations. As a result, the effect of statin use on breast cancer risk is still controversial among epidemiology studies. There are several studies that do not demonstrate a chemoprotective effect. For example, two retrospective studies,

the Nurses' Health Study[107] and Women's Health Initiative[108], did not find a protective effect with statins and ER– breast cancer but evaluated lipophilic and hydrophilic statins collectively. Furthermore, there have not been epidemiological analyses on statin use and obesity-related cancers, therefore, there is no literature that could determine if premenopausal obese women would be a population to benefit from statin chemoprevention. Ultimately, the epidemiological complexities/ should not be a deterrent against statins as chemoprevention. Because of statin tolerability and cardiovascular benefits, statins should not be overlooked when it comes to their potential to reduce the risk of ER– breast cancer[101].

Scope of the Project

The introduction has described the current knowledge gaps surrounding obesity-associated ER– breast cancer and fluvastatin as a potential chemopreventive agent. In summary, it is unknown how VAT stimulates transformation of ER– breast epithelial cells nor how HMGCR is involved in this process. The preceding rationale was used to formulate the central hypothesis that FGF2/FGFR1 is a critical mechanism in ER-independent VAT-associated transformation and is a potential target for chemoprevention strategies. This dissertation went beyond this initial hypothesis and investigated the mechanisms of fluvastatin, the lead candidate identified for chemoprevention, and determine the role of the mevalonate pathway in VAT-stimulated transformation of ER– breast epithelial cells.

CHAPTER 2

A role for FGF2 in visceral adiposity-associated mammary epithelial transformation

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Abstract (120 words)

Obesity is a leading risk factor for post-menopausal breast cancer, and this is concerning as 40% of cancer diagnoses in 2014 were associated with overweight/obesity. Despite this epidemiological link, the underlying mechanism responsible is unknown. We recently published that visceral adipose tissue (VAT) releases FGF2 and stimulates the transformation of skin epithelial cells. Furthermore, obesity is differentially associated with many epithelial cancers, and this mechanistic link could be translational. As FGF2 and FGFR1 are implicated in breast cancer progression, we hypothesize that VAT-derived FGF2 plays a translational role in promoting adiposity-associated mammary epithelial cell transformation. In this brief report, data suggest that FGF2/FGFR1 signaling is a potential mechanistic link in VAT-stimulated transformation of breast epithelial cells.

Introduction

Obesity is a well-established risk factor for post-menopausal breast cancer. [109] A greater waist to hip ratio [indicative of a higher content of visceral adipose tissue (VAT)] increases the risk of post-menopausal breast cancer [110-113]. In pre-menopausal breast cancer, when adjusted for weight or body mass index (BMI), women with the smallest waist to hip ratios have a 37% lower risk [114]. Thus, visceral obesity, an increase in adipose tissue surrounding the intra-abdominal organs, directly relates to the magnitude of obesity-related breast cancer risk [110-113]. However, the underlying mechanisms responsible for the VAT-breast cancer link are not fully elucidated [115].

Anatomical and physiological differences between VAT and subcutaneous adipose tissue (SAT) determine the extent of how these depots contribute to obesity, metabolic syndrome (MetS), and cancer [23,116]. In obese individuals, adipocytes become hypertrophic, which makes them dysfunctional and insulin resistant. The pathophysiology of obesity-induced insulin resistance has been attributed to ectopic fat deposition, increased inflammation, oxidative stress, adipose tissue hypoxia and mitochondrial dysfunction, and impaired adipocyte expansion and angiogenesis [23,116]. Excess VAT contains a greater number of large adipocytes in contrast to SAT, which contains smaller, insulin sensitive adipocytes [23]. Moreover, expanding adipose tissue can induce hypoxia from insufficient vasculature and oxygen supply [23]. This hypoxia can induce immune cell infiltration, causing low-grade chronic inflammation [117]. Adipocyte hypertrophy and

immune cell infiltration alters the release of adipokines (cytokines derived from adipose tissue) that can exacerbate the immune response and induce systemic release of adipokines that can act on neighboring and distant targets [115,117]. These obesity-related changes are associated with insulin resistance, which in turn leads to hyperglycemia, hypertension, dyslipidemia, and other metabolic abnormalities [116,118]. Similarly, MetS is characterized by a cluster of three or more metabolic abnormalities including visceral obesity, insulin resistance, dyslipidemia, hypertension, and hyperglycemia. However, these similar physiological aspects, they are not mutually exclusive; for example, not everyone who is obese has inflammation and metabolic syndrome [119]. Regardless, many studies have concluded the rise in visceral obesity has led to an increase in MetS [116]. Epidemiological studies show both obesity and MetS are breast cancer risk factors [111,120]. Bridging the link between obesity, MetS and breast cancer risk, Kabat *et al*. showed obesity is associated with increased breast cancer risk, and metabolically unhealthy obese individuals had the highest risk [119]. However, epidemiological associations and obesity-related changes fall short of explaining the biological mechanisms by which adiposity contributes to cancer promotion and malignant transformation (a change a cell undergoes to become malignant).

Animal models have given insight into the mechanistic link between visceral adiposity and cancer. In a rat model of intestinal cancer, removing VAT significantly attenuated obesity-associated intestinal tumorigenesis [81]. In addition, we previously demonstrated removing VAT in HFD-fed mice significantly reduces UVB-induced squamous cell carcinomas by 75-80% when compared to sham-operated control animals [82]. These data suggest VAT-derived factors are critical for carcinogenesis [81,82]. We also utilized an *ex vivo* model to evaluate the ability of VAT-derived growth factors to stimulate transformation of non-tumorigenic JB6 P^+ mouse skin epithelial cells. Cellular transformation as indicated by anchorage-independent growth in soft agar is a well-established, stringent method for detecting the tumorigenic potential of transformed cells [121-123]. JB6 P^+ cells can not proliferate in an anchorage-independent manner but have the ability to transform upon treatment of tumor promoters [121-123]. Using this model, we identified fibroblast growth factor 2 (FGF2) as the critical VAT-derived factor in stimulating JB6 P^+ growth in soft agar assay[79]. JB6 P^+ cells that lacked the fibroblast growth factor receptor 1 (FGFR1), FGF2's receptor, failed to transform in the presence of VAT, suggesting the FGF2/FGFR1 signaling axis is critical in VAT-stimulated transformation of epithelial cells at distant targets. How generalizable this mechanism is to other tissues

and human cells is unknown. Therefore, we hypothesize that VAT-stimulation of skin carcinogenesis through the FGF2/FGFR1 signaling is translational to VAT-associated breast cancer.

The objective of this study is to determine the effects of human VAT on the transformation of MCF-10A human mammary epithelial cells. MCF-10A cells are non-tumorigenic and do not exhibit anchorage-independent growth in soft agar. We hypothesized that VAT will stimulate the transformation of MCF-10A cells and this activity will be dependent on FGFR1. Establishing a human model of VAT-stimulated transformation will strengthen support for the direct role of VAT in adiposity-promoted carcinogenesis. There are fundamental differences in the transformation susceptibility of human and mouse cells specifically in the greater number of events required to transform human cells than those required for non-tumorigenic mouse cells [124,125]. Consequently, establishing a human model of VAT-stimulated transformation of mammary epithelial cells shows adiposity-promoted carcinogenesis is relevant to both mouse and human models and is translational to obesity-associated breast cancer.

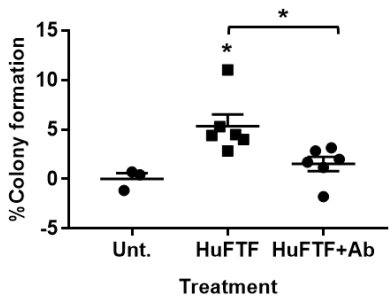

Figure 2.1. Inhibition of FGFR1 attenuates HuFTF-stimulated transformation of MCF-10A cells. HuFTF and HuFTF+Ab significantly stimulated colony formation above the untreated control. Cells were treated with 200 µg/mL of HuFTF protein. HuFTF-stimulated growth in soft agar was significantly attenuated by the FGFR1 Ab (p=0.02). MCF-10A ells were treated with a FGFR1 neutralizing antibody (FGFR1 Ab) at 2 µg/mL and treated with HuFTF from six different donors. The percent of colony formation was calculated as described in Methods, MCF-10A cells were cultured as described in Methods. Data is presented as mean ± six biological replicates. Each biological replicate had three technical replicates. Confidence intervals (CI) were calculated for HuFTF treated MCF-10A cells (95% CI 3.024-7.658) and for HuFTF+Ab (95% CI 0.093-2.929). Both treatments did not contain the untreated control (0% colony formation). Statistical significance between HuFTF and HuFTF+FGFR1 Ab was determined by unpaired t-test (* $p<0.05$).

Results

To test the effects of VAT on mammary epithelial transformation, human fat tissue filtrate (HuFTF) was generated from VAT of six different human donors. VAT was obtained from omental tissue of cancer-free female obese human subjects purchased from Spectrum Health Universal Biorepository (SHUB, Grand Rapids, MI). These subjects were undergoing surgery for gastrointestinal conditions. Table 2.1 describes the human donor characteristics including age, BMI, gender, and ethnicity. We were not able to obtain information on menopausal status, metabolic status or serum metabolites. To determine if HuFTF stimulates transformation of human mammary epithelial cells, MCF-10A cells were incubated with 200 µg/mL of HuFTF in soft agar and scored for colony formation. Colonies (8 cells or greater) were visually counted and a percent of colony formation was obtained by relating the number of colonies with the number of cells plated (750 cells/well). While MCF-10A cells are non-tumorigenic epithelial cells, they have a low level of spontaneous transformation in contrast to tumorigenic epithelial cells, which have almost 100% transformation. Using this low, baseline, spontaneous transformation, tumor promoters can be added to stimulate transformation, inducing colony formation to increase above the control. HuFTF significantly stimulated colony formation above the control with statistical significance ($p<0.05$) (Figure 2.1).

Table 2.1. Clinical characteristics of donors

Donor ID	FGF2 (pg/mL)	Age	BMI	Gender	Ethnicity
01	1.0034	52	30.8	F	African American
02	1.1472	76	28.1	F	White
03	0.61684	70	26.0	F	White
04	0.67632	82	27.6	F	White
05	0.50780	62	22.0	F	White
06	0.35249	50	24.0	F	White

Abbreviation: F, Female

To determine the role of FGFR1 signaling in HuFTF-stimulated transformation, MCF-10A cells were incubated with a tyrosine kinase fibroblast growth factor receptor 1 antibody antagonist (FGFR1 Ab). Inhibiting FGFR1 receptor activity attenuated HuFTF-stimulated transformation of MCF-10A cells (Figure 2.1). The FGFR1 Ab (2 µg/mL) significantly decreased HuFTF-stimulated colony formation, indicating FGFR1 signaling is required for optimal HuFTF-stimulated transformation.

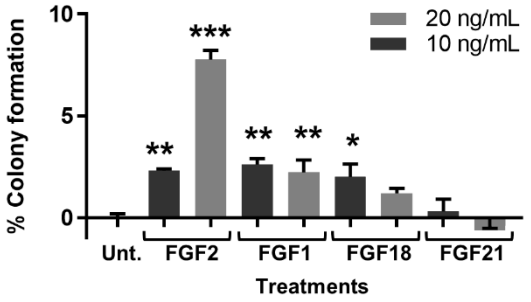

Figure 2.2. FGF2 transforms MCF-10A cells in a concentration-dependent manner. FGF1 and FGF2 significantly stimulated transformation of MCF-10A cells at 10 and 20 ng/mL. FGF18 significantly stimulate transformation at 10 ng/mL but not 20 ng/mL and FGF21 was not statistically significant. MCF-10A cells were cultured as described in Methods, control cells were untreated. Data is presented as mean ± SE of triplicate values. Statistical significance was determined by one-way ANOVA with multiple comparisons (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). The percent of colony formation was calculated as described in Methods.

FGFR1 is a receptor for many FGF ligands and to determine the optimal ligand for stimulating MCF-10A transformation, MCF-10A cells were incubated with FGF1, FGF2, FGF18, and FGF21. FGF1 binds to all four FGFR receptors, FGF18 has the highest affinity for FGFR3 IIIc, with some affinity for FGFR4, FGF21 has the highest affinity for FGFR4, followed by FGFR2 IIb and FGFR IIIc, and FGF2 has the highest affinity for FGFR1 IIIc, FGFR3 IIIc, and FGF4 with some affinity for FGFR2 IIIc [126,127]. While FGF1, FGF2, and FGF18, significantly increased colony formation in MCF-10A cells, FGF2 was the only ligand to induce a concentration response at 10 and 20 ng/mL (Figure 2.2).

A colony of FGF2-stimulated MCF-10A cells was isolated from soft agar and grown in traditional cell culture plates. The FGF2-transformed MCF-10A cells have a fibroblastic-spindle morphology compared to the parent MCF-10A cells that are more epithelial-like with a polygonal shape (Figure 2.3). After several passages this spindle morphology remained and the FGF2-transformed MCF-10A cells demonstrated an irreversible ability to grow in soft agar (data not shown).

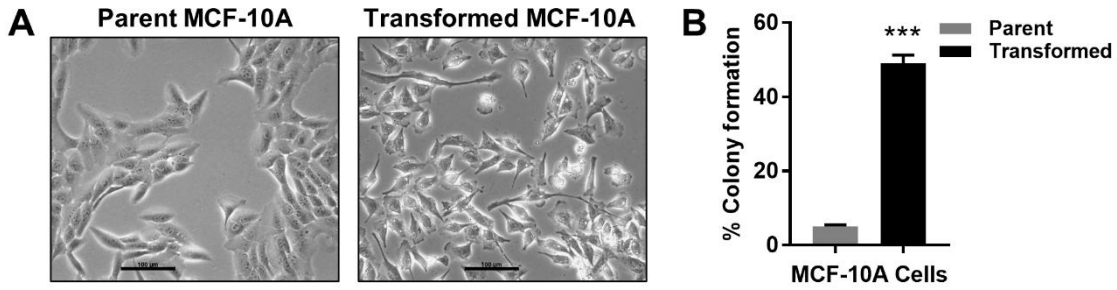

Figure 2.3. FGF2-tranformed MCF-10A cells are morphologically and functionally distinct from parent MCF-10A cells. (A) MCF-10A cells have epithelial-like morphology and transformed MCF-10A cells have spindle-like morphology. Transformed MCF-10A cells were obtained by treating MCF-10A cells with FGF2 in soft agar. After 14 days, a colony was isolated and cultured in traditional cell culture conditions for several passages, making transformed MCF-10A cells. Untreated transformed MCF-10A cells formed over 44% more colonies in soft agar compared to untreated parent MCF-10A cells (data not shown).

The concentration of FGF2 in each HuFTF donor was determined by ELISA and related to the percent of colony formation and BMI (Figure 2.4). The transforming activity of each HuFTF in the soft agar assay was moderately associated with FGF2 concentration in the filtrates ($R^2 = 0.45$) (Figure 2.4A) and with BMI ($R^2 = 0.64$) (Figure 2.4B). Additionally, there was a moderate association between BMI and HuFTF FGF2 concentrations ($R^2 = 0.64$) (Figure 2.4C).

Discussion

Visceral adiposity is significantly associated with breast cancer risk, and despite this strong association, the mechanism is unknown [128,129]. We previously showed visceral obesity promoted skin tumor formation, with our mechanistic finding that VAT-derived FGF2 stimulates skin epithelial cell transformation through FGFR1[79]. As obesity is associated with many different cancers, we hypothesized our mechanistic finding may be translational to other obesity associated cancers, like breast cancer. Visceral obesity, as measured by large waist circumferences and waist-to-hip ratios, is strongly correlated with pre- and post-menopausal breast cancer risk [110-113]. Herein, we describe a translational role for VAT-derived FGF2 in stimulating mammary epithelial cell transformation through FGFR1. These findings highlight FGF2/FGFR1 signaling as a potential link between VAT and breast cancer risk.

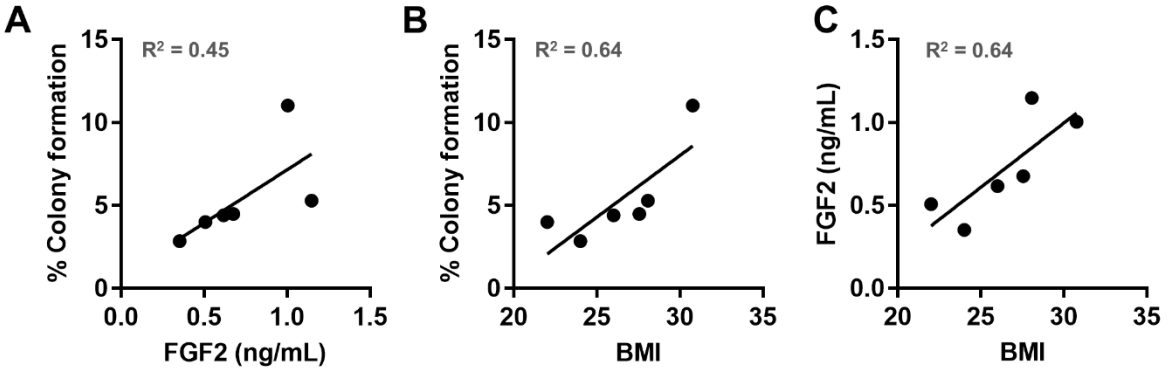

Figure 2.4. HuFTF-stimulated transformation of MCF-10A cells is moderately associated with the HuFTF FGF2 concentration and BMI. (A) HuFTF with higher FGF2 concentrations more potently stimulated MCF-10A transformation compared with HuFTF with lower FGF2 concentrations (R2 = 0.45). (B) HuFTF from donors with a higher BMI more potently stimulated MCF-10A transformation compared to HuFTF from donors with a lower BMI (R2 = 0.64). (C) Higher HuFTF FGF2 concentrations is moderately associated with a higher BMI ((R2 = 0.64). The % colony formation, HuFTF FGF2 concentration, and BMI of six HuFTF were used. MCF-10A cells were cultured as described in Methods. Data were analyzed with Linear regression (performed in PRISM).

Previous research implicates FGF2/FGFR1 signaling in breast cancer [130]. Constitutive activation of FGFR1 in normal mouse mammary epithelium induced proliferation, invasive lesions, and antiapoptotic effects [131]. In breast cancer cells, FGF2 is a strong mitogen and potent antiapoptotic and induces invasiveness while subverting various chemotherapeutic agents, leading to drug resistance [132-134]. In addition, FGFR1 activation increases proliferation and invasion of breast cancer cell lines [135,136]. Clinical studies have shown that FGF2 levels in serum, nipple aspirate fluid, and tumor samples are higher in patients with cancerous breast tumors as compared with benign breast diseases/tumors [137-140]. In breast cancer patients, FGFR1 amplification is seen in up to 10-15% of all breast cancers and is associated with early relapse and poor survival [120,141]. Likewise, tumors overexpressing FGFR1 exhibited increased proliferation and decreased distant metastasis-free survival [141]. The role of FGF2/FGFR1 in breast cancer onset is less clear. One study demonstrated that genetic variants in FGFR1, FGFR3, or FGFR4 had no impact on breast cancer risk, [142] whereas an intronic single-nucleotide polymorphism (SNP) in the FGFR2 gene was associated with an increased risk of breast cancer, particularly estrogen receptor (ER) positive disease [142]. A separate study demonstrated no significant associations with SNPs in FGF2 and breast cancer risk [143]. However, the functional relevance of these FGF2 polymorphism for function are unknown.

Our data demonstrate that FGFR1 activation is critical for optimal VAT-stimulated MCF-10A cell transformation. These data add additional relevance to the previous findings that FGFR1 activation by inducible dimerization of the receptor induced growth in soft agar of MCF-10A cells [135]. Moreover, we showed VAT FGF2 concentrations were associated with VAT transforming activity (Figure 2.4A). Collectively, these data suggest that FGF2 from VAT stimulates mammary epithelial cell neoplastic transformation through FGFR1 activation. The downstream effects of FGFR1 activation of transformation are unknown, but it would be interesting to observe a potential subtype or breast cancer signature in tumors that arise from visceral adiposity-promotion. Breast cancer is characterized into different subtypes based on expression of ER, progesterone receptor (PR), and human epidermal growth factor receptor (HER). Studies show FGFR1-overexpressing tumors are frequently ER positive, the primary subtype associated with obesity [120,144]. Therefore, we would hypothesize that FGF2 activation of FGFR1 would promote ER positive tumors in viscerally obese individuals.

Evaluating individual characteristics including age, BMI, gender, and ethnicity were not associated with transforming capacity of the HuFTFs. As obesity is more strongly associated with post-menopausal breast cancer, we requested samples from individuals of post-menopausal age. Menopause occurs on average in women at 51 years old, however, we were not able to confirm the menopausal status of the individual donors as two donors were 52 and 50 years old at the time of surgery. It would be interesting to investigate if menopause affects the quality of VAT, in turn affecting FGF2 levels. In our study, there was no relationship between age and the transforming capacity of the HuFTF (R^2= *0.09*). For BMI, there is a moderate association with BMI and the transforming capacity of the HuFTF, giving an R^2 value of 0.64 (Figure 2.4B). This suggests BMI might be an indicator of VAT FGF2 levels. We were not able to investigate ethnicity as a variable. Additionally, we were not afforded any information on serum metabolites or the metabolic status of each donor.

There are 22 structurally similar FGF ligands that mediate effects through activation of receptor tyrosine kinases (RTK), fibroblast growth factor receptors (FGFR) 1-4. FGFs can have affinities for more than one receptor and each receptor can bind multiple FGFs. Similarities between receptors has resulted in receptor redundancy as they can converge on key downstream signaling cascades [145,146]. All four FGFRs activate PLCγ/PKC, PI3K/AKT, RAS/MAPK, and STAT pathways [145]. Activation of these pathways play important roles in migration, survival, differentiation, and proliferation [146]. However, studies suggest the strength and specificity of each signaling cascade can vary depending on the type of FGFR and FGF [145]. Additionally, pharmacologically inhibiting FGFR1 partially attenuated VAT-stimulated mammary epithelial transformation (p=0.02) (Figure 2.1). This partial attenuation suggests either ligands, receptors, and/or signaling cascades are influencing colony formation or the FGFR1 Ab does not have complete inhibition of FGFR1.

Furthermore, we found FGF1 and FGF18 induces colony formation, but not in the concentration-dependent manner as seen with FGF2 (Figure 2.2). One study using MCF-10A cells revealed phenotypic distinctions in 3D growth stimulated by different RTKs, including EGFR and MET [147]. Other FGFRs or RTKs activated by other ligands could be inducing signaling pathways that attribute to VAT-stimulated transformation [146]. Therefore, while other RTKs could contribute to transformation, these data suggest biased agonism associated with FGF2/FGFR1 is optimal for VAT-stimulated MCF-10A transformation.

HuFTF from donor 02 had the highest level of FGF2 at 1.14 pg/mL. Independently FGF2 required at least 10 ng/mL to stimulate transformation of MCF-10A cells. This could be due to a potential difference in the FGF2 isoform in the HuFTF compared to the recombinant protein. FGF2 exists in five different isoforms that are divided into two groups low molecular weight (LMW) and high molecular weight (HMW) proteins. Studies have suggested there are distinct biological activities of LMW and HMW proteins. For example, one study showed that overexpressing LMW FGF2 enhanced bone mineral density (BMD) whereas overexpressing HMW FGF2 lowered BMD [148], and another study showed LMW FGF2 suppressed hepatic fibrosis and HMW enhanced hepatic fibrosis [149]. In contrast, other studies have showed FGF2 isoforms exhibit different potencies. Kole *et al*. showed that all FGF2 isoforms exhibited mitogenic activity in dermal fibroblasts, however, HMW isoforms were less efficient [150]. Additionally, a study by Mydlo *et al*. showed that FGF2 derived from omental VAT demonstrated greater mitogenic and angiogenic activity than FGF2 derived from either benign and cancerous renal tissue [151]. The recombinant protein used is an LMW FGF2 (18 kDa), and the ELISA used to detect FGF2 is nonspecific regarding FGF2 isoforms. Therefore, the recombinant protein might not be representative of the most active type of FGF2 in the HuFTF. Additionally, HMW FGF2 could be more potent than LMW FGF2, accounting for the difference in dose of FGF2 in HuFTF and the recombinant FGF2 used. Currently, only LMW FGF2 is commercially available, and isolating FGF2 from HuFTF would provide a more accurate representation of the transformative capabilities of VAT-derived FGF2.

FGF2 is classically considered to function in both an autocrine and paracrine manner, however, our research suggests FGF2 functions in an endocrine manner acting on distant targets. Our previous study showed an induction of serum FGF2 in HFD-fed mice compared to LFD-fed mice [79]. This serum induction was depleted following lipectomy of VAT suggesting the circulating levels of FGF2 are of adipose tissue origin [79]. Circulating levels of FGF2 in these animals were associated with UVB-induced squamous cell carcinomas, suggesting that FGF2 secreted from VAT influences tumor promotion at distant sites [79]. In tandem, one study found FGF2 concentrations in serum increased with higher BMIs [77], and another found plasma FGF2 levels of obese Chinese men were correlated with adipose tissue mass [78]. Our recent study demonstrates that human serum samples with elevated FGF2 had greater efficacy in stimulating JB6 P^+ cell growth in soft agar. Future studies are needed to assess circulating FGF2 concentrations in relation to

visceral obesity and breast cancer risk to ascertain potential associations and a role for FGF2 as an endocrine growth factor and as a biomarker for at risk individuals.

Obesity and breast cancer are independently complex diseases with multiple factors potentially influencing their etiology. FGF can be secreted from many different tissues along with other FGFR1 ligands. For example, FGF2 is secreted from skin [152], heart [153], liver [149], lungs [154], and SAT [155] and could contribute to circulating FGF2 levels. The contribution of FGF2 from VAT and other sources to mammary tumorigenesis will be determined *in vivo* in future investigations. Although VAT is more strongly correlated with breast cancer risk than its subcutaneous counterpart, there is an intimate and bidirectional interaction between mammary epithelium and adjacent subcutaneous mammary adipose tissue (MAT). The total absence of MAT in transgenic mice prevents non-tumorigenic mammary gland development and MAT supports and amplifies breast cancer progression [156]. Dialog between MAT and mammary epithelium might persist and influence breast cancer onset as a potential source of FGF2 [156,157]. This exposes a limitation in our study as assessing VAT and mammary epithelial cells in our *in vitro* model does not evaluate whole body effects *in vivo*.

In summary, we demonstrate FGF2 from human VAT stimulates transformation of non-tumorigenic mammary epithelial cells. Our data suggests differences the transformative ability of human VAT is associated with FGF2 levels and that inhibiting FGFR1 attenuated this transformation. These findings highlight FGF2/FGFR1 signaling as a potential link between visceral adiposity and elevated breast cancer risk. Future studies will use *in vivo* mouse models to determine the tumorigenicity of transformed MCF-10A cells, the ability of HFD to promote mammary tumorigenesis, and the effect of lipectomy on mammary tumorigenesis. FGF2/FGFR1 signaling could be a therapeutic target for breast cancer prevention strategies and/or a biomarker for identifying at risk individuals.

Methods

Cell Culture

MCF10A cells (human mammary epithelial cells) were obtained from ATCC (Manassas, VA, USA). Cells were cultured in DMEM/ Ham's F12 media supplemented with 5% horse serum (HS), 1% penicillin/streptomycin, 100 ng/mL cholera toxin, 20 ng/mL epidermal growth factor (EGF), 10 µg/mL insulin,

0.5 mg/mL hydrocortisone, 7.5% sodium bicarbonate, 15mM HEPES, and 2 mM L-Glutamine (growth media). MCF-10A cells were trypsinized with 0.05% trypsin and quenched in DMEM/ Ham's F12 media with 20% horse serum and antibiotics (resuspensions media). The FGFR1 Ab was purchased from R&D Systems (Minneapolis, MN, USA #MAB765).

Human fat tissue filtrate

VAT was homogenized in equal volume of serum free media on ice for 30 seconds using Tissue Ruptor (Qiagen, Hilden, Germany) on medium speed. Homogenates were filtered through a hanging transwell insert 15-mm wide 0.4 um filter (Millicell, cat# MCHT06H48) positioned in 6-well plates filled with 750 µL of serum free media. Plates were incubated on a rocker for 1 hour to allow fat derived factors to diffuse into the media. After incubation, filtrates were centrifuged at 4500 rpm for 5 minutes and the supernatant was collected. Protein concentrations were quantified using BCA Assay. An aliquot of 200 µg/mL concentration of HuFTF was used for respective experiments.

Anchorage-Independent Colony Formation Soft Agar Assay

MCF10A cells were seeded at 750 cells per well in a 24-well plate in 200 µL of DMEM/Ham's F12, 5% HS, and 0.33% agar with or without HuFTF and/or inhibitors which was overlaid onto 350 µL of DMEM/Ham's F12, 5% HS, and 0.5% agar. Soft agar plates were left at room temperature for 30 minutes before 200 µL of growth media was gently added to each well and then stored at 37°C. Every 3-4 days, the growth media was removed from each well and replenished with 200 µL of growth media. After two weeks, the colonies were fixed in 70% ethanol (EtOH) and stained with 150 µL of 0.01% crystal violet. Colonies were visually counted and used to calculate the percent of colony formation from the number of cells plated ([Colonies counted x 100] / 750 cells). The % colony formation was then normalized to the control to determine the increase in % of colony formation. (% Colony formation of treatment – the % colony formation of untreated control).

FGFR1 Ab Treatment

FGFR1 Ab was added directly into the top layer of the soft agar assay. MCF-10A cells were pre-incubated with the monoclonal FGFR1 Ab (2 µg/mL) in 37°C for 1.5 hours before being added to the top soft agar layer.

Statistics

Six biological and three technical replicates were used to ensure adequate power to detect a significant change in growth in soft agar. Data are presented as mean ± s.e. Unpaired t-test and one-way ANOVA for multiple comparisons were used appropriately. For all statistical tests, 0.05, 0.01, and 0.001 level of confidence, were accepted for statistical significance.

FGF2 Quantification: FGF2 concentrations in HuFTF was measured by ELISA kit according to the manufacturer's protocol using R&D Systems Quantikine ELISA kit' (Cat# DFB50). The lowest detectable FGF2 concentration was 0.625 pg/mL.

CHAPTER 3

Identifying chemopreventive agents for obesity-associated cancers using an efficient, 3D high-throughput transformation assay

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Abstract

Obesity is associated with ~40% of cancer diagnoses but there are currently no effective preventive strategies, illustrating a need for chemoprevention. We previously demonstrated that fibroblast growth factor 2 (FGF2) from adipose tissue stimulates malignant transformation, as measured by growth in soft agar, the gold-standard *in vitro* transformation assay. Because the soft agar assay is unsuitable for high throughput screens (HTS), we developed a novel method using 3D growth in ultra-low attachment conditions as an alternative to growth in agar to discover compounds that inhibit transformation. Treating non-tumorigenic, skin epithelial JB6 P^+ cells with FGF2 stimulates growth in ultra-low attachment conditions analogous to growth in the soft agar. This transformation HTS identified picropodophyllin, an insulin growth factor 1 receptor (IGF1R) inhibitor, and fluvastatin, an HMG-CoA reductase inhibitor, as potential chemopreventive agents. These compounds were validated for efficacy using two non-tumorigenic cell lines in soft agar. Another IGF1R inhibitor and other statins were also tested and several were able to inhibit growth in soft agar. This novel 3D HTS platform is fast, robust and has the potential to identify agents for obesity-associated cancer prevention.

Introduction

Obese individuals are a specific high-risk population that would benefit from targeted chemoprevention strategies. Obesity is associated with 13 different types of cancers and is associated with over 40% of cancer diagnoses[47]. Because of the swelling obesity epidemic, over 38% of American adults were considered overweight/obese in 2014[158,159]. Rising obesity rates illustrate an immediate need for effective primary prevention strategies for obesity-associated cancers. Current prevention strategies like lifestyle changes are ineffective due to non-compliance. Although there are theories to explain the obesity-cancer association, the underlying mechanisms are poorly elucidated. While investigating this mechanism, our studies demonstrated that circulating fibroblast growth factor 2 (FGF2) from visceral adipose tissue (VAT) stimulates fibroblast growth factor receptor 1 (FGFR1) on epithelial cells to drive malignant transformation[79,160,161]. We demonstrated that VAT depleted of FGF2 failed to transform epithelial cells and epithelial cells lacking FGFR1, the primary receptor for FGF2, also failed to exhibit VAT-induced transformation[79]. Although FGF2 is considered a paracrine growth factor, its circulating levels correlate with

adipose tissue mass in humans and are at levels sufficient to induce transformation *in vitro* [78,79]. This suggests that VAT-secreted FGF2 has a systemic role. In animals, we found that a high-fat diet increased serum FGF2 levels and that removing VAT via lipectomy depleted serum FGF2 levels[79]. Therefore, the FGF2/FGFR1 signaling axis is a potential chemopreventive target for obesity-associated epithelial cancers.

Compounds that lower cancer risk by delaying or preventing cancer, whether synthetic or natural, have the potential to significantly reduce cancer burden[51]. As the World Health Organization estimates that 30–50% of all cancer cases are preventable, lowering cancer incidence will in turn lower cancer mortality[84]. Effective chemopreventive agents either eliminate premalignant cells or protect normal/initiated cells from undergoing malignant transformation (i.e. the changes a non-transformed or normal cell undergoes to become carcinogenic)[83,84]. There are, however, drawbacks to implementing chemoprevention[162]. As these agents would benefit only a subset of the treated population, they must be efficacious and have minimal to no side effects so that they are tolerated for a long duration[163]. Cumulatively, these improbable standards are a challenge for the development and implementation of chemoprevention. Applying an approach of precision medicine to chemoprevention can help overcome these shortfalls[83]. Target-driven strategies to risk-stratify individuals would reduce strict restraints on side effects because the clinical benefits would presumably outweigh the risks [83]. Therefore, implementing targeted chemoprevention strategies in conditions such as obesity has the potential to greatly reduce cancer burden.

Methodological limitations make identifying compounds that prevent obesity-associated cancers challenging. Transformation is commonly modeled by the soft agar assay, a well-established technique that measures transformation by assessing anchorage-independent growth[122,164-167]. Non-transformed cells must be anchored to an extracellular matrix (ECM) or a treated-culture plate to proliferate. In contrast, tumorigenic cells, which have undergone transformation, lose their anchorage-dependence[168]. 3D models like the soft agar assay do not provide an ECM-like environment, so cells suspended in soft agar only proliferate and form colonies if they are transformed[168]. This gives 3D models a distinct advantage for modeling transformation over 2D culture methods and can distinguish transformed and non-transformed cells[168,169]. We previously used the soft agar assay to show that factors derived from VAT as well as FGF2 itself can stimulate epithelial cells to transform[79,160,161]. This experiment can identify chemopreventive compounds that prevent or inhibit FGF2-stimulated transformation, but the soft agar assay is unsuitable for

high-throughput screening because it is laborious and inefficient, using a 6-24 well format with a 2-week incubation period.

Identifying compounds for precision chemoprevention requires a targeted high-throughput screening platform that models malignant transformation of non-transformed cells. However, this is an underdeveloped area of cancer prevention as current models use cancer cells lines and 2D culture. Currently, there are not any high throughput assays that model the process of transformation[168,169]. Therefore, the objective of these studies was to develop a high-throughput 3D model of transformation to screen for chemopreventive agents and then validate hits in soft agar with two cell lines, JB6 P^+ and MCF-10A. These are, respectively, non-tumorigenic mouse epidermal cells and non-tumorigenic human breast epithelial cells. In a recent publication, Rotem *et al.* describe an HTS assay (384-well) where growth in ultra-low attachment conditions in a round bottom plate (3D growth) is strongly correlated to growth in soft agar [166]. Investigators measured growth of non-transformed and transformed cell lines in soft agar and in low attachment conditions and demonstrated a strong correlation with an R^2 value of 0.82[166]. In this manuscript, we used growth in ultra-low attachment conditions to develop a novel phenotypic transformation HTS assay using FGF2/FGFR1 signaling as the target-based mechanism to identify chemopreventive agents for obesity-associated epithelial cancers. Screened compounds that prevent growth in ultra-low attachment conditions may further the mechanistic understanding of malignant transformation and have the potential to be developed as precision chemopreventive therapies. While this screen could identify novel inhibitors of FGF2/FGFR1 signaling, it also has the potential to find compounds that interrupt the transformation process itself.

Results

Development and optimization of transformation HTS

The transformation HTS was developed using JB6 P^+ mouse skin epithelial cells. JB6 P^+ cells cannot proliferate in an anchorage-independent manner, but they have the ability to transform upon treatment with tumor promoters.[167] JB6 P^+ cells are used below passage 15 to prevent spontaneous transformation. We previously demonstrated that visceral adipose tissue (VAT)-derived FGF2 stimulates JB6 P^+ cell growth in soft agar (transformation) FGF2 was used in the HTS assay to stimulate transformation.[79] Using mouse fat

tissue filtrate (MFTF), a filtrate that contains factors from the VAT of high-fat diet (HFD)-fed mice, might be more physiologically relevant and constitute a screen with better face validity to identify chemopreventive compounds in obesity-associated transformation. However, using biological matrices in HTS often result in significant variability. Therefore, the assay was developed and optimized with FGF2 as the stimulus to increase rigor and reproducibility. We used CellTiter-Glo (Promega) to quantitate the ATP content in the cells, a surrogate marker for proliferation, as the amount of ATP is generally proportional to the number of cells (Figure 3.1A).

The transformation HTS assay parameters, including number of cells plated per well, incubation time, and FGF2 concentration were optimized. To determine the optimal cell number, JB6 P^+ cells were plated in 384-well round-bottom, ultra-low-attachment plates at 50, 100, and 200 cells/well with FGF2 at 30 ng/mL. 200 cells/well gave an optimal z-factor of 0.56 (Figure 3.1B). Using 200 cells/well, a concentration response study was performed. FGF2 at 30 ng/mL gave the optimal z-factor of 0.64 (Figure 3.1C). The highest concentration of FGF2 (100 ng/mL) increased the variability, decreasing the Z-factor (Figure 3.1C). Figure 1D illustrates FGF2-stimulated growth over a 96-hour period, whereas untreated or vehicle-treated JB6 P^+ cells congregate at the bottom of the well and fail to proliferate (Figure 3.1D). Overall, the transformation HTS was developed in a 384-well, round bottom, ultra-low attachment plates and optimized to have 200 JB6 P^+ cells/well, 30 ng/mL of FGF2, 96-hour incubation at 37°C, resulting in a Z-factor of 0.503 over eight independent assays. DMSO, the solvent for the compound libraries, was used below 0.05% because higher concentrations negatively impacted the Z-factor. Therefore, the compounds were screened with a final DMSO concentration of 0.025%.

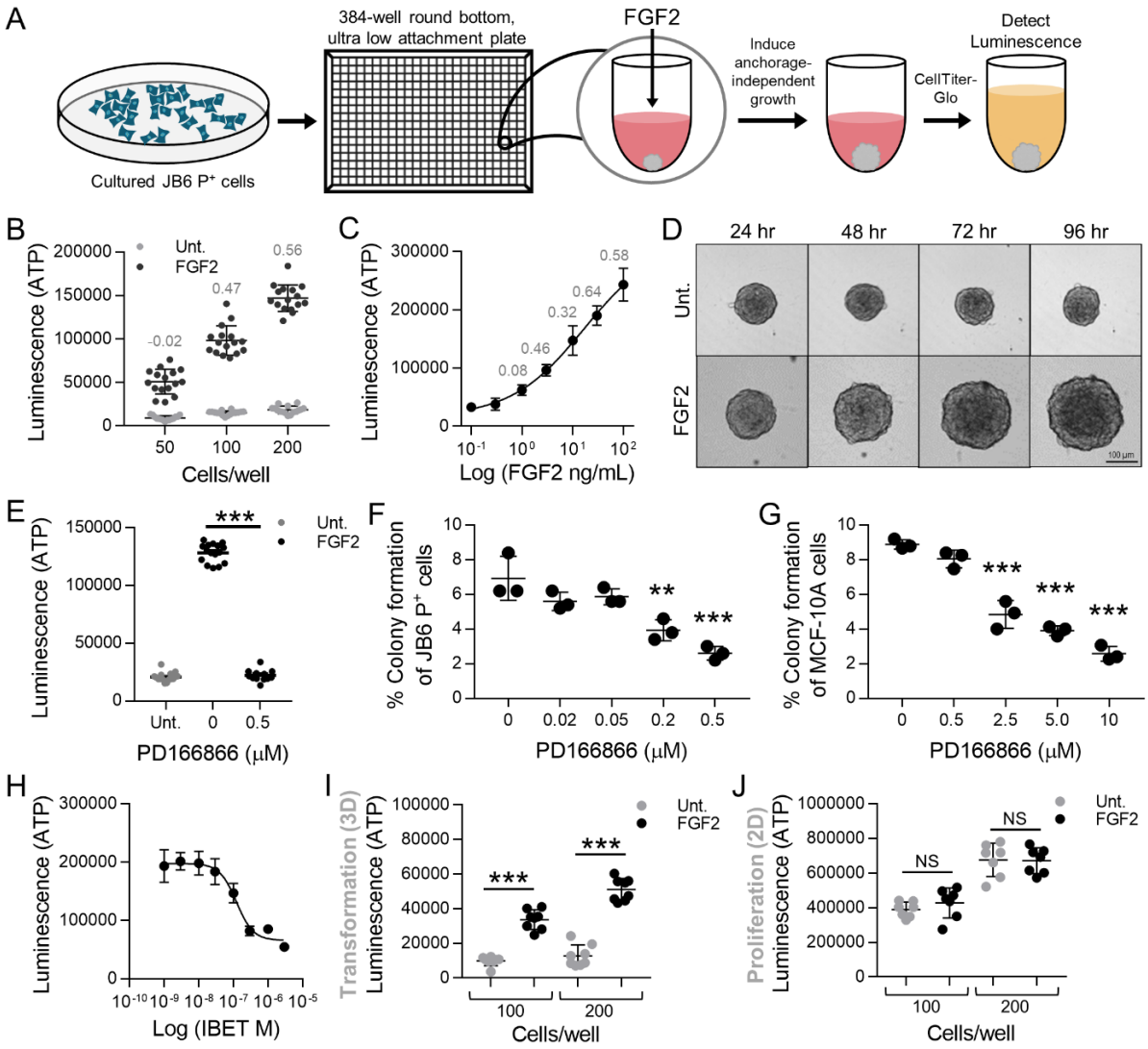

Figure 3.1. Optimization and development of the transformation HTS. A) Methodology schema of the transformation HTS. JB6 P+ cells were plated in 384-well round bottom ultra-low attachment conditions and stimulated with FGF2. JB6 P+ cell growth in ultra-low attachment conditions was measured with CellTiter-Glo that gives a luminescent signal stimulated by binding to ATP. ATP levels are proportional to the number of cells, and thus used as a measure of proliferation. B) JB6 P+ cells were plated in 384-well round bottom, ultra-low attachment plates and treated with 30 ng/mL of FGF with either 50, 100, or 200 cells/well. 200 cells/well was the optimal cell density giving a Z-factor of 0.56. Fifty (50) and 100 cells/well gave Z-factors of -0.023 and 0.47 respectively. Each treatment group had 16 technical replicates. C) A concentration response of FGF2 was performed with JB6 P+ cells at 200/cells per well. The EC_{50} was 15 ng/mL, however, 30 ng/mL gave the optimal Z-factor 0.644. Each treatment had 7 technical replicates. The concentration response stimulation was statistically analyzed using a nonlinear regression, dose-response with PRISM. D) FGF2-stimulated transformation of JB6 P+ cells can be visually observed with JB6 P+ cells at 200 cells/well with or without FGF2 at 30 ng/mL over the 96-hour incubation. JB6 P+ cells congregate at the bottom of the round-bottom wells and untreated, do not grow, but with FGF2, do proliferate. E) PD166866, a FGFR1 inhibitor, at 0.5 μM completely prevented FGF2-stimulated JB6 P+ cells growth in ultra-low attachment conditions. Untreated (Unt.) and FGF2 controls had 16 technical replicates and PD166866 had seven technical replicates. F) PD166866 attenuates FGF2 (0.5 ng/mL)-stimulated JB6 P+ cell growth in soft agar. The soft agar assay was performed as described in Material/Methods. G) PD166866 attenuates FGF2 (20 ng/mL)-stimulated MCF-10A cell growth in soft agar.

Figure 3.1 (cont'd). The soft agar assay was performed as described in Material/Methods. Data are presented as mean ± S.D., statistical significance was determined using a one-way ANOVA, multiple comparisons (**p<0.01, ***p<.0001). H) IBET concentration-dependently inhibits FGF2 stimulated growth in ultra-low attachment conditions. JB6 P^+ cells were plated at 200 cells/well with FGF2 at 30 ng/mL. The IC50 of IBET inhibition is 0.12 μM. Each treatment had seven technical replicates. The concentration response inhibition was statistically analyzed using a nonlinear regression, dose-response with PRISM. I) JB6 P^+ cells were plated in 384-well round-bottom, low attachment plates (100 and 200 cells/well) and incubated at 37ºC for 48 hrs. FGF2 at 30 ng/mL significantly stimulates JB6 P^+ cell growth in low attachment conditions compared to untreated controls (Unt.). Each treatment had seven technical replicates and data was analyzed by one-way ANOVA. Data are presented as mean ± S.D. J) JB6 P^+ cells were plated in 384-well flat-bottom, cell culture treated plates (100 and 200 cells/well) and incubated at 37ºC for 48 hrs. FGF2 at 30 ng/mL did not stimulate growth in 2D culture conditions compared to untreated controls (Unt.). Each treatment had seven technical replicates and data was analyzed by one-way ANOVA. Data are presented as mean ± S.D. (**p<0.01, ***p<.0001) NS, not significant.

FGFR1 is critical in FGF2-stimulated transformation

The transformation HTS was developed to be a FGFR1 target-based, phenotypic screen. Therefore, this assay can identify hits that act directly on FGF2 and/or FGFR1, as well as ones that target the transformation process, including any part of the FGF2/FGFR1 signaling axis that promotes transformation. To demonstrate that FGFR1 is critical in FGF2-stimulated growth in ultra-low attachment conditions, we used PD166866, an FGFR1-selective inhibitor (SelleckChem), in the screen as a positive control. PD166866 at 0.5 μM completely inhibited FGF2-stimulated transformation in the HTS (Figure 3.1E), demonstrating that compounds which inhibit FGF2/FGFR1 signaling will inhibit JB6 P^+ cell transformation. This result was validated in soft agar with both JB6 P^+ and MCF-10A cells. PD166866 significantly inhibited JB6 P^+ colony formation at 0.2 and 0.5 μM and MCF-10A colony formation at 2.5, 5.0 and 10 μM (Figure 3.1F, G). These data demonstrate that FGFR1 is critical in FGF2 stimulated epithelial cell transformation.

To demonstrate efficacy and feasibility of this assay, we also tested I-BET-762 (IBET), a bromodomain inhibitor, which we have previously shown to prevent FGF2-stimulated transformation both *in vitro* and *in vivo*[170]*.* IBET concentration-dependently prevented FGF2-stimulated growth in low attachment conditions (Figure 3.1H).

2D vs 3D growth

Cell growth in 2D culture (proliferation) is not mechanistically analogous to cell growth in 3D culture (transformation) [168,169]. To demonstrate this, JB6 P^+ cells were plated in conventional 384-well flat-bottom, cell culture plates at 100 or 200 cells/well, with or without FGF2 (30 ng/mL) for 48 hours (Figure 3.1D). JB6 P^+ cells were also plated in 384-well round-bottom, ultra-low attachment plates at 100 or 200 cells/well, with or without FGF2 (30 ng/mL) for 48 hours (Figure 3.1I). 96 hrs in 2D growth was not optimal because all cells are proliferating and reach confluence before the 96 hours, a limitation that does not apply to ultra-low attachment cell growth because the cells are not restrained to the surface area of the well. FGF2 significantly stimulated proliferation in 3D conditions and not in 2D conditions with either 100 or 200 cells per well (Figure 3.1I). FGF2 is a known mitogen that stimulates growth of cancer cells in 2D culture[20]. However, in our assay with non-tumorigenic cells, FGF2 is not a mitogen as proliferation of JB6 P^+ was not increased by FGF2 (Figure 3.1J). This corresponds to findings from Rotem *et al.,* which demonstrate that

the oncogenic capacity of cells (growth in 3D) is independent of the proliferation rate[16]. Therefore, FGF2 stimulates anchorage-independent growth, a characteristic of malignant cells, which suggests that FGF2 stimulates JB6 P^+ cell transformation but does not enhance traditional 2D proliferation.

Table 3.1. Funnel Strategy - 2,532 compounds screened

Hit Parameter	Identified Hits	Hit Percentage
Transformation HTS - >50% inhibition	178	7.0
Cytotoxicity - <25% decrease in HEK293 cell viability)	105	4.2
Promiscuity - active in <20% assays listed in Pubchem	58	2.3
Commercially available compounds - DTP/CC	33	1.3
Fresh powder confirmation - >40% inhibition	7	0.27

Abbreviations: HEK293- human embryonic kidney; DTP- Developmental Therapeutics Program; CC- Cayman Chemical

Screening of compound libraries

Over a thousand compounds from the Prestwick Chemical Library®, the National Cancer Institute (NCI) Natural products library, and the Michigan State University (MSU) Chemistry library of MSU-made analogs of natural products were screened. These libraries were used at 0.5 μM, a relatively low concentration to identify chemopreventive agents with a higher potency and to reduce potential for toxicity. The screen gave an average Z-factor of 0.503. Compounds that attenuated transformation 50% or more (which is 3-4 standard deviations from the mean) were considered primary hits. Compounds were then assessed for general cytotoxicity and eliminated if HEK293 cell viability was decreased by more than 25% at 10 μM (MSU screening core, unpublished). Next, compounds were assessed for promiscuity and eliminated if they demonstrated activity in more than 20% of the bioassays listed in PubChem, determined by number of hits/total assays screened (data acquired in August 2017). Promiscuous compounds are problematic for chemoprevention due to the increased potential for side-effects. These parameters narrowed hits down to 58 compounds for validation and concentration response (Table 3.1).

Hit confirmation and soft agar validation

Fresh powder was obtained from the Developmental Therapeutics Program (DTP) at the NCI or commercially (Cayman Chemical) for 33 compounds to confirm hits and eliminate false positives. Not all 58 prioritized hits were commercially available for order. Confirmation tests with new powders ensure that inhibition of FGF2-stimulated transformation corresponds to the intact compound and not to an impurity or degraded compound in DMSO library stocks which can occur if compounds have undergone several freeze-thaw cycles over time[171]. Of the prioritized hits from the transformation HTS, 2 hits were confirmed: picropodophyllin (PPP) and fluvastatin. PPP is an insulin-like growth factor-1 receptor (IGF1R) inhibitor that is currently undergoing clinical trials as an adjuvant chemotherapeutic. PPP concentration-dependently attenuated FGF2-stimulated growth in low attachment conditions (Figure 3.2A). Fluvastatin, a statin drug used for lowering blood cholesterol and triglycerides, concentration-dependently attenuated FGF2-stimulated growth in low attachment conditions (Figure 3.2A). Both PPP and fluvastatin have been used clinically with no observed toxicity[172-175].

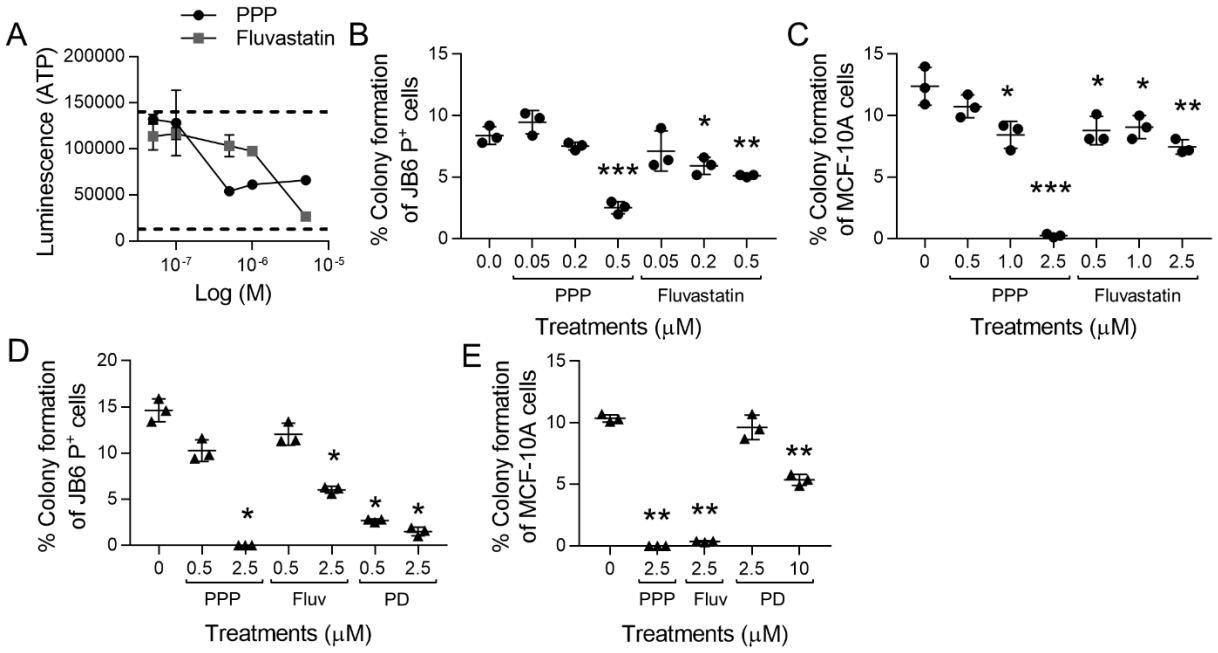

Figure 3.2. PPP and fluvastatin significantly attenuate FGF2-stimulated transformation of epithelial cells. A) PPP and fluvastatin concentration-dependently inhibit JB6 P^+ cell growth in low attachment conditions. Dotted lines indicated FGF2 (top) and untreated controls (bottom). Each treatment had three technical replicates. JB6 P^+ cells were cultured with the optimized parameters. **B)** PPP and fluvastatin attenuate FGF2 (0.5 ng/mL)-stimulated JB6 P^+ cell growth in soft agar at 0.5 µM and 0.2 and 0.5 µM respectively. The soft agar was performed as described in Materials/Methods with three technical replicates. **C)** PPP and fluvastatin attenuates FGF2 (20 ng/mL)-stimulated MCF-10A cell growth in soft agar at 1.0 and 2.5 µM and 0.5, 1.0, and 2.5 µM respectively. The soft agar was performed as described in Materials/Methods with three technical replicates. **D)** PPP and fluvastatin (Fluv) significantly attenuate MFTF (200 µg/mL)-stimulated JB6 P^+ cell growth (1000 cells/well) in soft agar at 2.5 µM and PD166866 (PD) was significant at 0.5 and 2.5 µM. The soft agar was performed as described in Materials/Methods with three technical replicates. **E)** PPP and fluvastatin (Fluv) significantly attenuate MFTF (200 µg/mL)-stimulated MCF-10A cell growth (1000 cells/well) in soft agar at 2.5 µM and PD166866 (PD) was significant at 10 µM. The soft agar was performed as described in Materials/Methods with three technical replicates. The soft agar assays were analyzed by one-way ANOVA with multiple comparisons (*$p<0.05$; **$p<0.01$; ***$p<0.001$).

PPP and fluvastatin were validated in the soft agar assay using both JB6 P^+ and MCF-10A cells to demonstrate their efficacy in preventing FGF2-stimulated transformation. MCF-10A cells are non-tumorigenic human mammary epithelial cells. We previously showed that MFTF and FGF2 can stimulate MCF-10A cells to transform[160,170]. Here, PPP and fluvastatin concentration-dependently attenuated FGF2-stimulated colony formation of JB6 P^+ (Figure 3.2B) and MCF-10A cells in soft agar (Figure 3.2C), analogous to the inhibition observed in the HTS. JB6 P^+ cells were stimulated with 0.5 ng/mL of FGF2 and PPP significantly attenuated colony formation at 0.5 µM compared to the FGF2-treated control; fluvastatin also significantly attenuated colony formation at 0.2 and 0.5 µM (Figure 3.2B). MCF-10A cells were stimulated with 20 ng/mL of FGF2 and PPP significantly attenuated colony formation at 1.0 and 2.5 µM; fluvastatin significantly attenuated colony formation at 0.5, 1.0, and 2.5 µM (Figure 3.2C). Overall, these data demonstrate that compounds discovered through the transformation HTS also inhibit colony formation in the gold-standard soft agar transformation assay.

The objective of these studies is to identify chemopreventive compounds for obesity-related cancers. Therefore, as a more relevant stimulating with FGF2, we tested the efficacy of PPP and fluvastatin for preventing MFTF-stimulated transformation. For JB6 P^+ cells were stimulated with 200 µg/mL of MFTF, both PPP and fluvastatin significantly attenuated colony formation at 2.5 µM (Figure 3.2D). The FGFR1 inhibitor PD166866 also significantly attenuated colony formation at 0.5 and 2.5 µM. For MCF-10A cells stimulated with 200 µg/mL of MFTF, PPP and fluvastatin significantly attenuated colony formation at 2.5 µM and PD166866 showed significant inhibition at 10 µM (Figure 3.2E).

Picropodophyllin and fluvastatin do inhibit 2D proliferation

Many anti-cancer agents inhibit cell proliferation and induce cell death, however, for chemoprevention, compounds need to exhibit little to no toxicity. To determine if PPP and fluvastatin affected cell proliferation and viability. JB6 P^+ cells were labeled with Cell Proliferation Dye eFluor™ 450 and plated in 2D culture with PPP, fluvastatin, PD166866, or DMSO for 48 hours and analyzed by flow cytometry. The eFluor™ 450 dye binds to cellular proteins containing primary amines. As the cells divide, the dye evenly distributes to both daughter cells. Reduction of the median fluorescence intensity (MFI) by approximately half indicates

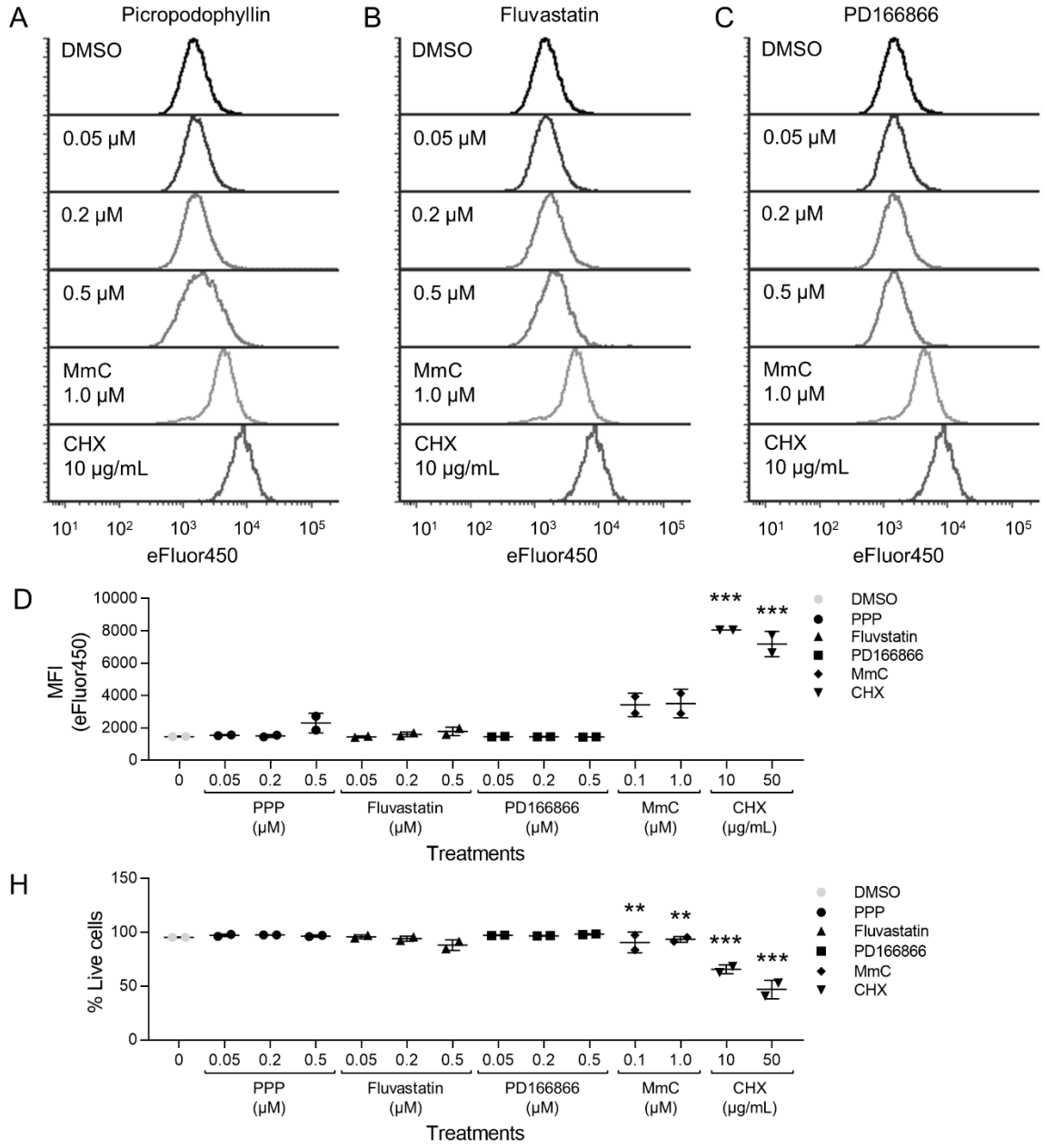

Figure 3.3. PPP, fluvastatin or PD166866 do not inhibit 2D proliferation of JB6 P⁺ cells. Histograms of JB6 P⁺ cells stained with Cell Proliferation Dye eFluor™ 450 were treated with **(A)** PPP, **(B)** fluvastatin, PD166866 **(C)** or the vehicle, DMSO. Inhibition of proliferation is indicated by higher fluorescent signal that is represented by visually distinct rightward shifted histograms as shown following treatment with mitomycin C (MmC) and cycloheximide (CHX). The same histograms for DMSO, MmC (1.0 µM), and CHX (10 µg/mL) are shown for comparison with PPP, fluvastatin, and PD166866. **(D)** The eFluor™ 450 MFI of JB6 P⁺ cells (singlet, SYTOX Red negative cells) stained with Cell Proliferation eFluor™ 450 and treated with PPP, fluvastatin, or PD166866 was graphically depicted. PPP, fluvastatin, and PD166866 did not significantly influence the eFluor™ 450 MFI compared to vehicle (DMSO) controls, whereas MMC and CHX (positive controls) had significantly higher eFluor™ 450 MFI values. **(E)** PPP, fluvastatin, or PD166866 did not significantly decrease the % of live cells (singlet, SYTOX Red negative cells). MmC and CHX significantly induced cell death at 0.1 and 1.0 µM and 10 and 50 µg/mL respectively. Each treatment group had two replicates and analyzed by one-way ANOVA with multiple comparisons (**p<0.01; ***p<0.001).

a successful division. Therefore, cells treated with compounds that inhibit proliferation would have a higher eFluor™ 450 MFI, represented by a rightward shift of the histogram compared to the lower MFI measured in cells that have undergone proliferation. PPP, fluvastatin, and PD166866 had minimal to no effect on JB6 P^+ cell proliferation in 2D culture, as there was no significant difference observed in eFluor™ 450 MFI between these treatments at any concentration and vehicle (DMSO) treatment. (Figure 3.3A-C). Mitomycin C (MmC), a chemotherapeutic agent that inhibits DNA synthesis, and cycloheximide (CHX), a protein synthesis inhibitor, are shown as positive controls. The same histograms for vehicle (DMSO), MmC at 1.0 µM, and CHX at 10 µg/mL are shown for comparison with PPP (Figure 3.3A), fluvastatin (Figure 3.3B), and PD166866 (Figure 3.3C) demonstrating the substantial shift expected when cell proliferation is strongly inhibited. The eFluor™ 450 MFI of the histograms were quantified in Figure 3.3D.

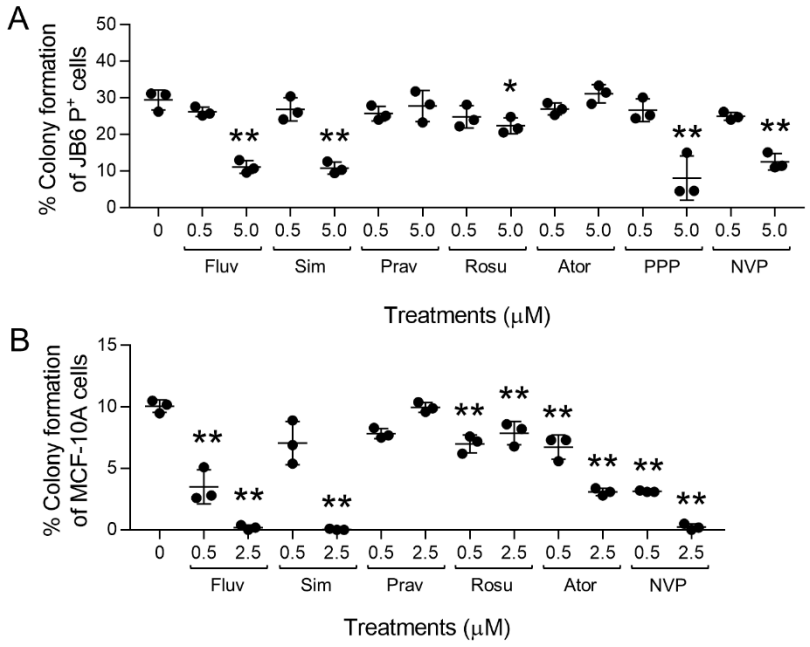

Figure 3.4. IGF1R inhibitors prevent FGF2-stimulated transformation whereas efficacy of HMG-CoA reductase inhibitors varies. A) Fluvastatin (Fluv), simvastatin (Sim), rosuvastatin (Rosu), PPP, and NVP-ADW742 (NVP) significantly attenuated FGF2 (5 ng/mL)-stimulated transformation of JB6 P^+ cells (2,000 cells/well) at 5.0 µM. Colonies were counted via automated counting using the Cytation 3 imaging reader from Biotek using Gen5 3.04 software. Seven pictures were taken every 100 microns and superimposed together by the zprojection function. **B)** Fluvastatin (Fluv), rosuvastatin (Rosu), atorvastatin (Ator), and NVP-ADW742 (NVP) significantly attenuated FGF2 (30 ng/mL)-stimulated transformation of MCF-10A cells (1,000 cells/well) at 0.5 and 2.5 µM. Simvastatin (Sim) was significant at 2.5 µM. Each treatment group had three replicates and analyzed by one-way ANOVA with multiple comparisons (*$p<0.05$; **$p<0.01$).

All eFluor™ 450 labeled samples were also stained with SYTOX Red dead cell stain at the end of the 48-hour incubation before flow cytometric acquisition of samples to determine whether any of the compounds induced cell death. PPP, fluvastatin, and PD166866 did not significantly induce cell death, as the percent of SYTOX Red negative cells were similar to DMSO vehicle (Figure 3.3H). Both MmC and CHX significantly induce cell death compared to DMSO-treated controls (0 μM), as indicated by reduction in the percent of SYTOX Red negative JB6 P^+ cells. Overall, PPP, fluvastatin, and PD166866 did not influence JB6 P^+ cell division or cell viability.

Efficacy of statins and IGF1R inhibitors

To determine if the mechanisms identified by PPP and fluvastatin were compound-specific or applicable to similar classes of drugs, NVP-ADW742 (NVP), an IGF1R inhibitor, and 4 statins (simvastatin, pravastatin, rosuvastatin, atorvastatin) were evaluated for efficacy for preventing FGF2-stimulated transformation. PPP and NVP both inhibited FGF2-stimulated significantly attenuated colony formation. For JB6 P^+ cells stimulated with 5 ng/mL of FGF2, both PPP and NVP significantly attenuated colony formation at 5.0 μM (Figure 3.4A). For MCF-10A cells stimulated with 30 μg/mL of FGF2, NVP significantly attenuated colony formation at 0.5 and 2.5 μM (Figure 3.4B). Fluvastatin, simvastatin, and rosuvastatin significantly inhibited FGF2-stimulated JB6 P^+ colony formation at 5.0 μM (Figure 3.4A). Fluvastatin, rosuvastatin, and atorvastatin significantly prevented FGF2-stimulated MCF-10A colony formation at 0.5 and 2.5 μM (Figure 3.4B). Simvastatin significantly prevented FGF2-stimulated MCF-10A transformation at 2.5 μM (Figure 3.4A).

Discussion

Primary prevention for high-risk patients is arguably the most effective tool for mitigating cancer burden. More effective chemotherapeutics and precise molecular targeting have contributed to a substantial decrease in worldwide mortality from 2005 to 2015[176]. In contrast, cancer incidence has steadily increased from 2005 to 2015, rising 33% and affecting more than 14 million people in 2015[176,177]. The cancers investigated in this manuscript, breast cancer and skin cancer, are two of the ten cancers that are still increasing in incidence[178,179]. Interestingly, obesity-associated cancers are preferentially on the rise, and

account for 40% of cancers diagnoses[47]. These epidemiological studies illustrate that cancer is still a major health burden and suggests a need to prevent obesity-associated cancer incidence. We previously demonstrated a role for FGF2/FGFR1 in visceral obesity-associated epithelial cell transformation[79]. Therefore, using this mechanism, we aimed to develop an HTS method to screen for compounds that have the potential to prevent or attenuate FGF2/FGFR1-stimulated transformation. Herein we describe the first HTS assay that models the process of transformation in an *in vitro* experimental setting. This transformation HTS assay is a tool to identify potential prevention strategies targeting obesity-associated epithelial cancers and if successful, could greatly reduce cancer burden.

The transformation HTS is a novel FGF2/FGFR1 target-based, phenotypic screen that can evaluate the ability of compounds to inhibit transformation. Target-based drug discovery may lack clinical efficacy because inhibiting a single target doesn't account for redundancy and/or compensatory crosstalk that may negate target inhibition[86]. Additionally, using phenotypic approaches with no mechanism of action can identify non-selective agents that influence a plethora of processes by acting on other cell types, receptors, or pathways. A screen that utilizes both target-based and phenotypic qualities creates a mechanism-informed, phenotypic screen that overcomes the individual limitations of each screen type and thus can identify compounds that are more likely to be efficacious *in vivo* [86]. The transformation HTS can identify hits that act directly on FGF2 and/or FGFR1 or can target critical factors in the FGF2/FGFR1 signaling axis required to stimulate transformation. This HTS can identify potential chemopreventive agents and serve as a tool to further elucidate the mechanisms of transformation. PD166866, a selective FGFR1 inhibitor, inhibited FGF2-stimulated transformation, in both ultra-low attachment conditions and the soft agar assay, which confirms FGFR1 as the critical receptor in FGF2-stimulated transformation (Figure 3.1E-G). Furthermore, the transformation HTS identified PPP and fluvastatin as effective chemopreventive agents (Figure 3.2). However, based on our knowledge based on published literature, these two compounds do not directly interact with FGF2 or FGFR1. Therefore, compounds like PPP and fluvastatin can be used to gain mechanistic understanding on how FGF2/FGFR1 stimulates transformation. Screening more compound libraries should reveal more efficacious compounds that would further aid in elucidating the mechanisms of obesity-associated transformation.

The transformation HTS method is the first to stimulate a non-tumorigenic cell to transform *in vitro* while overcoming limitations of the soft agar assay and 2D cell proliferation/apoptosis assays for chemoprevention drug discovery. The soft agar assay in its traditional 6-24 well plate format is laborious, inefficient, and costly, and is not usable for high-throughput screening [79,180,181]. However, studies have scaled up the soft agar assay to a 96- or 384-well format to screen for chemotherapeutic compounds[182-184]. For example, Horman *et al*. developed an HTS-compatible 3D colony formation assay in a 384-well plate by incubating 150 HCT116 human colorectal carcinoma cells with compounds for 5 days, quantifying colonies with a laser-scanning fluorescence cytometer[183]. Methods such as this successfully identify chemotherapeutics but are not suitable for identifying chemopreventive agents for two reasons. First, these soft agar assays use cancer cell lines which are functionally different from non-transformed cells regarding activated/inhibited signaling pathways, changing the druggable pathways in each cell type. Because the methodology for true prevention assays is underdeveloped, inhibiting colony formation of transformed cells is used to investigate both chemotherapeutic[185] and chemopreventive[186] compounds, highlighting a need for more effective models for chemoprevention that target the transformation process. Second, using non-transformed cell lines in a high-throughput soft agar assay results in only a small fraction of these cells forming colonies over the course of 10-14 days making visualizing colony formation and inhibition a challenge. Furthermore, achieving a Z-factor of 0.5 or above would be highly improbable. While traditional 2D prevention assays are advantageous because they are easily scaled up to 384-1536-well formats, mechanisms of 2D growth are different from mechanisms of 3D growth[169]. For example, Figure 3.1I-J demonstrates that FGF2 stimulates 3D growth of JB6 P^+ cells, but not 2D growth. Moreover, these traditional 2D prevention assays use cancer cells. Using cancer cells to gain mechanistic insight may not be fully representative of the mechanism(s) to prevent carcinogenesis[185,186].

One limitation to the transformation HTS, as with many anti-cancer screens, is that cytotoxic compounds will show up as hits. For example, Roridin A and CHX, cytotoxic compounds that inhibit protein synthesis, inhibited 3D growth by more than 60% in the initial screen and were selected as primary hits. Since our libraries consisted of known and/or FDA approved compounds, those compounds could be eliminated based on published/known toxicities in non-transformed cells. CHX was used in the eFluor™ 450 proliferation experiment as a control that inhibits 2D proliferation (Figure 3.3). For unknown or novel

compounds, following cytotoxicity studies should be performed to ensure that compounds are preventing the process of transformation and are not simply inducing cell death or inhibiting vital cell functions like protein synthesis.

Our transformation HTS resulted in the identification of picropodophyllin (PPP) as an inhibitor of FGF2-stimulated growth in ultra-low attachment conditions. PPP is a cyclolignan alkaloid from the mayapple plant family. PPP has been suggested to have anti-neoplastic activity by inhibiting IGF1R[187], a receptor tyrosine kinase that is a key regulator of energy metabolism ant tumor growth. PPP inhibits the IGF1R by inducing the activation loop-specific inhibition of tyrosine phosphorylation. Although it has been suggested that PPP is specific for IGF1R at nanomolar concentrations, it is unknown if PPP at micromolar concentrations will inhibit FGF1R, a mechanism that will be explored in future studies. A role of IGF1R itself in transformation is supported by recent investigations into metformin as a chemopreventive agent. Metformin inhibits insulin like growth factor 1 (IGF1)/IGF1R signaling[188]. Furthermore, an additional IGF1R inhibitor, NVP-ADW742 attenuated FGF2-stimulated transformation of JB6 P^+ cells suggesting a potential role for IGF1R in FGF2/FGFR1-driven transformation. Interestingly, there are elevated circulating and tissue levels of both insulin growth factor and insulin in obesity, suggesting that a combination of elevated growth factors may increase cancer risk[174,188].

PPP has demonstrated both safety and efficacy in clinical studies and mouse models of tumorigenesis. In a phase I/II trial of four patients with squamous cell lung carcinoma, PPP treatments induced necrosis in the tumor and disease progression was halted for seven months[172]. None of the patients in this study showed dose-limiting toxicity[172]. These studies showed that PPP is a potential chemotherapeutic and has good tolerability[172]. *In vivo* mouse models demonstrated that PPP decreased tumorigenesis with no associated toxicity. In a mouse model of Benzo(a)pyrene (BaP)-induced lung tumorigenesis, PPP decreased tumor volume, increased apoptosis (caspase-3) and decreased proliferation (Ki-67) in the tumor[173]. Additionally, these A/J mice were treated with PPP once a day, five times a week for 20 weeks and there were no changes in body weight and no overt side effects[173]. In another study using a xenograft model of multiple myeloma (MM), PPP was subcutaneously administered to mice with established MM tumors. PPP significantly decreased tumor burden and inhibited tumor- associated angiogenesis and osteolysis. PPP also significantly prolonged the life of the mice from 100 days to 150 days[174]. It is important

to note that current published studies examine effects of PPP on established tumors, whereas this manuscript investigates PPP as a chemopreventive agent, we test its ability to prevent the process of transformation, revealing a new clinical target for prevention that has not been previously explored. Collectively, these studies show that PPP has oral clinical efficacy in humans and overall is well tolerated, suggesting that PPP has the potential to have utility for cancer prevention.

Our transformation HTS also identified fluvastatin as an inhibitor of FGF2-stimulated growth in ultra-low attachment conditions in the primary screen. Fluvastatin is one of several 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, cholesterol lowering agents that treat dyslipidemia and prevent cardiovascular disease[189]. Statins work by competitive inhibition of HMG-CoA reductase, the rate-limiting step in cholesterol biosynthesis, causing reductions in cholesterol and low-density lipoproteins (LDL) and an increase in high-density lipoproteins (HDL), that carry cholesterol from other parts of the body to the liver for removal[189,190]. Fluvastatin is a good candidate for chemoprevention because it has a favorable safety profile and has been shown to have anti-cancer activity[190]. Fluvastatin inhibits breast cancer cell proliferation and with a greater potency in estrogen receptor (ER) negative breast cancer cells[191,192]. Interestingly, fluvastatin inhibited FGF2-stimulated transformation of MCF-10A cells, which are ER negative. Recently, FGFR1 activation was identified as the primary mechanism by which obesity drives estrogen receptor positive mammary tumor progression following endocrine deprivation[193]. These studies suggest that fluvastatin may be efficacious for inhibiting obesity-promoted mammary tumor progression and a potential compound for secondary prevention in obese patients.

Epidemiological studies that evaluate statins and cancer risk have been inconclusive. A 2006 meta-analysis by Browning *et al*. reviewed the association between statins and cancer risk investigating 42 studies and concluded statins use is not associated with short-term cancer risk. However, these studies had relatively short follow-ups that were too brief to capture a true association between statin use and cancer incidence or mortality[194]. In more recent analyses, Yang *et al*. (2017)[195] analyzed four articles and came to the tentative conclusion that fluvastatin may reduce breast cancer risk but further high-quality research is needed to confirm this. Likewise, Liu *et al*. (2017) investigated seven studies and suggested that lipophilic statins (like fluvastatin, simvastatin, and atorvastatin) were more protective than hydrophilic stains (like pravastatin and rosuvastatin) but due to high heterogeneity between the studies made it difficult

to see an advantageous benefit for this population[196]. Ultimately, long-term data is lacking to support the role of statins in primary chemoprevention[197,198]. We investigated four additional statins including simvastatin, rosuvastatin, pravastatin, and atorvastatin to determine if the effects on colony formation were specific to fluvastatin. Not all the statins attenuated colony formation, nor did efficacy correlate with statin lipophilicity. Additionally, similar effects were observed in both cell lines, except for atorvastatin which had no effect on colony formation of JB6 P^+ cells but attenuated colony formation of MCF-10A cells (Figure 3.4). Follow-up studies focused on mechanisms underlying the efficacy of statins are warranted.

Overall, we optimized a novel HTS of FGF2-stimulated transformation utilizing growth in ultra-low attachment conditions. This is the first screen to stimulate non-tumorigenic cells to transform *in vitro.* Additionally, this assay has the potential to be optimized with other tumor promoters such as hepatocyte growth factor, epidermal growth factor, and phorbol esters, as well as with complete carcinogens such as BaP. The transformation HTS identified PPP and fluvastatin as potential chemopreventive agents. After these compounds were confirmed to concentration-dependently inhibit FGF2 stimulated transformation, they were validated in soft agar with two non-tumorigenic cell lines, JB6 P^+ and MCF-10A cells. Future studies will test these compounds *in vivo* and evaluate their mechanism of action. Overall, the transformation HTS is a fast, robust and uniquely adept 3D screen to identify potential chemopreventive compounds.

Materials/Methods

Cell culture

JB6 P^+ cells (mouse skin epidermal cells) were obtained from ATCC (Manassas, VA, USA). Cells were cultured in minimum essential medium (MEM) supplemented with 5% fetal bovine serum (FBS) and 1% penicillin/streptomycin (p/s) (JB6 P^+ growth media). JB6 P^+ cells were trypsinized with 0.05% trypsin and quenched with MEM with 5% FBS and 1% p/s. JB6 P^+ cells are used below passage 15 prevent spontaneous transformation.

MCF-10A cells (human mammary epithelial cells) were obtained from ATCC (Manassas, VA, USA). Cells were cultured in DMEM/Ham's F:12 media supplemented with 5% horse serum (HS), 1% p/s, 100 ng/mL cholera toxin, 20 ng/mL epidermal growth factor (EGF), 10 µg/mL insulin, 0.5 mg/mL hydrocortisone,

7.5% sodium bicarbonate, 15mM HEPES, and 2 mM L-Glutamine (MCF-10A growth media). MCF-10A cells were trypsinized with 0.05% trypsin and quenched in DMEM/ Ham's F12 media with 20% HS and antibiotics (resuspension media). MCF-10A cells are used below passage 20 prevent spontaneous transformation.

Reagents

Mouse FGF2 is a recombinant protein purchased from Prospec (CYT-386). Fresh powder for confirmation was ordered from the NCI's DTP or from Caymen Chemical. Picropodophyllin and fluvastatin were obtained from the DTP (Figure 2). Fluvastatin (10010337), simvastatin (MK-733), rosuvastatin (ZD 4522), pravastatin (10010342), atorvastatin (10493), cycloheximide (14126), and mitomycin C (11435) were ordered from Caymen Chemical. PD166866 (S8493) and NVP-ADW742 (S1088) was purchased from SelleckChem. eBioscience™ Cell Proliferation Dye eFluor™ 450 (65-0842-85) and SYTOX™ Red Dead Cell Stain, for 633 or 635 nm (S34859) were purchased from ThermoFisher. I-BET-762 was purchased from JSTAR Research Inc.

Generating fat tissue filtrate

Mouse fat tissue filtrate (MFTF) was made as previously described[79]. Briefly, the parametrial fat pad was removed from 13-week-old mice that had been on a HFD for 4 weeks. This fat was placed in a transwell insert above serum free media to collect factors from the fat (MFTF). This fat was used in the soft agar assay at 200 µg/mL.

All animal experiments were performed in adherence to principles stated in the Guide for the Care and Use of Laboratory Animals (NIH publication, 1996 edition). The IACUC and Animal Care Program of Michigan State University (East Lansing, MI, USA) approved all animal experiments under AUF 02/15-027-01.

Soft agar assay

JB6 P^+ cells were plated at 500 cells/well (or otherwise indicated) in a 24-well plate in 200 µL of MEM media with 10% FBS in 0.33% agar overlaid onto 350 µL of MEM media with 10% FBS in 0.5% agar. FGF2

(Prospec, CYT-386) was incubated with the cells at 0.5 ng/mL and compared to untreated controls. Soft agar plates were left at room temperature for 30 minutes then incubated at 37°C.

MCF-10A cells were seeded at 750 cells/well (or otherwise indicated) in a 24-well plate in 200 µL of DMEM/Ham's F12, 5% HS, and 0.33% agar overlaid onto 350 µL of DMEM/Ham's F12, 5% HS, and 0.5% agar. FGF2 (prospec, CYT-386) was incubated with the cells at 20 ng/mL and compared to untreated controls. Soft agar plates were left at room temperature for 30 minutes before 200 µL of MCF-10A growth media was gently added to each well and then stored at 37°C. Every 3-4 days, the growth media was removed from each well and replenished with 200 µL of MCF-10A growth media.

After two weeks, JB6 P^+ and MC2HitF-10A soft agar plates were fixed in 70% ethanol (EtOH) and stained with 150 µL of 0.01% crystal violet. Colonies were visually counted and used to calculate the percent of colony formation from the number of cells plated ([Colonies counted x 100] / number of cells plated).

Transformation HTS

JB6 P^+ cells were plated in 384-well round-bottom low attachment plates at 200 cells/well in 40 µL of JB6 P^+ growth media. FGF2 (30 ng/mL) was used to stimulate JB6 P^+ cell growth in ultra-low attachment conditions. Cells were manually added to each plate with a multichannel, repeater pipette. Compounds were plated using a dual arm Biomek FX liquid handling robot with 384-well pintool liquid handling system. Plates were incubated at 37°C for 96 hours. Then 40 µL of CellTiter Glo (Promega) was added to each well. Plates were shaken at 300 rpm for 5 mins on a plate shaker and then a Biotek synergy Neo HTS Multi-Mode Microplate Reader detected the luminescence signal of each well. Untreated cells were used as the negative control. For all compound screening, FGF2 and untreated controls were treated with the vehicle, DMSO.

A Z-factor was used to evaluate the quality of a HTS. A Z-factor is a screening window coefficient that qualitatively assesses the ability of a screen to identify active compounds or hits while screening large compounds libraries. The Z-factor considers the means and standard deviation of the positive and negative controls ($Z\ factor = 1 - (3(\sigma_p + \sigma_n)) / | \mu_p - \mu_n |$). A high quality HTS assay has a Z-factor that ranges between 0.5 and 1.0. A Z-factor was calculated during method development to optimize the parameters of the screen to achieve a Z-factor of 0.5 or above during the screening assay.

Compound libraries

The Prestwick Chemical Library®, the National Cancer Institute (NCI) Natural products library, and a Michigan State University (MSU) library of MSU-made compounds were used for screening in the MSU Assay Development and Drug Repurposing Core (ADDRC). The Prestwick Chemical Library® is a unique collection of 1280 diverse, small molecules consisting of mostly FDA approved drugs, with known bioavailability and safety. The NCI Natural products library consists of 419 compounds selected from the Developmental Therapeutics Program (DTP) open repository and has a variety of scaffold structures with multiple functional groups. The MSU compounds were synthesized by Dr. Jetze Tepe (MSU) and designed to mimic the diverse structural features found in natural products.

Cell viability and proliferation assessment by flow cytometry

JB6 P^+ cells were stained with Cell Proliferation Dye eFluor™ 450, resuspended in JB6 P^+ growth media and were plated at 50,000 cells/well in 6-well plates. After 24 hours, cells were treated with PPP, fluvastatin, PD166866, or DMSO (vehicle control). After an additional 48 hours, cells were prepped for flow cytometric analysis. Cells were trypsinized, washed, resuspended in PBS, and filtered. Prepared JB6 P^+ cells were stained with SYTOX ™ Red Dead Cell Stain at a concentration of 5 nM, which was added approximately 15 minutes before analyzing samples on a BD FACS Aria IIu located in the MSU South Campus Flow Cytometry Core. Flow cytometry data was analyzed using FCS Express (DeNovo Software). Viability was assessed as a percentage of singlet, SYTOX Red negative cells. Proliferation was assessed by measuring the eFluor™ 450 median fluorescence intensity (MFI) of live JB6 P^+ cells (singlet, SYTOX Red negative cells).

Statistical analysis

Data are presented as mean ± SD. For soft agar experiments, three technical replicates were used and analyzed by one-way ANOVA, multiple comparisons. For HTS method development, Z-factor was used to define optimal parameters giving a quality HTS able to define primary hits. For all statistical tests, the 0.05, 0.01, and 0.001 level of confidence was accepted for statistical significance.

CHAPTER 4

Investigating the mechanistic connection between visceral adipose tissue and the mevalonate pathway in ER– breast epithelial cell transformation

Abstract

Cancer prevention has the potential to drastically reduce the number of breast cancer cases. However, cancer prevention strategies are difficult to develop and implement because compliance can be difficult to maintain in a general population because of the prevention paradox. The prevention paradoz states that while a population sees the benefits of generalized prevention, it often times goes unnoticed on an individual basis. Therefore, identifying a high-risk population further encourages compliance resulting in more successful implementation prevention strategies. For example, pre-menopausal women who are obese are high-risk for developing ER– breast cancer and would benefit from cancer prevention strategies. However, there are no pharmacological means to prevent for ER– breast cancer. My previous studies identified FGF/FGFR1 signaling as a mechanistic target for the inhibition of ER– breast epithelial cell transformation. Our previous data demonstrated that FGF2 in VAT can stimulate malignant transformation of breast epithelial cells by activating FGFR1. Furthermore, I revealed that fluvastatin, an HMGCR inhibitor, could prevent FGF2-assciated transformation, identifying fluvastatin as a potential chemopreventive agent. HMGCR is the rate limiting enzyme in the mevalonate pathway and interestingly, literature has not demonstrated a mechanistic connection between the mevalonate pathway and FGF2/FGFR1 signaling. Therefore, the objective of these studies is to investigate how an HMGCR reductase inhibitor mechanistically inhibits FGF2/FGFR1 stimulated transformation. Herein, I demonstrated that factors from VAT upregulates enzymes in the mevalonate pathway, suggesting that VAT does affect the mevalonate pathway. However, more research is needed to fully understand the relationship between FGF2/FGFR1 signaling and the mevalonate pathway.

Introduction

Women who are high risk for obesity-associated pre-menopausal ER– breast cancer are in need chemopreventive strategies. Obesity is now a global pandemic and obesity rates are expected to rise. The prevalence of obesity in the United States was 42.4% in 2018, a ~12% increase from 2000[13]. Epidemiologically, high BMI/increased abdominal obesity is strongly associated with premenopausal triple negative breast cancer (TNBC)[54,199,200]. TNBC is partly defined by absence of the estrogen receptor (ER) and current breast cancer chemoprevention compounds are only effective against breast cancers

expressing the ER. Tamoxifen, a selective ER modulator (SERM), reduces the risk of ER+ breast cancer by almost 50%,[46] but does not reduce the risk ER– breast cancer. Therefore, premenopausal obese women are a potential high-risk target population that would benefit from chemoprevention strategies.

Elucidating the mechanisms of how obesity promotes ER– breast cancer would reveal targets for chemopreventive compounds. Our published and supporting data revealed a key role for visceral adipose tissue (VAT)-derived fibroblast growth factor 2 (FGF2) via fibroblast growth factor receptor 1 (FGFR1) signaling in estrogen receptor (ER) negative (ER–) tumor initiation and progression. In addition, there is a high rate of FGFR1 amplifications[201-203] and sustained activation of the Ras/MAPK pathway in TNBC and basal-like breast cancer (as opposed to other subtypes)[204,205]. This suggests breast carcinomas and their normal epithelial precursors may use the Ras/MAPK pathway for survival. The Ras/MAPK pathway is activated by receptor tyrosine kinase receptors like FGFR1. Therefore, FGFR1/Ras signaling could be a viable target for obesity-associated ER– breast cancer prevention. However, directly targeting FGFR1/Ras signaling is difficult. While preclinical FGFR1 inhibitors (and one clinical FGFR inhibitor, Balversa) exist, they likely lack the high safety threshold chemoprevention requires[206,207] and oncogenic Ras is considered an 'undruggable' drug target, which means an effective chemopreventive agent will likely interact indirectly with FGFR1/Ras. While activating Ras mutations in breast cancer are rare, Ras expression and activity is often enhanced in breast cancer[208].

To discover potentially safe chemopreventive and therapeutics for obesity-associated pre-menopausal ER– breast cancer, I developed a novel phenotypic high throughput screen (HTS) targeting FGFR1/Ras-driven malignant transformation to identify compounds that inhibit FGF2-stimulated 3D growth[209]. Using our HTS, fluvastatin emerged as the lead candidate, showing concentration-response efficacy in our soft agar assay (inhibiting and FGF2-stimulated transformation in MCF-10A cells)[209]. Chemoprevention strategies must effectively reduce cancer burden and have minimal to no side effects and fluvastatin is a clinically approved drug that is relatively safe[210]. Fluvastatin inhibits 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (HMGCR), the rate limiting enzyme in the mevalonate pathway. Before the transformation HTS screen, there has not been a described relationship between FGFR1 signaling and the mevalonate pathway.

Our HTS was not the first to implicate HMGCR in malignant transformation and breast cancer. Clendening *et. al.* demonstrated that deregulated HMGCR expression (increased HMGCR activity) in MCF-10A (non-tumorigenic ER– human breast epithelial) cells stimulated transformation as determined by the soft agar assay[211]. Furthermore, another study demonstrated that a microRNA (MiRNA) which represses HMGCR and HMGCS1, decreased through an *in vitro* model of breast cancer progression, starting with normal-like MCF10A to preneoplastic MCF10.AT1, and invasive MCF10.Ca1d cells[30]. This means that HMGCR and HMGCS1 activity increased in this *in vitro* model of breast cancer progression[30]. Epidemiology also corroborates mevalonate involvement in early transformation events — 93% of ductal carcinomas in situ (DCIS) express HMGCR[212]. Furthermore, mevalonate pathway activity is linked to visceral obesity as visceral obesity, and not subcutaneous obesity, is positively associated with elevated serum levels of squalene, which is cholesterol's precursor[213]. Additionally, the mevalonate pathway synthesizes sterol isoprenoids (e.g., cholesterol, farnesyl pyrophosphate (FPP), geranylgeranyl pyrophosphate (GGPP)) and non-sterol isoprenoids (e.g., dolichol, heme-A, isopentenyl tRNA and ubiquinone[90]). Isoprenoids like FPP and GGPP are used to prenylate Rho and Ras family proteins which induces Rho/Ras plasma membrane localization enabling GTPase activation. Therefore, inhibition of HMGCR has the potential to alter posttranslational modifications of signaling molecules[90,91]. Dysregulation of HMGCR and the mevalonate pathway may make a causal contribution to obesity-associated ER– cancer, suggesting statins may be useful for prevention[211]. However, studies have not identified how mevalonate pathway dysregulation can promote transformation.

Unraveling the connection between VAT-derived growth factor stimulation and malignant transformation will provide new targets for chemoprevention. Identifying fluvastatin further informs on the mechanisms involved in ER– breast epithelial cell transformation as it suggests a role for HMGCR dysregulation and the mevalonate pathway. Therefore, the objective of these studies is to determine the role of the mevalonate pathway in VAT-stimulated ER– breast carcinogenesis. We hypothesize that FGF2 and factors from VAT activate the mevalonate pathway through FGFR1 activity and subsequent Ras protein prenylation/activation drives ER– transformation.

Herein, we demonstrate that VAT stimulation of MCF-10A cells induced protein expression of mevalonate pathway enzymes HMG-CoA synthase 1 (HMGCS1), farnesyl transferase (FNTA), squalene

synthase (FDFT1) and Harvey rat sarcoma viral oncogene homolog (HRas). In addition, this protein upregulation was prevented by PD166866, a selective FGFR1 inhibitor. While HRas induction was measured by both western blot and immunofluorescence by VAT and FGF2, we failed to confirm its activation status.

Results

Statins have varying efficacy in preventing MFTF-stimulated transformation

Previous data demonstrated that fluvastatin attenuated FGF2-stimulated transformation of MCF-10A cells as measured by colony formation in the soft agar assay[209]. Mouse fat tissue filtrate (MFTF), made from factors derived from VAT, was used in soft agar with MCF-10A cells to stimulate transformation. Fluvastatin also prevented MFTF-stimulated MCF-10A cell transformation. To evaluate if other statins can prevent MFTF-stimulated transformation simvastatin, atorvastatin, pravastatin, and rosuvastatin were also tested for efficacy (Figure 4.1). Fluvastatin and simvastatin significantly attenuated MFTF-stimulated transformation. Pravastatin and rosuvastatin, hydrophilic statins, did not attenuate MFTF-stimulated transformation. Atorvastatin is the most lipophilic statin, followed by simvastatin, fluvastatin, pravastatin, and then rosuvastatin[214]. While fluvastatin and simvastatin are both lipophilic statins, atorvastatin is the most lipophilic statin, thus demonstrating lipophilicity does not determine efficacy, but efficacious statins have a tendency to be lipophilic.

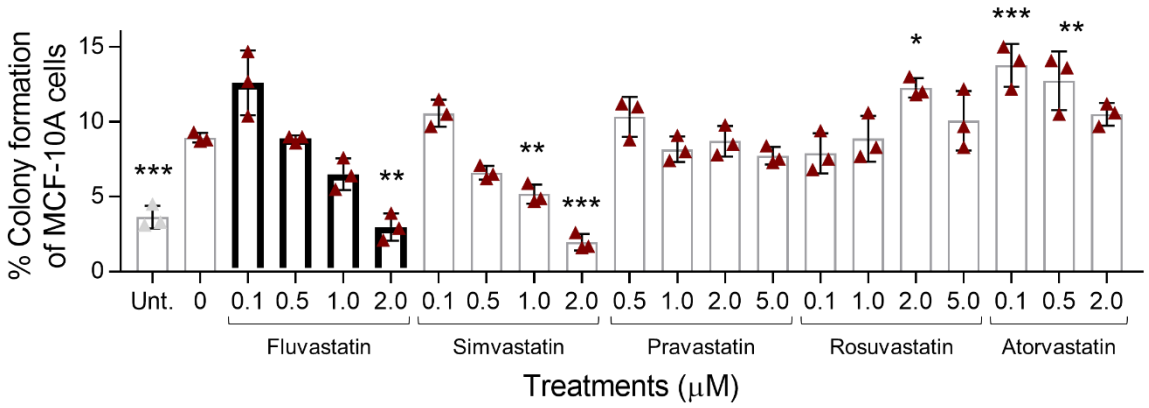

Figure 4.1. Lipophilic statins, fluvastatin and simvastatin concentration-dependently inhibit MFTF-stimulated transformation of MCF-10A cells. Fluvastatin significantly attenuated MFTF (200 µg/mL)-stimulated transformation of MCF-10A cells (1,000 cells/well) at 2.0 µM. Simvastatin significantly attenuated MFTF-stimulated transformation of MCF-10A cells at 1.0 and 2.0 µM. Pravastatin, rosuvastatin, and atorvastatin did not attenuate MFTF-stimulated transformation of MCF-10A cells. Rosuvastatin and atorvastatin enhanced MFTF-stimulated transformation. Colonies were counted via automated counting using the Cytation 3 imaging reader from Biotek using Gen5 3.04 software. Seven pictures were taken every 100 microns and superimposed together by the zprojection function. Treatments (3 biological replicates) were analyzed by one-way ANOVA with multiple comparisons (*p<0.05, **p<0.01, ***p<0.001 vs 0 µM). This experiment was repeated one time.

MFTF upregulates protein expression of enzymes in the mevalonate pathway

To determine if the mevalonate pathway is upregulated by MFTF, MCF-10A cells were treated with MFTF for 24 hours with and without fluvastatin or PD166866, a selective FGFR1 inhibitor. Protein lysates were probed for proteins involved in the mevalonate pathway as indicated in Figure 4.2. At 24 hours, MFTF upregulated HMGCS1, FNTA, and HRas protein expression. PD166866 prevented this MFTF-induced protein upregulation compared to untreated control. Fluvastatin upregulated HMGCS1, which is in line with published literature. Inhibiting HMGCR induces a feedback loop that upregulates the HMGCR in an attempt to gain HMGCR activity[215,216]. Fluvastatin also prevented MFTF-upregulation of HRas and FNTA.

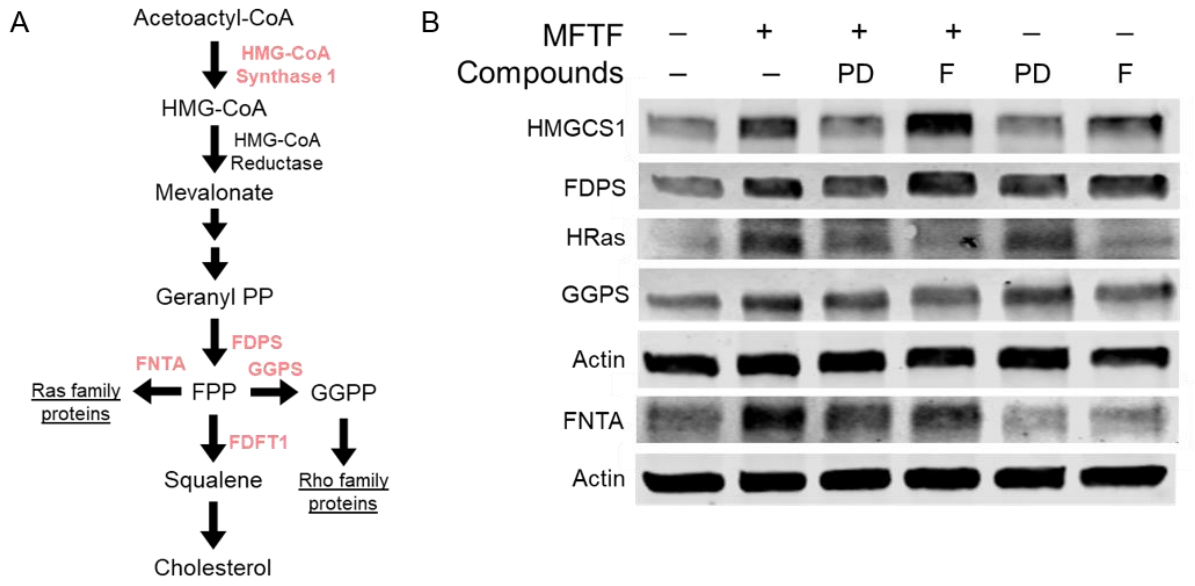

Figure 4.2. MFTF upregulates protein expression of enzymes in the mevalonate pathway and this upregulation is prevented by PD166866, a selective FGFR1 inhibitor. A) Diagram of the mevalonate pathway First, two Acetyl-CoA molecules are condensed into acetoacetyl-CoA by acetoacetyl-CoA thiolase. From here HMG-CoA synthase 1 (HMGCS1) produces the metabolite HMG-CoA. Next, HMG-CoA reductase, the rate limiting enzyme, converts HMG-CoA to mevalonate. Through multiple steps, this pathway produces geranyl pyrophosphate (GPP) and then farnesyl pyrophosphate synthase (FDPS) adds an isopentenyl-5- pyrophosphate (IPP) to create FPP. FPP can either be converted to GGPP by geranylgeranyl pyrophosphate synthase 1 (GGPS1) or FPP can be converted to squalene by squalene synthase (FDFT1) which ultimately leads to cholesterol biosynthesis. B) MCF-10A cells were treated with MFTF (200 µg/mL) and/or fluvastatin (1 µM), PD166866 (10 µM) for 24 hours (fluvastatin and PD166866 were pre-treated 30 minutes before MFTF). The same lysates were run twice at 22 µg of protein per sample. Membranes were re-probed for proteins. The protein probes appear above their respective actin bands. This experiment was repeated once.

To determine if proteins upregulation is transient or sustained, MCF-10A cells were treated with 200 µg/mL of MFTF over 8, 24, 48, and 72 hours (Figure 4.3). The relative intensity for each protein was normalized to actin (Figure 4.3 B-F). HMGCS1 is upregulated at 24 hours and this protein upregulation is maintained for an additional 48 hours (Figure 4.3 B). FDFT1, squalene synthesis is upregulated at 24 hours, but returns to baseline levels after 72 hours (Figure 4.3 C). The relative protein intensity of FDPS (Figure 4.3 D) and FNTA (Figure 4.3 F) moderately increased (Figure 4.3 A). GGPS1 protein expression remained unchanged (Figure 4.3 A, E).

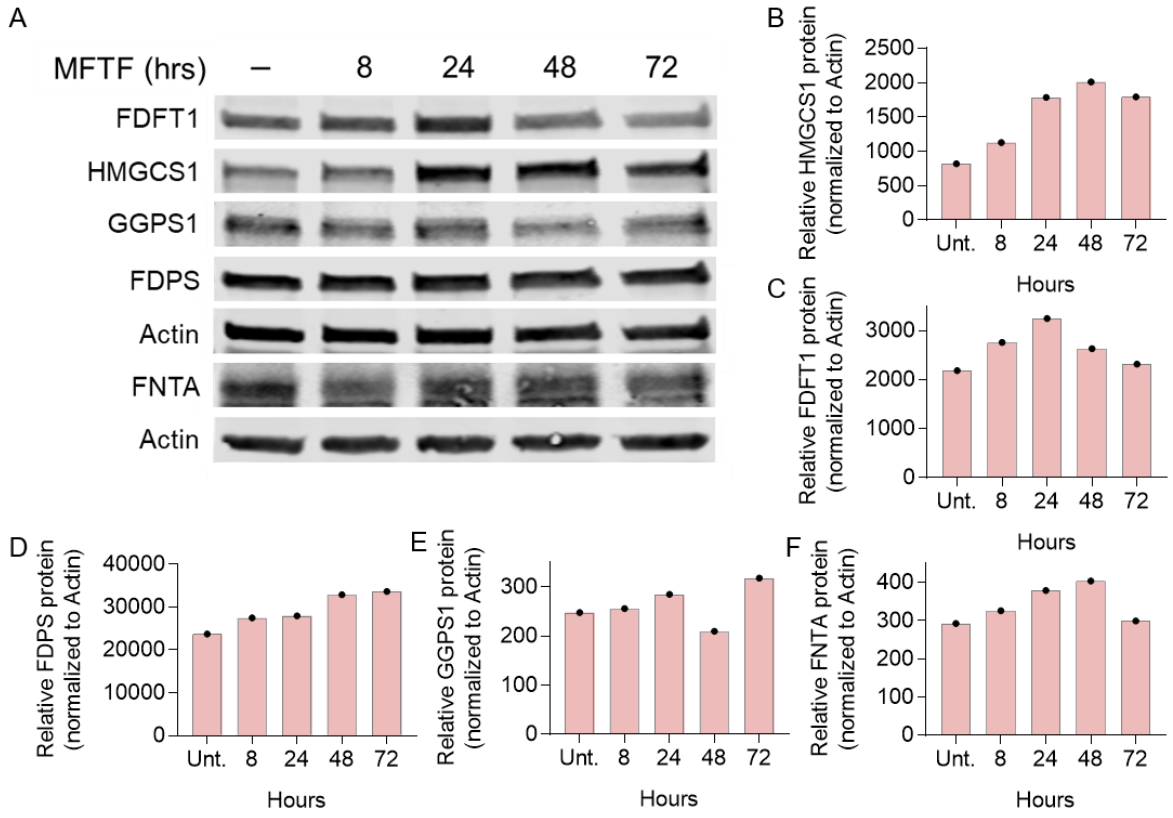

Figure 4.3. MFTF induces sustained upregulation of HMGCS1 protein and transient upregulation of FDFT1 protein at 24 hours. B) MCF-10A cells were treated with MFTF (200 µg/mL) for 8, 24, 48 and 72 hours. The same lysates were run twice at 20 µg of protein per sample. Membranes were re-probed for proteins. The protein probes appear above their respective actin bands. This experiment was performed once.

FGF2/MFTF does not alter mRNA expression of mevalonate pathway related proteins

RT-PCR was used to investigate if MFTF-stimulated protein expression was a product of MFTF-activated gene transcription (Figure 4.4). FGF2 after 1, 4, and 24 hours did not affect mRNA expression of HMGCR, FDPS, GGPS1, FNTA, HRas, and SREBF2. Fluvastatin upregulated mRNA of HMGCR, FDPS, and SREBF2 after 24 hours, which is also a result of the afore mentioned positive feedback loop in line with

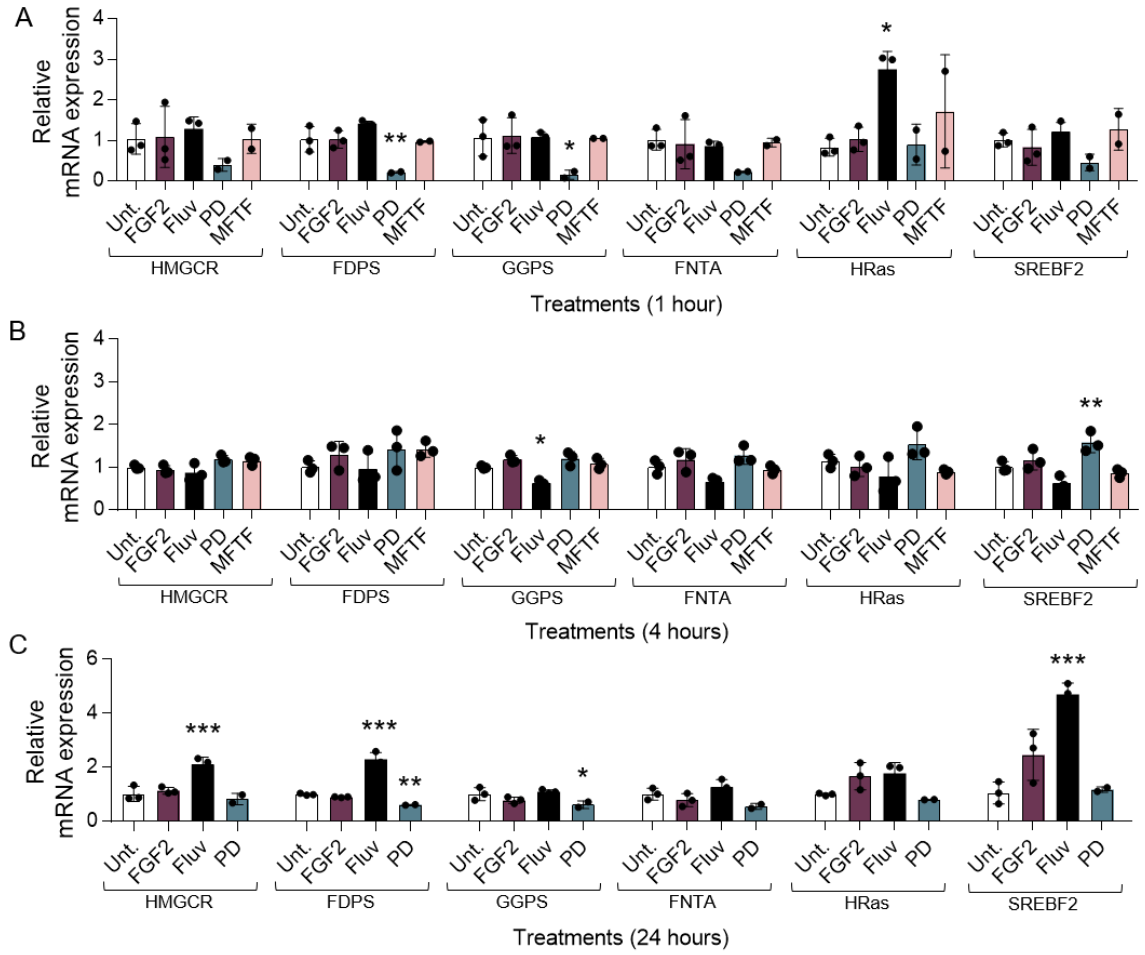

Figure 4.4. FGF2 and MFTF do not alter mRNA expression of enzymes in the mevalonate pathway in MCF-10A cells. MCF-10A cells were untreated (Unt.) or FGF2 (30 ng/mL) treated, fluvastatin (Fluv) (2.5 µM), PD166866 (PD) (10 µM), or MFTF (200 µg/mL) for 1, 4, or 24 hours. mRNA expression levels of HMGCR, FDPS, GGPS1, FNTA, HRas, and SREBF2 were normalized to GAPDH. Fluvastatin upregulated HRas at 1 hour and HMGCR, FDPS, and SREBF2 at 24 hours. Fluvastatin downregulated GGPS at 4 hours. PD166866 downregulated FDPS and GGPS at 1 hour and 24 hours. PD166866 upregulated SREBF2 at hours. Each treatment group has 3 biological replicates, 2 technical replicates. Treatment groups with 2 biological replicates did not have enough RNA recovered during sample prep. Data is presented as mean ± SD (*p<0.05, **p<0.01, ***p<0.001 vs Unt. control)

published literature. Interestingly, Fluvastatin upregulated HRas mRNA at 1 hour. PD166866 downregulated FDPS and GGPS mRNA at 1 hour and 24 hours and upregulated SREBF2 mRNA at 4 hours. This upregulation indicates fluvastatin is inhibiting HMGCR, its known mechanism of action.

Acute FGF2 or VAT stimulation failed to stimulate Ras activation in MCF-10A cells

The mevalonate pathway begins with acetyl coenzyme A (Acetyl-CoA) which and can produced products like cholesterol and isoprenoids. Isoprenoids like farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP) are used for proteins that undergo prenylation, the attachment of a moiety onto proteins like Rho or Ras, stimulating membrane localization and in turn activation (Figure 4.2). To determine if isoprenoid production is critical in FGF2/FGFR1-stimulated transformation, geranyl pyrophosphate (GPP) and mevalonate (MVL) were exogenously added to MCF-10A cells in the soft agar assay. As a positive control, FGF2 was used to stimulate transformation. PD166866 attenuated FGF2-stimulated transformation, as previously demonstrated[209]. GPP and MVL did not stimulate transformation of MCF-10A cells at 10 or 20 ng/mL and these isoprenoids did not rescue the inhibitory effects of PD166866 (Figure 4.5).

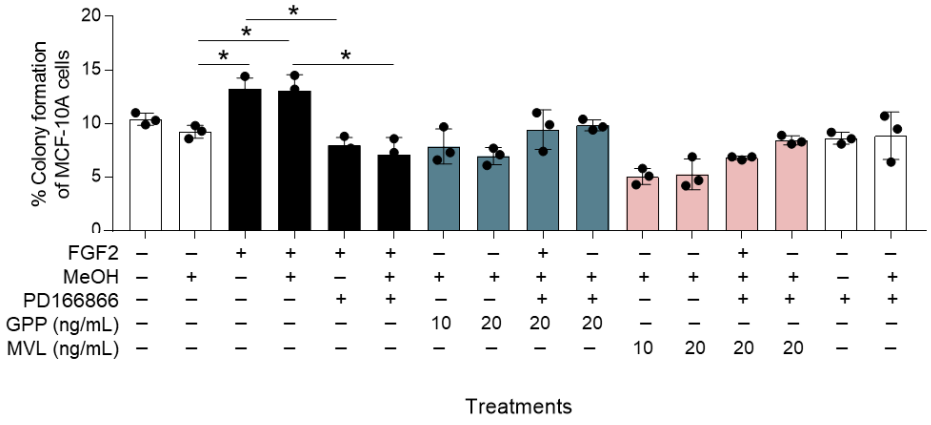

Figure 4.5. Isoprenoids GPP and MVL failed to stimulate transformation of MCF-10A cells and do not rescue the attenuating effects of PD166866. A) MCF-10A cells were treated with FGF2 (30 ng/mL), PD166866 (10 µM) and either 10 or 20 ng/mL of GPP or MVL. GPP and MVL are constituted in methanol (MeOH) and is the vehicle for this experiment. Colonies were counted via automated counting using the Cytation 3 imaging reader from Biotek using Gen5 3.04 software. Seven pictures were taken every 100 microns and superimposed together by the zprojection function. Treatments (3 biological replicates) were analyzed by one-way ANOVA with multiple comparisons (*p<0.05).

Isoprenoid production leads to protein prenylation which localizes Ras to the membrane where its GTPase activity activates signaling cascades[88]. Therefore MCF-10A cells were treated with FGF2 to determine if FGF2 induced Ras membrane localization and/or Ras activation. MCF-10A cells were treated with FGF2 for 24 hours and Ras expression patterns were evaluated using immunofluorescence (Figure 4.6A). FGF2 increased HRas protein expression but did not induce HRas localization. A Ras activation assay (Ras GTPase Chemi ELISA Kit) demonstrated that FGF2 (30 ng/mL) failed to activate Ras in MCF-10A cells (Figure 4.6 B).

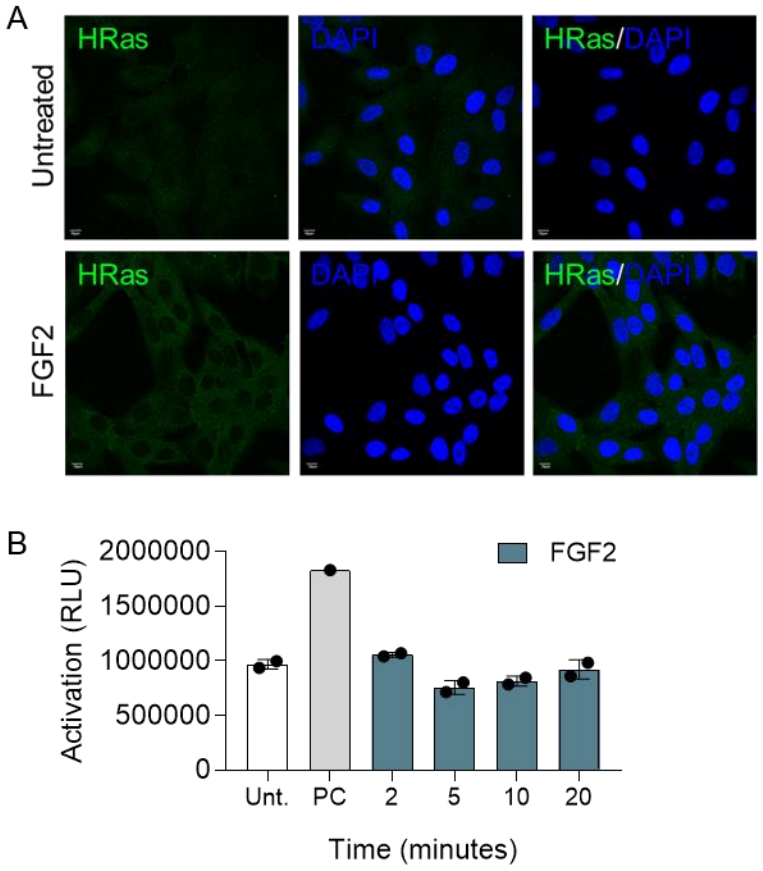

Figure 4.6. FGF2 upregulates HRas protein expression but does not induce membrane localization or activation. A) MCF-10A cells were treated with FGF2 for 24 hours and immunofluorescence was used to detect HRas protein. B) MCF-10A cells were treated with FGF2 (30 ng/mL) for 2, 5, 10, and 20 mins and did not stimulate Ras activation as determined by luminescence, as shown as relative light units (RLU). PC is a supplied positive control of HeLa cells treated with EGF.

Discussion

Fluvastatin was identified as a chemopreventive compound in obesity associated ER– breast epithelial cell transformation with our recent HTS. The transformation HTS models FGF2/FGFR1 stimulated transformation, a critical signaling pathway in VAT-stimulated ER– breast epithelial cell transformation. Herein, fluvastatin was confirmed and validated as the lead chemopreventive hit. Before the transformation HTS screen, there has not been a described relationship between FGFR1 signaling and the mevalonate pathway, which begs the questions, how does an HMG-CoA reductase inhibitor prevent FGF2/FGFR1 stimulated transformation? Therefore, the objective of these studies is to determine the role of the mevalonate pathway in VAT-stimulated ER– breast carcinogenesis.

First, I investigated whether fluvastatin's chemopreventive effects are achieved by other statin types. These data revealed that lipophilic statins, simvastatin and fluvastatin, concentration dependently prevents MFTF-stimulated ER– breast epithelial cell transformation whereas hydrophilic statins, pravastatin and rosuvastatin did not. However, atorvastatin, the most lipophilic statin was not efficacious in preventing transformation unlike the other lipophilic statins. This suggests lipophilic statins are more likely to have cancer prevention efficacy, but lipophilicity alone does not guarantee efficacy. These data also do not suggest efficacy is specific to Type I or Type II statins. Statin type is determined by the molecular structure, and fluvastatin is a Type II statin and simvastatin is a Type I statin[217]. All statins are HMGCR competitive inhibitors and bind to the active site. Structural differences between statins are thought to account for potency differences. Rosuvastatin is the most potent HMGCR inhibitor, followed by simvastatin, atorvastatin, and then fluvastatin (this study did not evaluate pravastatin). All statins have an HMG-like moiety that binds to the active site of HMGCR. The methylethyl group and decalin ring structure on Type II and Type I statins respectively, involve equivalent and numerous hydrogen binding interactions. The fluorophenyl groups of Type II statins exhibit additional binding interactions (Figure 4.7)[217]. Rosuvastatin and atorvastatin form additional hydrogen bonds. However, structural differences and their respective binding interactions do not predict or associate with chemopreventive efficacy as both statin types did and did not prevent transformation. While these data do not indicate that chemopreventive efficacy is specific to statin lipophilicity or molecular structure, fluvastatin and simvastatin are potential are candidates for ER– breast tumorigenesis prevention.

Since statins are relatively safe, they are promising candidates for chemoprevention. Chemopreventive agents, like tamoxifen, can effectively reduce ER positive (ER+) breast cancer but not ER– breast cancer. TNBC, defined as being ER–, progesterone receptor (PR) negative (PR–), with no human epidermal growth factor receptor 2 (HER2) overexpression, constitutes 10% to 20% of all breast cancers. TNBC is a heterogenous, aggressive form of breast cancer with high tendency of metastasis, poor prognosis, and has no approved targeted therapy[48,49], and no targeted chemopreventive strategies[50]. The ER– breast cancer subtype would an ideal candidate for statin chemoprevention.

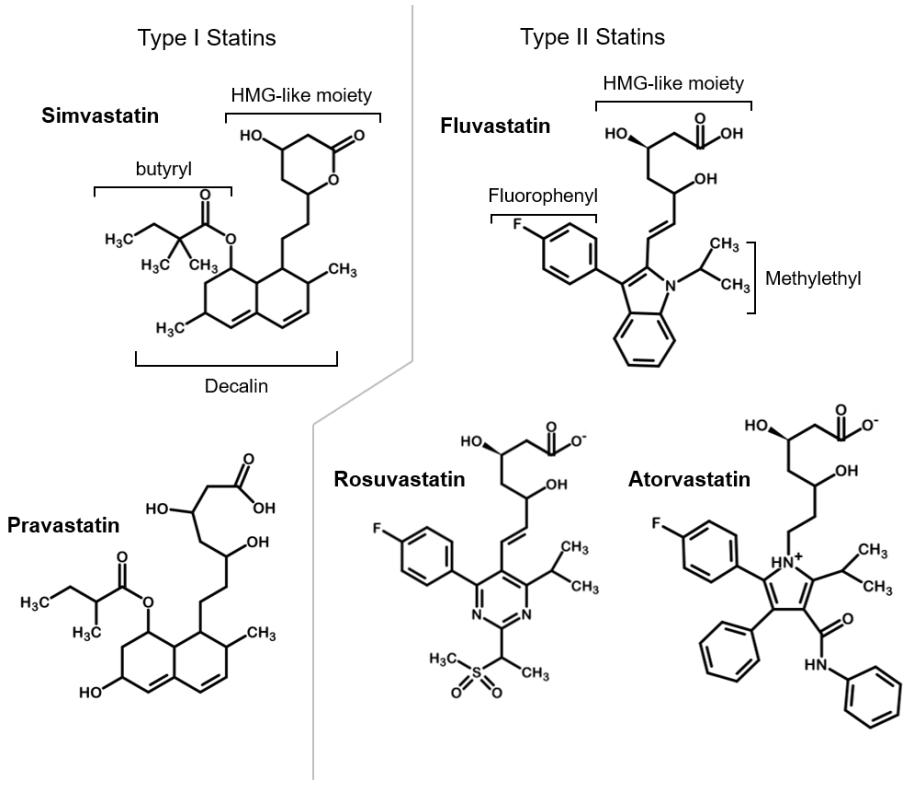

Figure 4.7. The molecular structure of statins determines if they are Type I or Type II statins. Type I statins have an HMG-like moiety group, a butyryl group, and a decalin group. Simvastatin and pravastatin are Type I Statins. Type II statins have their HMG-like moiety, a fluorophenyl group, and a methylethyl group. Fluvastatin, rosuvastatin, and atorvastatin are Type II statins.

Statins have a challenging history when it comes to cancer risk because epidemiology does not collectively conclude that statins are chemopreventive[210]. Initial retrospective analyses agreed statins did not increase cancer risk, a crucial finding if statins were to be used universally as a cholesterol lowering agent, but these retrospective studies did not demonstrate a chemoprotective effect either[94]. However, these initial retrospective epidemiological studies were designed to evaluate cardiovascular outcomes rather than cancer prevention, cancer reoccurrence, and/or cancer mortality[94]. Once studies were designed to look at cancer outcomes and stratified by breast cancer subtype, statin lipophilicity, and menopausal status, they demonstrated lipophilic statins have a protective against premenopausal ER– breast cancer and not ER+ breast cancer[100,101,103]. Studies that do not stratify by lipophilicity, breast cancer subtype, or menopausal status give inconsistent results[107,108]. However, because of statins safety thresholds and health benefits, they should not be overlooked for their ability to reduce the cancer burden of aggressive ER– breast cancers[101].

In vitro and *in vivo* studies positively support statins as chemopreventive agents. Using a N-methyl-N-nitrosourea (NMU)-induced mammary carcinogenesis in female Sprague–Dawley rats, fluvastatin suppressed mammary tumor frequency by 63% and tumor incidence by 33% in comparison with the controls[218]. *In vitro* studies also reveal deregulated HMGCR activity promotes/and is associated with transformation. HMGCR is composed of two contiguous domains, the N-terminal is integrated into the endoplasmic reticulum (EndoR) membrane and the c-terminal projects into the cytosol which is responsible for its enzymatic activity. When HMGCR is expressed in a truncated form with only it's cytosolic C-terminal domain, HMGCR has stable catalytic activity[219]. MCF-10A cells that express HMGCR in this deregulated state form colonies in soft agar, indicating that constitutive HMGCR activity induces MCF-10A transformation[211]. Additionally, one study demonstrated that miR-140-3p-1, a microRNA that represses HMGCR and HMGCS1, decreased through an *in vitro* modeled of breast cancer progression, starting with normal-like MCF10A to preneoplastic MCF10.AT1, and invasive MCF10.Ca1d cells. As the cell lines became more cancer like, there is more HMGCR and HMGCS1 activity[30]. Therefore, these studies suggest HMGCR activity promotes MCF-10A cell transformation and supports our data showing fluvastatin prevents MCF-10A cell transformation.

Furthermore, our data suggests that the mevalonate pathway is altered in obesity-associated ER– breast epithelial cell transformation. Factors from VAT upregulated protein expression of mevalonate pathway-involved enzymes including HMGCS1, FDFT1, and FNTA and PD166866, an FGFR1 inhibitor prevented this upregulation (Figure 4.1). As MFTF/FGF2 did not regulate the gene expression of these enzymes as determined by RT-PCR, MFTF must regulate protein translation and/or post-translational modifications of these enzymes. This suggests that FGF2/FGFR1 signaling stimulated by MFTF alters the mevalonate pathway and in turn cholesterol biosynthesis.

Along with cholesterol biosynthesis, the mevalonate pathway also produces isoprenoids, like FPP and GGPP are used for protein prenylation. The Rho and Ras superfamilies are examples of prenylated proteins and mevalonate pathway dysregulation can promote oncogenesis through this process. Prenylation is necessary to maintain malignant activity of oncogenic Ras. All Ras isoforms (KRas, NRas, and HRas) are farnesylated, but NRas and HRas are then palmitoylated, the attachment of one and two fatty acids to NRas and HRas respectively. Farnesylated and palmitoylated Ras has a 100-fold higher membrane affinity than Ras that is only farnesylated. Membrane localization places Ras in proximity with cell surface receptors and effector proteins which is required for Ras activity. As statins deplete isoprenoid production by reducing cellular mevalonate levels, this isoprenoid depletion could inhibit farnesylation preventing oncogenic Ras membrane localization and activation. The mevalonate pathway is the only metabolic pathway that produces FPP and GGPP.

Ras is a known oncogene and previous literature demonstrated that constitutive HRas activation induces MCF-10A cell transformation. Moon *et al*. transfected MCF-10A cells with a mutant HRas (HRas MCF-10A cells) or mutant NRas (NRas MCF-10A cells) using retroviral vectors[220], and both mutant cell lines produced colonies in soft agar. However, constitutively active HRas induced an invasive phenotype but constitutively active N-Ras did not (as determined by transwell invasion assay)[220]. Furthermore, simvastatin treatment markedly decreased prenylated HRas in the membrane and increased unprenylated HRas in the cytosol of HRas MCF-10A cells[221]. Simvastatin was also able to concentration-dependently attenuate the invasiveness of HRas MCF-10A cells and reintroducing exogenous FPP rescued the invasive phenotype of HRas MCF-10A cells[221]. The ability of exogenous FPP to restore simvastatin inhibited migration implicates HRas farnesylation in simvastatin's mechanism of action in MCF-10A cells. Therefore,

if FGF2/FGFR1 requires HRas farnesylation in MFTF-stimulated transformation, FGF2/FGFR1 activity would need HRas membrane localization and exogenous isoprenoids would rescue inhibitory effects of PD166866.

Because HRas is an oncogenic driver in MCF-10A cells, FGFR1 signaling activates Ras, and HRas is a prenylated protein, we hypothesized that HRas protein prenylation is critical in FGF2/FGFR1-stimulated ER– breast epithelial cell transformation (Figure 4.8). However, we did not see HRas activation or prenylation in MCF-10A cells following FGF2 treatment (Figure 4.5). In addition, exogenous isoprenoids did not rescue the effects of PD166866, a selective FGFR1 inhibitor. This suggests that fluvastatin inhibition of HRas prenylation is not how fluvastatin prevents FGF2/FGFR1-stimulated ER– breast epithelial cell transformation.

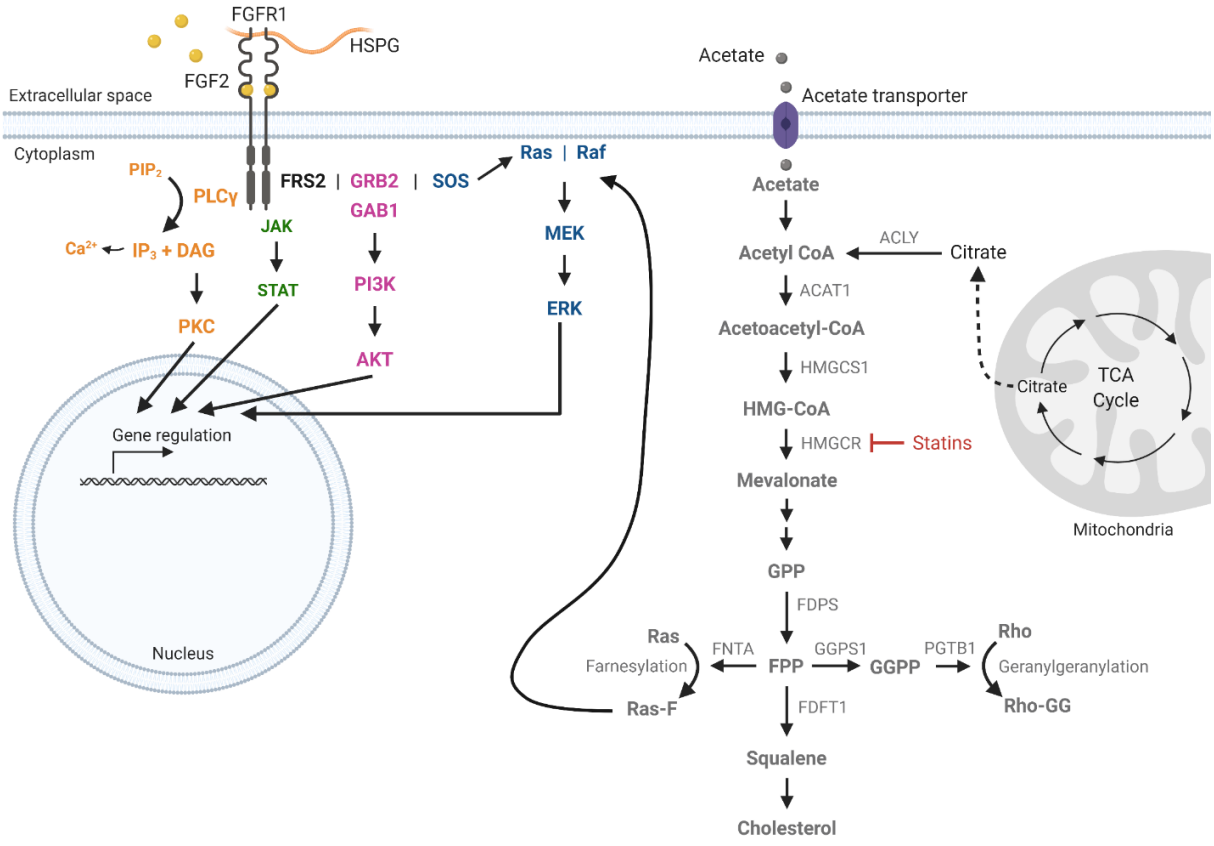

Figure 4.8. The FGF2/FGFR1 signaling pathway and the mevalonate pathway. This figure is a combination of Figure 1.2 and Figure 1.4. Farnesylated HRas undergoes membrane localization and can activate FGFR1 downstream signaling pathways. Previous literature has not described a relationship between FGF2/FGFR1 pathways.

There are potential limitations to our studies. While we did not see HRas activation/prenylation, these assays can be technically challenging. For exogenous isoprenoids to rescue a phenotype, isoprenoids must cross the plasma membrane. While previous literature has demonstrated exogenous isoprenoids like mevalonate and FPP cross the plasma membrane to rescue phenotypes[222], this has not been demonstrated in soft agar. The Ras Activation assay is technically involved, and technical issues could have resulted in the negative data. Ultimately, it is still unclear how statins inhibit FGF2/FGFR1 stimulated ER– breast epithelial cell transformation and more studies are needed to clarify the underlying molecular mechanisms.

In conclusion, our data demonstrated that factors from VAT upregulated the protein expression of HMGCS1, FDFT1, FNTA, and HRas through FGFR1 signaling. More studies are needed to investigate the role of the mevalonate pathway in ER– breast epithelial cell transformation to determine if the induction of these enzymes are drivers of transformation or bystanders. In addition, studies to knock out HMGCS1 are needed to determine if this protein is necessary, sufficient, or dispensable for transformation by VAT. Ultimately, FGF2/FGFR1 and the mevalonate pathway are potential pathways by which cells transform breast epithelial cells independent of ER signaling that can be targeted for prevention.

Materials/Methods

Cell culture

MCF-10A cells were cultured as previously described.

Mouse fat tissue filtrate

Mouse filtrate was prepared as previously described.

Soft agar assay

The soft agar assay was performed as previously described.

Western blot

MCF-10A cells were plated in 6-well plates and incubated at 37°C. After 24 hours and/or time required for cells to reach the appropriate density, cells were treated according to each experimental parameter.

Each well was washed with PBS and cells were trypsinized with 0.05% Trypsin-EDTA. Trypsin was quenched with resuspension media and cells were centrifuged at 600 g for 5 mins. Supernatant was decanted and cells were resuspended in chilled PBS. Cells were centrifuged at 600 g for 5 mins. All the PBS was removed, and cells were resuspended in lysis buffer (RIPA buffer and cocktail or protease and phosphatase inhibitors). Samples were frozen in the -20°C and then thawed at 4°C. Samples were sonicated 3 times (10 seconds each, 30 second break) and then centrifuged at 12,000 rpm for 10 mins at 4°C. The supernatant was transferred to new tubes and used the Pierce™ BCA's Protein Assay Kit (Thermo Fisher, 23225) to determine protein content.

Equal amounts of protein and volume were used, with 8X loading dye. Samples were heated at 100°C for 5 mins. Samples were running at 60 V for ~20 mins and the 100 V for about 60 minutes. Protein from the gel was transferred to nitrocellulose membranes and washed with 1X tris-buffered saline, 0.1% Triton X (TBST). Membranes were blocked in 4% bovine serum albumin (BSA) in TBST or 5% non-fat dairy milk (NFDM) in TBST. Membranes were washed in 1X TBST (5 mins on rocker at room temperature) and incubated with primary membranes on the rocker either for 3 hours at room temperature or overnight at 4°C. Next, membranes were washed and incubated with secondary antibody (IRDye® 800CW Donkey Anti-Rabbit IgG, Invitrogen, 926-32213) at 1:10000 in 1% BSA in TBST for 1 hour on the rocker. Membranes were washed and fluorescence was read on the Li-Cor Odyssey CLX.

Table 4.1. Antibody parameters used for western blot protein detection

ANTIBODY	Company	Catalog #	Species	DF	Blocking buffer	1° Dilution Buffer
Actin	Sigma	A5060	Rabbit	1/500	5% NFDM/TBST	4% BSA/TBST
FDFT1	Proteintech	13128-1-AP	Rabbit	1/1000	5% NFDM/TBST	3% NFDM/TBST
FDPS	Proteintech	16129-1-AP	Rabbit	1/1000	5% NFDM/TBST	5% NFDM/TBST
FNTA	Proteintech	12274-1-AP	Rabbit	1/1000	5% NFDM/TBST	3% NFDM/TBST
GGPS1	Proteintech	14944-1-AP	Rabbit	1/1000	5% NFDM/TBST	3% NFDM/TBST
HMGCS1	Cell Signal	176431-1-APWB	Rabbit	1/1000	5% NFDM/TBST	3% NFDM/TBST
HRas	Proteintech	18295-1-AP	Rabbit	1/1000	5% NFDM/TBST	3% NFDM/TBST

Abbreviations: FDFT1- squalene synthase; FDPS- farnesyl pyrophosphate; FNTA- farnesyl transferase; GGPS1- geranylgeranyl pyrophosphate synthase 1; HMGCS1- HMG-CoA synthase 1; HRas- Harvey rat sarcoma viral oncogene

RT-PCR

Gene expression for the following genes were evaluated using RT-PCR: farnesyl transferase (FNTA), farnesyl pyrophosphate (FDPS), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), geranylgeranyl pyrophosphate 1 (GGPS1), HMGCR, Harvey rat sarcoma viral oncogene (HRas), and sterol regulatory element binding transcription factor 2 (SREBF2). Table 4.1 lists the genes with their corresponding forward and reverse primer sequences. MCF-10A cells were plated in 12-well plates and incubated at 37°C. After 24 hours and/or time required for cells to reach the appropriate density, cells were treated according to each experimental parameter. Each well was washed twice with PBS. 200 µL of lysis buffer from RNAeasy Mini Kit (Qiagen, 74104). After 2-3 minutes, lysis buffer/cell contents were transferred to a microcentrifuge tube and stored at -20°C RNA was isolated according to the Qiagen Quick-start protocol, Part 1, RNeasy Mini Kit. Ribonucleic acid (RNA) was quantitated on a nanodrop. cDNA was synthesized according to AppliedBiosystems Protocol for High Capacity cDNA Reverse Transcription Kid (4368814). cDNA was used during reverse transcription polymerase chain reaction (RT-PCR) in a SYBR® Green reaction. Primers were designed and ordered through Integrated developmental technologies (IDT) (Table 4.2). Each sample was run in a 384-well format with 10 µL of sample and RT-PCR master mix. Target gene values were normalized to the housekeeper gene GAPDH and graphed as $2^{-\Delta\Delta CT}$ [$\Delta\Delta CT = \Delta CT_{\text{Target gene}} - \text{Average}(\Delta CT_{\text{GAPDH Rep 1}}, \Delta CT_{\text{GAPDH Rep 2}}, \Delta CT_{\text{GAPDH Rep 3}})$].

Table 4.2. Primer sequences for RT-PCR

Gene	Forward Sequence	Reverse Sequence
FNTA	CACACGAGAGTGGTCCTTCC	GCACCCAAGGAGCATCAGTT
FDPS	TGTGACCGGCAAAATTGGC	GCCCGTTGCAGACACTGAA
GAPDH	CTGGGCTACACTGAGCACC	AAGTGGTCGTTGAGGGCAATG
GGPS1	ACAGCATCTATGGAATCCCATCT	CAAAAGCTGGCGGGTAAAAAG
HMGCR	TGATTGACCTTTCCAGAGCAAG	CTAAAATTGCCATTCCACGAGC
HRas	GCTACGGCATCCCCTACATC	TTGTGCTGCGTCAGGAGAG
SREBF2	AACGGTCATTCACCCAGGTC	GGCTGAAGAATAGGAGTTGCC

Ras Activation Assay

MCF-10A cells were plated in 6-well plates and incubated at 37°C. After 24 hours and/or time required for cells to reach the appropriate density, cells were treated according to each experimental parameter. The protocol for Ras GTPase Chemi ELISA Kit (Active Motif, 52097) was followed for Ras Extraction and the ELISA.

Immunofluorescence

MCF-10A cells were plated in 24 well-pates on top of glass coverslip and treated accordingly to experimental parameters. Cells were fixed with formaldehyde at a 1:10 dilution. After 15 minutes, media/formaldehyde was aspirated and washed with PBS then incubated in blocking buffer (1% bovine serum albumin in PBST) for 1 hour. Wells were incubated with a 1:500 dilution of HRas (same antibody used for western blot) and incubated on a rocker overnight at 4°C. Wells were incubated in secondary antibody for 1 hour. Wells were washed with PBS and coverslips were placed on DAPI on glass slides and allowed to adhere. Images were acquired by a confocal microscope.

CHAPTER 5

General Conclusions

The data presented in this dissertation reveals that factors from VAT can stimulate ER– mammary epithelial cell transformation. My studies suggested that FGF2 may be one of the most critical factors in VAT for inducing this response and FGF2 exerts its effects through FGFR1. Therefore, these data addressed the first part of my objective which is to determine the mechanism of visceral obesity driven ER– breast cancer and use this mechanism to identify chemopreventive compounds. To address the second part of the objective, I developed a HTS that utilized FGF2-stimulated epithelial cell growth in ultra-low attachment conditions and used this tool to discover compounds that have the potential to inhibit VAT-stimulated transformation. From this HTS, fluvastatin was identified as a potential chemopreventive agent. These data support the overall hypothesis that FGF2/FGFR1 is a critical mechanism in ER-independent VAT-associated transformation and is a potential target for chemoprevention strategies and these data revealed a potential role for the mevalonate pathway in transformation.

To determine the mechanism of visceral obesity driven ER– breast cancer, I identified factors in VAT that stimulated malignant transformation. First, I demonstrated that factors from VAT in HuFTF stimulates transformation of MCF-10A cells, non-tumorigenic ER– breast epithelial cells[160]. Previous data I generated also demonstrated HuFTF stimulates NMuMG cells, non-tumorigenic mouse mammary epithelial cells[79]. As determined by ELISA, the FGF2 protein levels in HuFTF is positively associated with HuFTF-stimulated MCF-10A and NMuMG cell colony formation. Furthermore, exogenous FGF2 concentration-dependently stimulated MCF-10A transformation. To verify that the transformative effects are specific to FGF2, the transformative ability of other FGF ligands were investigated. First, we compared the effects of FGF2, FGF1, FGF18, and FGF22 in soft agar and determined that FGF2 was the only ligand that concentration-dependently stimulated MCF-10A colony formation. As FGF2 has the highest affinity for FGFR1, two inhibitors were used, an FGFR1 antibody and PD166866, a selective FGFR1 inhibitor, to determine if FGF2 stimulates transformation through FGFR1. The FGFR1 Ab attenuated HuFTF-stimulated MCF-10A colony formation in soft agar and PD166866 concentration-dependently attenuates FGF2 and MFTF-stimulated colony formation in soft agar. PD166866 also attenuates FGF2-stimulated growth in ultra-low attachment conditions. These studies demonstrate that FGF2 may be the most important factor in VAT that activates FGFR1, the critical receptor in VAT-stimulated ER– breast epithelial cell transformation.

The next section of this dissertation addresses the second part of the objective which was to identify chemopreventive agents. There are currently no targeted treatments for ER– breast cancer prevention or therapy. This is in large part due to lack of targets as ER– breast cancer is defined by a lack of a receptor and the etiology of obesity-associated premenopausal ER– is unknown. Moreover, there was not a high-throughput method that modeled the transformation process which means the methodology did not exist to screen for compounds for chemoprevention. Because the soft agar assay is not suitable for a high-throughput format, we used an alternative model of anchorage-independent growth, ultra-low attachment conditions. I developed and optimized the first high-throughput screen to model the process of transformation using growth in low attachment conditions in a 384-well format. First, I demonstrated FGF2 concentration dependently stimulated growth of skin epidermal cells in ultra-low attachment conditions over 96 hours. MCF-10A cells were not suitable for this method and were used to validate hits in soft agar. Growth in low attachment conditions was quantified by CellTiter Glo, a luminescent signal proportional to the ATP content, a measure of proliferation. To ensure the HTS could identify chemopreventive agents, PD166866 was used to attenuate FGF2-stimulate growth in low attachment conditions. This was further supported with I-BET-726 (IBET), a bromodomain inhibitor that we previously demonstrated prevents HuFTF-stimulated transformation of NMuMG and MCF-10A cells[170]. IBET attenuated FGF2-stimulated growth in low-attachment conditions. Furthermore, FGF2 did not stimulate growth of skin epithelial cells in 2D culture conditions. After screening three libraries and confirmation and validation studies, fluvastatin emerged as the lead candidate for chemoprevention. Fluvastatin is an ideal candidate for prevention because of its relatively known safety profile. Overall, identifying fluvastatin as the lead candidate for chemoprevention, fulfilled the second part of the objective.

The last section of this dissertation investigates the role of the mevalonate pathway in VAT-stimulated transformation. Fluvastatin inhibits HMGCR, the rate-limiting enzyme in the mevalonate pathway but there has not been a described relationship between FGFR1 signaling and the mevalonate pathway. First, I established fluvastatin is working by its known mechanism of action. Inhibiting HMGCR triggers feedback loops that upregulates HMGCR protein expression and mRNA. After MCF-10A were treated with fluvastatin for 24 hours, HMGCS1 protein was upregulated and HMGCR mRNA was significantly elevated. Furthermore, fluvastatin decreased the cholesterol content of MCF-10A cells after 24 hours (Appendix A).

Therefore, fluvastatin is inhibiting HMGCR in MCF-10A cells. Next, it was determined that MFTF upregulated enzymes in the mevalonate pathway including HMGCS1, FNTA, FDFT1, and HRas and PD166866, a selective FGFR1 inhibitor prevented this upregulation. Fluvastatin treatment is known to up upregulate both HMGCS1 and HMGCR protein expression. HMGCS1 produces HMG-CoA, the substrate for HMGCR. Interestingly, an FGFR1 inhibitor was able to block VAT-stimulated upregulation of HMGCS1, which would reduce the amount of HMG-CoA produced. This suggests that HMGCS1, and in turn HMG-CoA production, may be part of the mechanism by which VAT stimulates ER– mammary epithelial cell transformation. Furthermore, aberrations in FGFR1, FNTA, and FDFT1 have significant co-occurrence in breast cancer patients, meaning these aberrations have functional synergy (Appendix B). These data suggest FGF2 and FGFR1 signaling influences the mevalonate pathway by upregulating protein expression of enzymes. Experiments to further understand this mechanistic connection gave negative data. We did not see HRas activation or membrane localization and exogenous isoprenoids did not rescue the effects of PD166866 on FGF2-stimulated MCF-10A transformation in soft agar. These data suggest mevalonate pathway HRas prenylation is not critical in VAT-associated ER– breast epithelial cell transformation. Overall, it is not clear how FGFR1-stimulated upregulation of HMGCS1 contributes to VAT-stimulated ER– breast epithelial cell transformation (Figure 5.1).

Lastly, this dissertation has made significant contributions to the development of three-dimensional (3D) platforms by creating the first high-throughput assay to model the transformation of a non-tumorigenic cell. This is a breakthrough in the technological development of cancer prevention HTSs. The studies described within this dissertation are important steps in understanding the obesity-cancer link as *in vitro* experiments are fundamental in deciphering mechanisms behind cell behavior *in vivo*[169]. However, *in vitro* methodology has predominantly consisted of two-dimensional (2D) cell culture. While 2D models have made significant contributions to understanding cell behavior, 2D systems deviate considerably from cell behavior *in vivo*. Physiologically, cells are influenced by interactions with their microenvironment. Unlike 2D systems, 3D models more closely resemble the *in vivo* microenvironment by more effectively inducing *in vivo*-like cell fates[63]. The soft agar assay is well-accepted as a model for transformation that uses a 3D experimental setting. Suspending single cells in agarose only allows cells to grow in an anchorage dependent manner, thus distinguishing between transformed and non-transformed cells. Previous cancer

prevention HTSs use cancer cells which reveals a significant limitation with cancer prevention models as cancer cells behave differently than normal cells. Overall, the transformation HTS further extends the range of processes 3D platforms can model. This has the potential to be adapted and modified to model transformation by carcinogens and other factors.

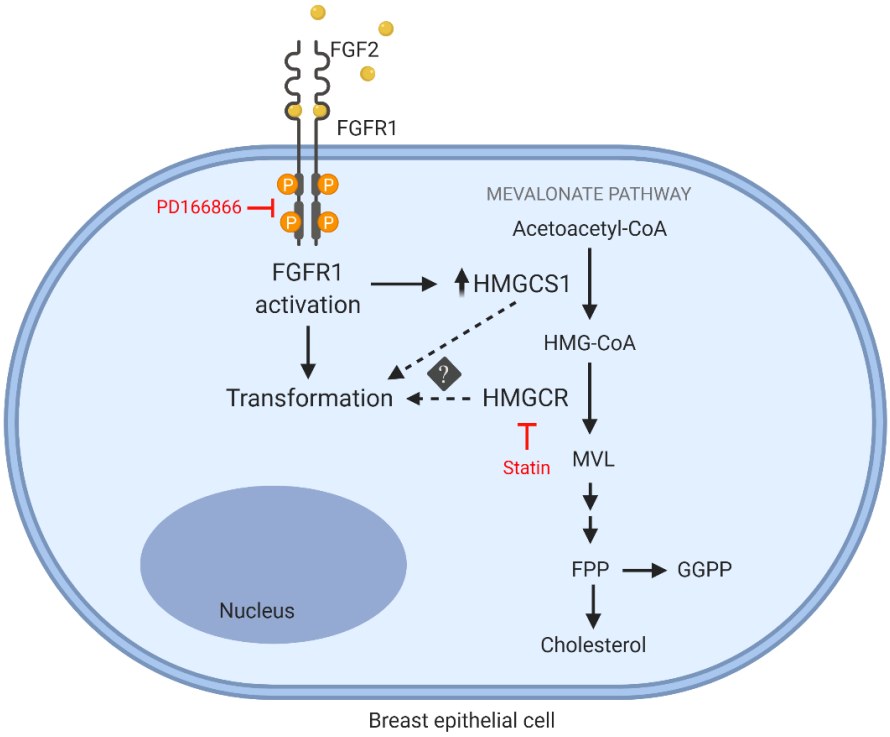

Figure 5.1. Overall findings: FGFR1 and HMGCR inhibition prevents VAT-stimulated ER– breast epithelial cell transformation and VAT upregulation of HMGCS1 may be part of this mechanism. It is unclear exactly how FGFR1 signaling and mevalonate pathway interconnects to stimulate ER– breast epithelial cell transformation.

Limitations

The experimental settings used in this dissertation were primarily *in vitro* assays. Using MFTF, made from the VAT of mice or humans mimics the factors from VAT in the *in vitro* environment. The soft agar assay is the gold standard assay of transformation as the ability for cells to form colonies in soft agar directly correlates to the cells ability to form tumors *in vivo*. Regardless, MFTF-treated cells in soft agar does not fully mimic tumorigenesis *in vivo*. The premise of using VAT is based on SAT not being as biologically active in promoting transformation. Subcutaneous mammary adipose tissue (MAT) is an important component of the mammary gland as complete ablation of MAT in transgenic mice prevents mammary gland development. Furthermore, MAT can augment breast cancer progression[156,157]. This bidirectional dialog between MAT and breast epithelium reveals another limitation of these studies as they do not model the role of MAT in ER–breast epithelial transformation. Overall, the findings of these studies need to be validated in an in vivo model of FGF2/FGFR1 stimulated ER– breast epithelial cells.

The previous chapter investigating the role of the mevalonate pathway in FGF2/FGFR1 stimulated transformation only used one cell line, MCF-10A cells. One-cell line studies can be problematic because the effects could be cell line specific and not representative of primary breast epithelial cells. In addition, the premise of this dissertation focuses on ER– transformation based on the epidemiology data that premenopausal obesity increases the risk for ER– breast cancer. Complementary studies using an ER+ breast epithelial cell would reveal if this mechanism is specific to ER– transformation. For a more comprehensive view, other non-tumorigenic breast epithelial cells should be analyzed for MFTF-induced upregulation of mevalonate pathway related enzymes.

Negative data regarding HRas protein prenylation limits data interpretation and conclusions. Interpreting negative data can be difficult because this can be a result of technical issues instead of being truly negative data. First, the Ras activation assay is a technically challenging assay, and technical errors could cause negative data as there isn't a positive control in the assay. The Ras activation baseline is high for the MCF-10A cells when compared to protocol examples. Further troubleshooting should be explored to verify the Ras activation assay results. In addition, exogenous isoprenoids did not rescue the effects of PD166866 on FGF2-stimulated MCF-10A cell colony formation in soft agar. While previous publications have shown that isoprenoids like mevalonate, FPP, GPP, and GGPP can cross the plasma membrane and

rescue stain-inhibited phenotypes, this hasn't been verified in MCF-10As and the soft agar assay. The inability of MVL or GPP to rescue the effects of PD166866 could be because of MVL or GPP didn't enter the cell. Additionally, MVL or GPP at 20 µM could be too low of a concentration to rescue the phenotype. Higher isoprenoid concentrations would likely see a vehicle effect due to the low stock concentrations and they are constituted in methanol, risking a vehicle effect. Additional experiments are needed in order to validate that isoprenoids can pass through the plasma membrane at these concentrations and induce prenylation.

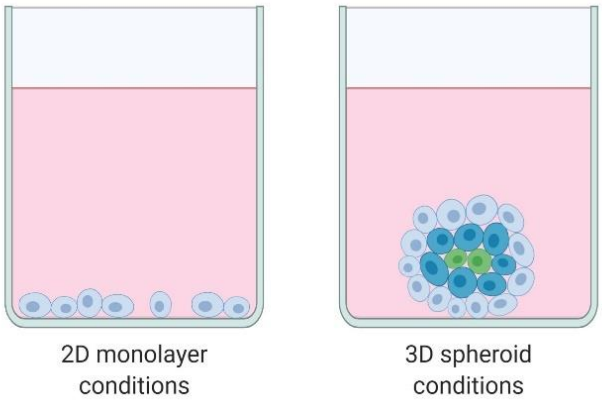

Figure 5.2. 2D and 3D cell culture methods. 2D monolayer conditions have unlimited access to the nutrients, oxygen, signaling molecules, etc. Spheroid structures form cell layers that limits exposure to internal cells as the outer layer are highly exposed to the medium whereas the core cells receive less growth factors, nutrients, and oxygen and are often quiescent or hypoxic. Image made in BioRender.

Investigating the mechanisms of VAT-stimulated transformation would preferably be performed in 3D culture conditions, however this proves to be challenging. As the transformation HTS was developed for the purpose of this dissertation, it is still a new technique and has not been adapted to investigate the underlying mechanisms of transformation. The cytotoxicity studies, western blots, RT-PCR, immunofluorescence, and Ras activation assay were all performed in 2D culture conditions. This deviates from the 3D experimental setting of the soft agar assay and transformation HTS. 2D culture conditions were used because the 3D equivalents have not been developed. This proves technically challenging because the premise of transformation assays is that non-transformed cells do no proliferate. Therefore, control or untreated cells would not produce enough cells to quantitate for assays like western blots or RT-PCR. Immunofluorescence assays would need to be optimized for 3D spheroids. Spheroids have diffusional limits so staining would consist of the outer cell layers, a potential limitation in 3D immunofluorescence (Figure 5.2). Ideally, mechanistic studies should be performed in 3D platforms. Future studies should look at adapting and optimizing methodologies like western blots and RT-PCR with 3D formats. This would give more representative mechanistic data regarding transformation. Furthermore, these 3D formats would enable more mechanistic studies comparing differences between 2D and 3D cell culture methods.

Unanswered questions

How does adipose tissue secrete FGF2?

Current literature has not identified the mechanism by which FGF2 is produced and secreted into the circulation. Studies have demonstrated that high BMI is associated with elevated circulating FGF2 levels, however, how FGF2 is secreted from adipose tissue, enters the circulation, and is transported to distance sites is a complex process[78]. FGF ligands are either paracrine, intracrine or endocrine signaling molecules[223]. Paracrine and endocrine FGFs have different FGFR binding mechanisms. Paracrine FGFs, like FGF2, interact with heparan-sulfate proteoglycans (HSPG) to stabilize the FGF/FGFR complex, whereas endocrine FGFs require klotho proteins and have very low affinity to HSPG[223]. Functionally, HSPGs regulate FGF availability, and their transport within tissues because HSPGs are covalently linked to cell surface proteins like FGFRs. Here, they modulate diffusion by sequestering FGFs that keeps them as local signals[224]. In contrast, endocrine FGFs require klotho proteins binding to stabilize the FGF/FGFR

complex and have low binding affinity to HSPG. This low affinity allows endocrine FGFs to enter the extracellular space and permeate freely through HSPGs and enter the blood. Because FGF2 is a paracrine growth factor, this goes against studies that attributes adipose tissue as the source of circulating FGF2 levels because HSPGs would prevent FGF2 systemic diffusion. Therefore, how FGF2 is secreted in an endocrine manner remains unclear.

Interestingly, FGF2 lacks a signal peptide and is secreted unconventionally. Typically, canonical protein transport to the extracellular space requires a signal-peptide that utilizes an endoplasmic reticulum/Golgi apparatus secretory mechanism[225]. FGF2 does not have a signal peptide and must undergo an alternative secretion mechanism. Unconventional secretion of FGF2 begins with phosphatidylinositol 4,5-bisphosphate (PIP_2)-dependent FGF2 oligomerization that forms a pore in the plasma membrane. Here, FGF2 exits the cytoplasm through this pore and once in the extracellular matrix, FGF2 is trapped and firmly bound by HSPG, keeping FGF2 proximal[225]. The intricacies of this unconventional secretory mechanism have yet to be fully understood. Regardless, this does not explain endocrine FGF2 transport. Another potential protein transport mechanism utilizes exosomes, vesicles that entrap intracellular molecules and travel to distant sites[226]. Exosomes are involved in cell-to-cell communication by transferring proteins, mRNAs, and microRNAs[225]. In addition, adipocyte secreted exosomes are altered in obesity[226]. Specifically, adipose tissue in obese mice release more than twice the amount of exosomes[227]. Another study demonstrated adipose-derived stem cells (ASCs) produced exosomes that contained FGF2[228]. Studies have not investigated circulating exosome FGF2 content in obesity compared to normal weight. FGF2-containing exosomes could be a mechanism of FGF2 secretion into the circulation, enabling FGF2 to act in an endocrine manner despite being a paracrine growth factor. Further studies are needed to understand how VAT produced FGF2 is released into the circulation.

Which FGF2 isoforms are produced and secreted by adipose tissue?

Human FGF2 can undergo alternative splicing that produces five FGF2 isoforms and our data does not inform as to which FGF2 isoforms are produced and secreted by VAT. FGF2 isoforms are referred to as either high molecular weight (HMW) (34 kDa) or low molecular weight (LMW) (24, 22.5, 22, and 18 kDa). Studies have described HMW and LMW FGF2 isoforms as having contrasting potencies and/or functions

on physiological processes. For example, HMW FGF2 overexpression lowered bone mineral density (BMD) while LMW FGF2 overexpression enhanced bone mineral density in mice[148]. Another mouse model demonstrated that HMW FGF2s enhanced hepatic fibrosis whereas LMW FGF2 suppressed hepatic fibrosis[149]. In addition, LMW isoforms protected mice from myocardial dysfunction following ischemia-reperfusions, whereas HMW isoforms had a detrimental effect[229]. This indicates that HMW and LMW FGF2 isoforms can have opposing functions with LMW isoforms promoting favorable health outcomes compared to HMW FGF2. In contrast, FGF2 isoforms can also demonstrate a range of potencies. LMW isoforms were more mitogenic than HMW isoforms in dermal fibroblasts[150]. Interestingly, FGF2 derived from omental VAT was more mitogenic than FGF2 from benign or cancerous renal tissue in endothelial cells[151]. In the soft agar assays, MCF-10A cells were treated with recombinant FGF2 that is exclusively 18 kDa, an LMW isoform and we did not investigate the type of FGF2 isoform in the FTF. Further investigations need to determine the efficacies/potencies of FGF2 isoforms in MCF-10A transformation and which FGF2 isoforms are in the circulation/produced in the VAT of normal weight and obese individuals. In tandem, studies have not looked at FGF2 isoforms in the breast cancer, so there could be a specific FGF2 isoform expressed in ER– breast cancer that promotes progression. Identifying specific FGF2 isoforms would reveal more specific targeting in ER– breast cancer progression and treatment.

Potential contraindications targeting FGFR1

Ablating FGFR1 in a healthy population could have significant unwanted side effects because FGF signaling controls a variety of processes in embryonic development and in adult organisms. FGFR1 mutations cause genetic disorders that effect bone development and the hypothalamic-pituitary-gonadal axis (HPG axis): kallman's syndrome, osteoglophonic dysplasia, and Pfeiffer's syndrome. FGFR1-associated genetic disorders reveal FGFR1 is crucial in physiological processes. In addition, FGFR1 is essential for mammary gland development. The developing mammary bud expresses FGFR1 and FGFR1 is the primary FGFR during ductal morphogenesis in mammary epithelium. Prenatal FGFR1 deletion delays mammary gland development. Inducible FGFR1 activation increases lateral budding in mammary epithelium, induces alveolar hyperplasia, and invasive lesions[131,136]. While the target population are adults and are no longer undergoing mammary gland development, FGFR1 is crucial for mammary gland

homeostasis which is demonstrated by the structural changes generated by inducible FGFR1 (iFGFR1) activation. iFGFR1 activation induces proliferation, disrupts cell polarity, and promotes cell survival[136]. Furthermore, FGFR1 plays a role in osteogenesis and inhibiting FGFR1 could negatively affect this process. For example, MiR-214, a microRNA, inhibits FGFR1 signaling and attenuates osteogenesis[230]. Therefore, targeting FGF2/FGFR1 signaling could have negative effects on osteogenesis in a healthy population. Overall, attenuating FGFR1 activity in a healthy population should be approached with caution to ensure critical physiological functions like osteogenesis and mammary gland homeostasis are not impeded and do not cause unwanted and/or adverse side effects.

Currently, there are no clinically approved compounds that selectively inhibit FGFR1 for chemoprevention. Erdafitinib, a pan-FGFR1 inhibitor, is the only approved FGFR inhibitor. Erdafitinib is clinically approved for advanced bladder cancer with FGFR2 or FGFR3 genetic alterations. For some patients, Erdafitinib use caused ocular toxicity, an adverse side effect common to MAPK pathway inhibitors[231]. Unfortunately, this means erdafitinib lacks the high safety threshold required for chemoprevention[206,207]. Theoretically, selectively targeting FGFR1 would lower potential side effects because processes regulated by FGFR2-4 would not be affected. However, there are currently no clinically approved selective FGFR1 inhibitors.

Regardless of a lack of FGFR1 inhibitors, targeting the transformation process itself instead of FGF2/FGFR1 directly is a more robust way to identify efficacious chemopreventive compounds. HTSs have two different types of approaches: target-based and phenotypic. Target-based approaches identifying compounds that directly inhibit FGF2 or FGFR1. However, this does not guarantee they will be FGFR1 selective nor will this guarantee chemopreventive efficacy. Moreover, RTKs have high redundancy and compensatory crosstalk, and compounds identified using a target-based approaches have a high attrition rate due to lack of efficacy when moved to *in vitro* and/or *in vivo* models due to redundancy and compensatory crosstalk. Therefore, we used a target-based, phenotypic assay to identify chemopreventive agents that inhibit the transformation process. This uses FGF2/FGFR1 as the informed mechanism but is a phenotypic assay to increase hit efficacy. This allows hits to target downstream signaling pathways involved in transformation and may not have the same contradictions that a direct FGFR1 inhibitor might

have. Regardless, toxicity studies would have to determine any potential side effects and/or adverse outcomes.

Future Directions

Further studies are needed to examine the chemopreventive efficacy of fluvastatin *in vivo* against FGFR1-promoted tumorigenesis. This would be effectively demonstrated using HC-11 mouse mammary epithelial cells that have a transgenic iFGFR1 that is induced by a synthetic molecule in a Balb/c xenograft model[232]. This model has a slow tumor onset, making it well suited for prevention studies. It would be interesting to determine whether fluvastatin decreases the percentage of mice that develop adenocarcinomas and delay tumor onset. Between tumors that arose from control/vehicle or fluvastatin treatment, the expression of Ras isoforms could be compared to see if FGFR-stimulated mammary tumorigenesis increased Ras protein expression/activity compared to benign mammary tissue. While this in vivo experimental system models FGFR1-stimulated mammary tumorigenesis, there are drawbacks to this model as HC-11 cells are ER+[233]. The ideal *in vivo* model would be ER–, revealing a limitation of the HC-11 iFGFR1 model. An alternative model would use MCF-10A cells in a xenograft model in immunocompromised mice with exogenous FGF2 injections. This would be modeling FGF2-stimulated ER– tumorigenesis. This *in vivo* model is immune deficient mice and precludes any role the immune system might have. However, this MCF-10A xenograft model needs to be developed. While there are limitations to both models, they would both function to further this research with *in vivo* data.

More experiments are needed to assess mevalonate pathway activity. Protein upregulation does not guarantee pathway activation. Therefore, quantification of mevalonate pathway endpoints is needed to determine in FGF2/MFTF activated the mevalonate pathway. Quantifying cholesterol content in MCF-10A cells treated with FGF2, MFTF, fluvastatin, and/or PD166866 would determine the role of FGF2/MFTF on cholesterol biosynthesis through the mevalonate pathway. Additionally, metabolomics analyses of the aforementioned treatments would also inform on mevalonate pathway activation.

Our data demonstrated both fluvastatin and simvastatin prevented ER– breast epithelial cell and future studies should follow up with both fluvastatin and simvastatin to determine if they have equal

chemopreventive efficacies and/or if one statin is more favorable over the other. Thus, the role of simvastatin should be investigated with *in vitro* and *in vivo* with fluvastatin.

Alternative hypotheses

While the data did not show HRas activation, further studies should investigate other prenylated proteins for a potential role in ER– breast tumorigenesis. There are two types of prenylation: farnesylation and geranylgeranylation. Farnesylated proteins include the Ras superfamily (K/N/HRas) and geranygeranylated proteins include RhoA/B/C, Rap1A, Rac-1, and Cdc42. The data described focused on HRas however, the NRas and KRas isoforms could be responsible. If there was NRas or KRas activity, the Ras activation assay detects all Ras isoforms so this assay would have shown activation if FGF2 was activating NRas and/or KRas. In addition, other prenylated GTPases are deregulated in cancer and/or promote tumorigenesis. For example, Cdc42 activation induce transformation. While no activating Cdc42 mutations have been detected in human cancer, Cdc42 is reportedly overexpressed in breast cancer[234]. Intriguingly, one study implicated Cdc42 activity in promoting NIH 3T3 fibroblast transformation[234]. Furthermore, the oncoprotein Dbl is highly transforming and it activates Rho GTPases like Cdc42[234]. Additionally, transformation assays demonstrated RhoA NIH 3T3 mutants did not have the same robust transformation seen with Ras mutants[235]. Ultimately, there are many GTPase proteins that could be further investigated for a role in transformation.

IGF/IGF1R signaling

Mechanistically, there are several obesity-related alterations in the insulin and the insulin-like growth factor (IGF) system that may promote tumorigenesis. Along with fluvastatin, insulin-like growth factor receptor 1 (IGF1R) inhibitor picropodophyllin (PPP) was identified as a chemopreventive compound in the transformation HTS. Insulin resistance is one of the hallmarks of obesity which is often followed by hyperinsulinemia, a condition that may contribute to breast cancer development[236]. First, a hyper insulinemic state induces changes in circulating ligands of the IGF family. The IGF family comprises two ligands, IGF1 and IGF2, and three receptors (IGF1R, IGR2R, and IGF3R) and six IGF-binding proteins (IGFBP1-6)[236,237]. Insulin increases hepatic growth hormone (GH) receptor expression, leading to GH

mediated increases in IGF1 production as well as repression of IGFPB1,2[31,236]. IGFPBs normally binds to IGF1, inhibiting IGF1, and free IGF1 is the bioactive form. Obese individuals consistently have elevated free circulating IGF1 levels[236]. Additionally, the insulin receptor (IR) and IGF1R. have high homology meaning both insulin and IGF1 can interact with either IR or IGF1R[31,237]. Furthermore, epidemiology associated receptor expression with TNBC. One study demonstrated the IR was highly expressed in TNBC cases[238]. African American women, have higher IGF1R expression in normal breast tissue, are three times more likely to develop TNBC then non-African American women[237,239-243]. High levels of IGF1 has been associated with increased risk of premenopausal breast cancer in women. Overall, epidemiological studies support a role for insulin and/or IGF1/IGF1R in obesity associated TNBC. As obesity has a complex multifactorial pathophysiology it is unlikely that obesity-promotes tumorigenesis through one pathway. More studies should focus on multi-factors stimulating transformation and pathway synergy in tumorigenesis.

Estrogen metabolism

Estrogens are known to influence isoprenoid metabolism through the mevalonate pathway, but little is known about the molecular mechanisms. Estrogen should not be overlooked when investigating the mechanisms underlying pre-menopausal obesity and ER– breast cancer. First, BMI is positively associated with increased aromatase expression and estrogen levels[29,30]. In normal weight premenopausal women, ovarian granulosa cells are the main source of estrogens while a small fraction is produced by other tissues like adipose. However, in obese premenopausal women, estrogens are predominantly produce by adipose tissue as adipocytes express aromatase, the rate limiting enzyme in estrogen biosynthesis[31].

Previously, it was thought estrogen was only involved in the development and progression of ER+ breast cancer, however estrogen can play a role in ER– breast cancer as well[46]. Estrogen can act as a carcinogen through ER binding or estrogen metabolism.[46] First, estrogen can bind to ER and activate multiple pathways including the MAPK and PI3K/Akt pathways. Estrogen can also bind to nuclear ER, dimerize with a complementary estorgen/ER, and act as a transcription factor that alters gene expression. The second pathway is through estrogen metabolism by the catechol pathway. In short, estrogen is metabolized (Phase I metabolism) by P450 enzymes to catechol estrogens, metabolites that forms unstable depurinating DNA adducts, and may generate cancer initiating mutations and potentially lead to

transformation[46,244]. Since estrogen doesn't need to bind to ER to have carcinogenic effects, it is naïve to assume estrogen does not influence TNBC.

Furthermore, epidemiological studies suggest estrogen metabolism is involved in cancer initiation[244]. Key *et al*. demonstrated high circulating levels of estradiol had a strong association with pre-menopausal breast cancer[245]. Interestingly, breast cancer 1 (BRCA1) tumors are predominantly ER– and oophorectomy, which reduces estrogen levels, decreased the risk of BRCA1 breast cancer[246]. In tandem, ovariectomy inhibited the development of ER– breast cancer[247] and exogenous steroid hormones contributes to ER– breast cancer progression[248]. Additionally, Gupta *et al*. demonstrated circulating estrogens are required for the formation of ER– tumors using a breast cancer xenograft model[249]. Lastly, aromatase inhibitors resulted in lower ER– cancer incidence in the contralateral breast compared to women who received tamoxifen[46]. Collectively, these studies indicate estrogen promotes ER-independent tumorigenesis.

ER-independent estrogen carcinogenicity is also demonstrated *in vitro* and *in vivo*. For example, ER– MCF-10A cells are transformed by estrogen as measured by growth in soft agar, even in the prescence of tamoxifen[244]. In addition, estrogen-transformed MCF-10A cells produced tumors when injected into immuno-deficient mice, indicating estrogen promotes breast tumorigenesis independent of ER[244]. Therefore, just because TNBC do not express ER, doesn't negate estrogen as a potential mechanism of tumorigenesis. High circulating estrogen levels in obese individuals could be a mechanism of obesity-associated tumorigenesis.

However, mechanistic studies investigating estrogen and cholesterol metabolism, use ER-expressing cell lines/models. For example, one study analyzed the promoter region of SREBF2 for estrogen response elements (ERE), a sequence of DNA that binds to estrogen/ER and regulates gene transcription. They found an ERE-like sequence in the SREBF2 promoter region and demonstrated estradial promoted SREBF2 expression in HepG2 cells (human heptatoblastoma cells), and an ER antagonist inhibited this[250]. While this study demonstrated that estrogen can regulate transcription of SREBF2, this is in an ER-dependent manner. Overall, more studies are needed to further understand how estrogen regulates the mevalonate pathway in an ER-independent manner.

Conclusions

Obesity is a complex disease and understanding the molecular mechanisms underlying how obesity-promotes pre-menopausal ER– breast cancer will reveal targets for prevention and potential biomarkers for at-risk individuals. There is an immediate need to elucidate these molecular mechanisms as there are no current effective chemopreventive strategies for ER– breast cancer. Identifying FGF2 in VAT and the role of FGFR1 in transformation suggests the FGF2/FGFR1 may be important in the etiology of obesity associated ER– breast cancer. In tandem, identifying fluvastatin as a chemopreventive agent suggests HMGCR activity may also be important. Together, these data suggest this FGFR1/HMGCR axis may be potential target for prevention and FGF2 could function as a biomarker for at risk individuals. Furthermore, developing the first HTS to model transformation marks a new advancement for 3D cell culture models. The transformation HTS can evaluate the ability of compounds to inhibit transformation, making this is a breakthrough in the technological development of cancer prevention HTSs.

APPENDICES

Appendix A: Fluvastatin decreases cholesterol in MCF-10A cells

Statins are clinically used as cholesterol lowering agents. To determine if fluvastatin was inhibiting HMGCR in MCF-10A cells, lipids were isolated from MCF-10A cells treated with fluvastatin (1 μM) for 24 hours. Fluvastatin-treated MCF-10A cells had significantly reduced cholesterol content then vehicle controls (Figure A.1)

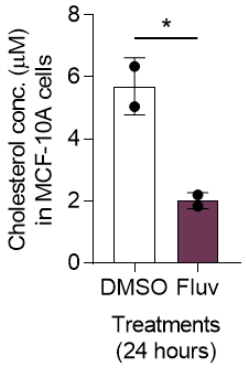

Figure A.1. Fluvastatin treatment for 24 hours significantly decreases the cholesterol content in MCF-10A cells. MCF-10A cells were treated with DMSO or fluvastatin (1 μM) for 24 hours. Cholesterol was quantitated as described in Methods. Significance was determined by Student's t-test (*$p < 0.05$).

Materials/Methods

Amplex Red cholesterol quantitation assay: MCF-10A cells were plated in 6-well plates and incubated at 37°C and after 24 hours/or time required for cells to reach the appropriate density, cells were treated according to each experimental parameter. Cells were washed 3X with PBS. Cells were scraped in 200 µL of chilled PBS and transferred to sterile glass vials and centrifuged at 3,000 x g. PBS was decanted and 370 µL of chilled (-20°C) methanol was added to each vial. Vials were sonicated for 10 second (twice) and then 270 µL of chloroform. Vials were vortexed for 1-2 minutes and then centrifuged for 5 minutes at 3,000 x g. The supernatant was transferred to new glass vial. For the glass vials with the pellet, 370 µL of chilled (-20°C) methanol was added to each vial. Vials were sonicated for 10 seconds (twice) and then 270 µL of chloroform. Vials were vortexed for 1-2 minutes and then centrifuged for 5 minutes at 3,000 x g. The supernatant was transferred the same vial as previous supernatant. Samples were run on a speedvac to evaporate the methanol:chloroform mixture. Lipids were resuspended in 200 µL of 1X reaction buffer from the Cholesterol Quantitation Kit (Invitrogen, A12216). The cholesterol quantitation assay was performed according to the protocol in the Cholesterol Quantitation Kit.

Appendix B: FNTA and FDFT1 have co-occurring mRNA alterations with FGFR1 in breast cancer patients

The mRNA of FGFR1 and mevalonate-related proteins was analyzed in breast cancer patients using the public cBioPortal for Cancer Genomics of four databases: TCGA, Firehose Legacy; TCGA, Nature 2012; TCGA, PanCancer Atlas; MetaBric, Nature 2012 & Nat Commun 2016 (Figure B.1). Figure A.1 is collectively summarized in Table B.1 listing the p values and how many data sets out of four reached significance. The mRNA of squalene synthase (FDFT1) was significantly reduced in FGFR1 amplified samples compared to FGFR1 diploid patients in three out of four data sets. FNTA mRNA was significantly elevated in FGFR1 amplified vs FGFR1 diploid patients in three datasets. Additionally, FDPS and GGPS1 mRNA was significantly reduced in FGFR1 amplified compared to FGFR1 diploid patients in three datasets.

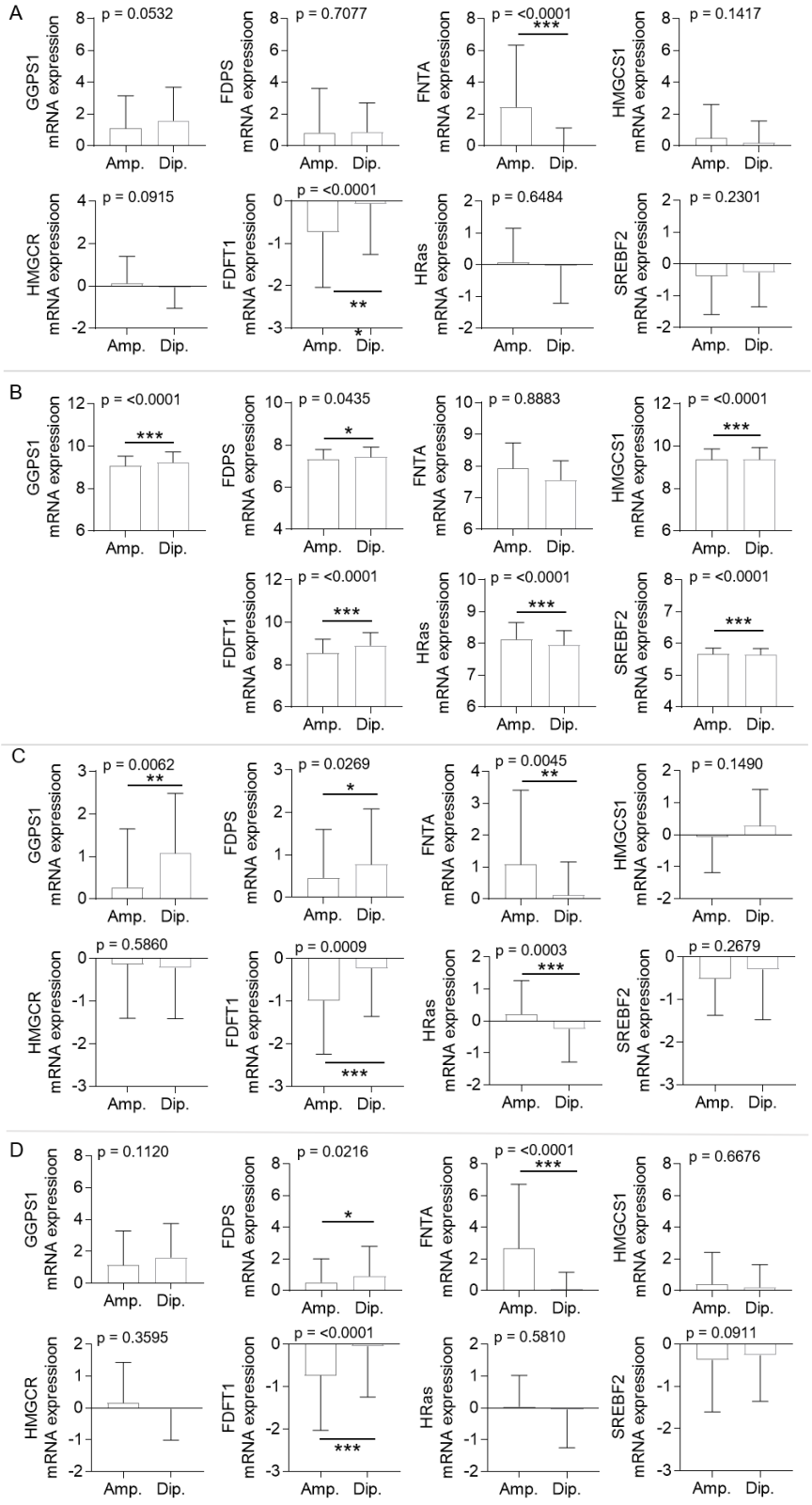

Figure B.1. Using four TCGA breast cancer databases, mRNA aberrations in FDPS, FNTA, and FDFT1 were associated with FGFR1 mRNA amplification in breast cancer patients. Breast cancer patients listed in the A) TCGA, firehose database, B) TCGA, METABRIC, Nature 2012 & Nat Commun

Figure B.1. (Cont'd). 2016, C) TCGA, Nature 2012, and D) TCGA PanCancer Atlas were analyzed for mRNA gene expression in amplified and diploid FGFR1. Statistics were analyzed by Pierson's Student's t-test (*p<0.05, **p<0.01, ***p<0.001).

Table B.1. P values calculated by Student's t-test comparing the mRNA of proteins in the Mevalonate pathway in FGFR1 Amplified vs Diploid Breast Cancer Samples

Gene	Firehouse p value	MetaBric p value	Nature p value	PanCancer p value	Significance
GGPS1	0.0532	<0.0001	0.0062	0.1120	2/4
FDPS	0.7077	0.0435	0.0269	0.0216	0/4
FNTA	<0.0001	0.8883	0.0045	<0.0001	3/4
HMGCR	0.0915	-	0.1490	0.6676	0/3
HMGCS1	0.1417	<0.0001	0.5860	0.3595	1/4
FDFT1	<0.0001	<0.0001	0.0009	<0.0001	4/4
HRas	0.6484	<0.0001	0.0003	0.5810	2/4
SREBF2	0.2301	<0.0001	0.2679	0.0911	1/4

Abbreviations: Firehose- TCGA firehose database; Metabric- TCGA METABRIC Nature 2012 & Nat Commun 2016; Nature- TCGA Nature 2012; PanCancer- TCGA PanCancer Atlas.

Furthermore, three of these datasets were used to analyze for patterns of co-occurrence or mutual exclusivity regarding the mRNA in breast cancer patients (the MetaBric dataset did not have co-occurrence/mutual exclusivity data available). Patterns of co-occurrence and mutual exclusivity can inform on functionally relevant mechanisms altered for oncogenesis.[251] Mutually exclusive alterations tend to suggest functional redundancy, meaning both alterations are unnecessary as the second will not be advantageous or could mean that both alterations induce synthetic lethality.[252] On the other hand, co-occurrent alterations suggest functional synergy. As calculated by cBioPortal, there is significant co-occurrence with alterations between FGFR1 / FDFT1, FGFR1 / FNTA, and between FDFT1 / FNTA (Table B.2). Interestingly, FDPS and GGPS1 tended to have co-occurring alterations. This suggest that FGFR1, FDFT1 and FNTA have functional synergy in breast cancer. FDPS and GGPS1 also had a tendency for co-occurrence, suggesting functional synergy

Table B.2. mRNA alterations of FGFR1 and/or proteins in the mevalonate pathway and their tendencies to co-occur

	A	B	None	A only	B only	Both	q-value
TCGA, Firehose	FGFR1	FNTA	309	38	93	59	<0.001
	FGFR1	FDFT1	326	56	76	41	<0.001
	FDFT1	FNTA	279	68	103	49	0.012
	FDPS	GGPS1	226	83	101	89	<0.001
TCGA, Nature 2012	FGFR1	FNTA	314	42	67	34	<0.001
	FGFR1	FDFT1	324	54	57	22	0.036
	FDFT1	FNTA	303	53	75	26	<0.001
	FDPS	GGPS1	262	62	98	35	0.185
TCGA, PanCancer	FGFR1	FNTA	829	75	40	52	<0.001
	FGFR1	FDFT1	833	113	36	14	0.035
	FDFT1	FNTA	863	41	83	9	0.210
	FDPS	GGPS1	856	41	45	54	<0.001

Abbreviations: Firehose- TCGA firehose database; Metabric- TCGA METABRIC Nature 2012 & Nat Commun 2016; Nature- TCGA Nature 201; PanCancer- TCGA PanCancer Atlas.

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