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Expression of adipocyte/macrophage fatty acid binding protein promotes tumor growth and metastasis.

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EXPRESSION OF ADIPOCYTE/MACROPHAGE FATTY ACID BINDING PROTEIN PROMOTES TUMOR GROWTH AND METASTASIS

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April 26, 2012

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DEDICATION

This dissertation is dedicated to my late mother

Georgetta Triplett,

and my grandmother,

Tommie Triplett.

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I would first like to express my deepest gratitude to my advisor, Dr. Jill Suttles. I am honored to have had the privilege to work with you. I truly appreciate your support and patience. You have always been so encouraging, enthusiastic, and supportive in all situations, and have truly made my Ph.D. experience positive and enjoyable. I admire you not only as a scientist, but as a woman. Throughout this difficult time, you have done a terrific job at balancing work and home life. As I continue on my career path, I will try to live up to the example that you have set, and hope to inspire future scientists that way that you have inspired me.

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ABSTRACT

EXPRESSION OF ADIPOCYTE/MACROPHAGE FATTY ACID BINDING PROTEIN PROMOTES TUMOR GROWTH AND METASTASIS

Ashley Triplett

April 26, 2012

It has been estimated that 30% of all cancer deaths in the U.S. are associated with obesity. It is well-established that obesity promotes low-grade chronic inflammation, however the mechanisms by which obesity-induced chronic inflammation may promote cancer development and progression are not well-defined. Fatty acid binding proteins (FABPs), which are intracellular lipid chaperones, regulate both metabolic and inflammatory pathways. Of the nine FABP family members, adipocyte/macrophage-FABP (A-FABP) has been found to be highly expressed in macrophages in both mice and humans and its expression is increased in response to a high-fat diet. In the present study we examined the influence of A-FABP expression on tumor growth and metastasis in mice under conditions of normal or high-fat feeding. Wild-type (WT) and A-FABP knockout (A-FABP KO) mice were placed on a normal or high-fat diet prior to the injection of Lewis Lung Carcinoma cells (LL/2). When fed a normal diet, LL/2 tumor metastasis was significantly reduced in A-FABP KO mice relative to WT mice, whereas tumor growth in A-FABP KO and WT mice was similar. However, a high fat diet resulted in a significant increase in both tumor growth and

metastasis in WT, but not A-FABP KO mice. Western blot and RT-PCR analysis demonstrated that tumor-infiltrating macrophages isolated from A-FABP KO mice on a normal or high-fat diet have reduced pro-inflammatory cytokine production, NF-kB activation, and decreased expression of metastasis-promoting proteins, MMP-9 and MMP-12. Immunohistochemical analysis showed reduced expression of CD31 in tumors from A-FABP KO mice on either diet compared to tumors from WT mice. Taken together, our data suggest that A-FABP contributes to tumor growth and metastasis and implicate A-FABP as a link between fat consumption and cancer progression.

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INTRODUCTION

General Background

Obesity is an increasing problem amongst children and adults in the United States **(Figure 1)** and it is estimated that 30% of all cancer deaths in the U.S. are associated with obesity. Obesity has been shown to increase the risk, incidence, and mortality of several types of cancers including those of the breast, prostate, colon, pancreas, kidney, liver, esophagus, endometrium, and many others [1, 2]. It is well established that obesity promotes systemic low-grade chronic inflammation, and chronic inflammation is also involved in the pathogenesis of 15-20% of human tumors. The main inflammatory component both within the tumor and the adipose tissue of obese humans are macrophages [3-5]. In an obese state, there is increased recruitment and accumulation of macrophages into the adipose tissue. In lean mice, 10-15% of cells in the adipose tissue express the macrophage marker F4/80, whereas 45-60% of cells are $F4/80^+$ in the adipose tissue of obese mice [6, 7]. Adipose tissue macrophages exhibit a polarized pro-inflammatory phenotype with increased production of various inflammatory cytokines such as interleukin-6 (IL-6) and

tumor necrosis factor-α (TNF-α), leading to a chronic inflammatory state. This, in turn, promotes the onset and progression of many diseases that collectively make up metabolic syndrome, including atherosclerosis, diabetes, and insulin resistance. In cancer, macrophages secrete a variety of growth factors, chemokines, and cytokines that potentiate inflammation, tumor cell growth, proliferation-and survival, angiogenesis, as well as invasion and metastasis [8-12]. However, the mechanism(s) by which obesity-induced chronic inflammation promotes cancer development and progression is not well defined. Herein, we demonstrate that adipocyte-fatty acid binding protein (A-FABP), which is expressed in macrophages and can influence their inflammatory phenotype, serves as a link between obesity and cancer via impacting the functional phenotype of tumor-infiltrating macrophages **(Figure 2)**.

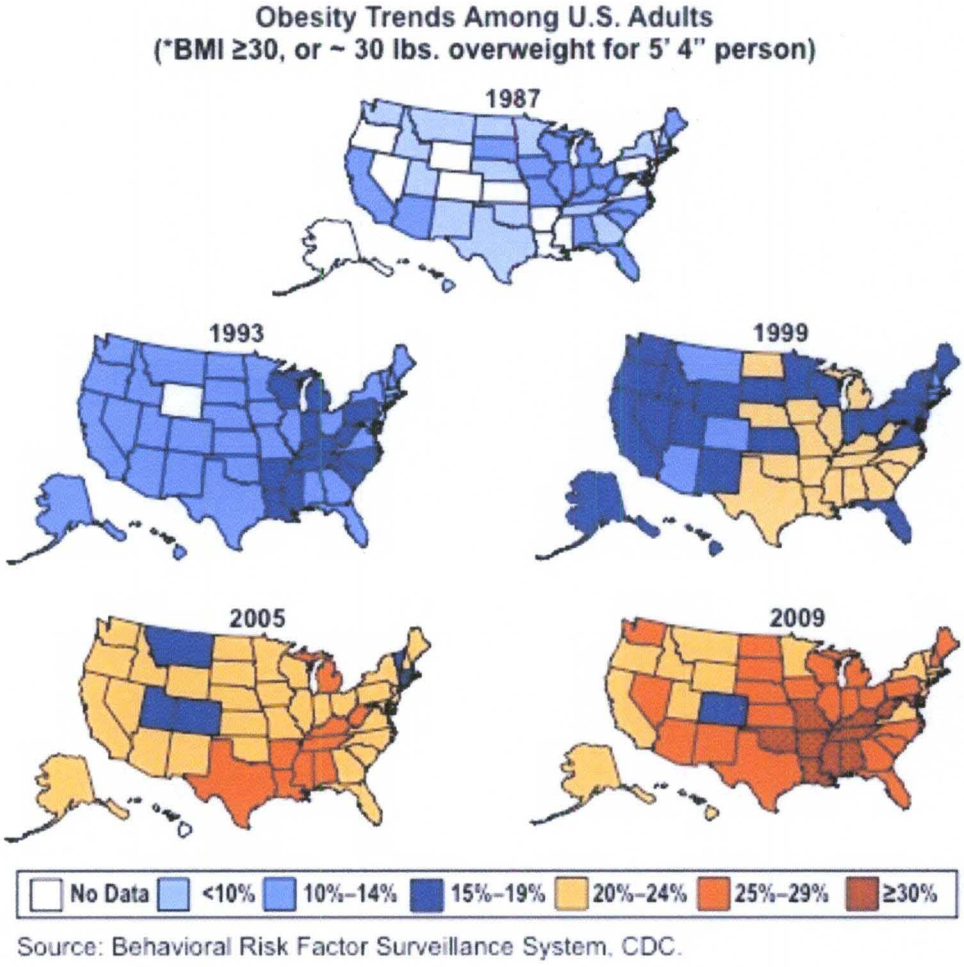

Figure 1. Statisical description of obesity trends among adults in the United States. The Center for Disease Control and Prevention (CDC) conducted a study where they followed obesity trends in U.S. adults over a 24-year period. In 1987, the U.S. was a relatively lean country with less than 14% of state populations considered to be obese. By 2009, however, majority of U.S. state populations had 25% or more adults that were obese. Adapted from Behavioral Risk Factor Surveillance System, CDC.

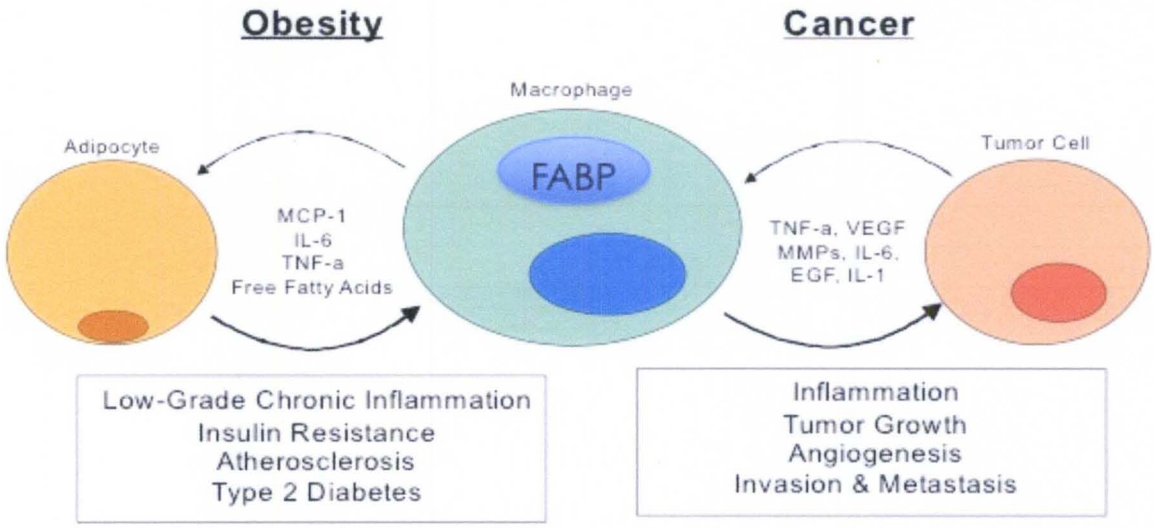

Figure 2. The role of macrophages in obesity-related diseases and cancer. Macrophages have a well-documented role in both obesity and cancer. Through the secretion of various cytokines, growth factors, fatty acids, and chemokines, macropahges promote the progression and development of several obesity-related diseases, including atherosclerosis and type 2 diabetes, as well as cancer. However the link between obesity-induced chronic inflammation and cancer progression remains elusive. Here, FABP expression in macrophages may serve as a link between a high fat diet and the progression of cancer.

Macrophage Function and Plasticity

The mononuclear phagocyte system is a subpopulation of immune cells that are generated from hematopoietic stem cells located in the bone marrow. Monocytes are released into the blood circulation and seed various tissues throughout the body. During inflammation and steady state, although the latter is less efficient, monocytes differentiate into macrophages or dendritic cells [13, 14]. Macrophages are highly versatile, multi-functional cells that are involved in the inflammatory response as well as common "janitorial" roles where they clear the interstitial environment of waste material [14, 15]. Because of the many duties that macrophages are involved in, they are able to take on distinct phenotypes [16, 17]. The acquisition of a distinct phenotype and activation status is highly dependent upon stimulatory factors that are present in the tissue microenvironment [18]. Several factors have been shown to alter macrophage phenotype and function including arachidonates, complement proteins, cytokines, stress hormones, apoptotic cells, catecholamines, and even fatty acids [18-24]. Several studies have used a characterization approach whereby gene expression profiles following cytokine or microbial stimulation of macrophages are used to classify macrophage subsets. From this, two subsets with distinct phenotypes have been described: classically activated M1 macrophages and alternatively activated M2 macrophages.

Classically activated macrophages are effector cells that employ pro-inflammatory and phagocytic functions during anti-microbial and anti-tumoral

immune responses. Upon activation by tissue cytokines and chemokines, notably IFN-γ, TNF-α, and TLR ligands, infiltrating macrophages will induce the activity of a combination of transcription factors, including signal transducer and activator of transcription (STAT) molecules and NF-kappa B (NF-κB) [25, 26]. These transcription factors, in turn, up-regulate genes involved in inflammation and pathogen clearance, such as reactive oxygen species and nitric oxide [27, 28]. Although the pro-inflammatory cytokines that are produced by classically activated macrophages are imperative for host defense, they can also cause considerable damage to the host. For example, reports have demonstrated that macrophage-derived IL-6, IL-23, and IL-1 are associated with the development and expansion of T_H17 cells, and the subsequent production of IL-17 has been shown to contribute to autoimmune disease, including rheumatoid arthritis and inflammatory bowel disease [29-33]. Nonetheless, classical activation of macrophages is vital for the protection of the host against viral and microbial pathogens, and even tumor development, as long as the response stays tightly controlled.

In contrast, alternatively activated macrophages display anti-inflammatory properties and are involved in resolution of the inflammatory response followed by wound healing. Alternative activation of macrophages is induced in the presence of IL-4 or IL-13, and produce anti-inflammatory cytokines including IL-10 and TGF-β, along with increased arginase [34, 35]. Promotion of wound healing and tissue repair is induced by the production of various proteases, growth factors, and angiogenic factors such as vascular endothelial growth factor

(VEGF), matrix metalloproteinases (MMPs), and epidermal growth factor (EGF) [36].

Macrophage activation, however, is much more complex and can not be linearly classified as classically or alternatively activated. Rather than discrete stable subpopulations, some scientists believe that macrophages represent a spectrum of phenotypes, and there are several documented studies demonstrating the flexibility in macrophage programming, with macrophages shifting from one functional phenotype to another in response to microenvironmental signals [37]. For example, upon clearance of bacteria, macrophages will begin to phagocytose apoptotic cells and down-regulate pro-inflammatory gene transcription in favor of a tissue reparative phenotype [38, 39]. This clearly demonstrates phenotypical adaptation rather than substituting subsets.

Macrophages are indeed crucial for tissue homeostasis and host defense, but they can also have pathologic roles. For example, macrophages are involved in intracellular lipid accumulation and foam cell formation, thus contributing to the development and progression of atherosclerosis [40]; macrophage-derived TNF and IL-23 mediates the pathology of Crohn's disease [41]; macrophages are key regulators in demyelinating disease of the central nervous system, such as experimental autoimmune encephalomyelitis (EAE) in mice and multiple sclerosis in humans [42]; and they are heavily involved in tumorigenesis [43]. Due to their plastic nature and their role in disease pathology, macrophages may make for effective therapeutic targets via the manipulation of their functional phenotype.

The Role of Macrophages in Cancer

The association between inflammation and cancer development and progression is well established [4, 44, 45]. It is now clear that virtually all tumors contain an array of immune cells at densities ranging from subtle inflammation to heavy infiltration [46]. Infiltrating immune cells were originally thought to be involved in the eradication of tumors, and although there is evidence demonstrating anti-tumor responses, many of these cells, particularly innate immune cells, display tumor-supporting phenotypes. One of the main inflammatory components within the microenvironment of primary and secondary tumors are macrophages [3]. High density of tumor-infiltrating macrophages (TIM) is associated with reduced patient survival in many different forms of cancer, including cancer of the breast, prostate, bladder, kidney, endometrium, esophagus, as well as follicular lymphoma, and squamous cell carcinoma [47-51]. This poor prognosis is primarily due to the ability of TIM to produce bioactive molecules including growth factors, cytokines, chemokines, and matrix-modifying enzymes that promote tumor cell proliferation and growth, survival, angiogenesis, activation of epithelial-mesenchymal transition, as well as invasion and metastasis [8-10, 12, 52].

During tumorigenesis, monocytes enter tumors through blood vessels. Monocyte and macrophage infiltration can be found in early-stage tumors that are beginning to vascularize and late-stage tumors that are invasive and metastatic [9, 11, 52]. The constant recruitment of monocytes into tumors can be attributed to tumor-derived chemoattractants, including colony-stimulating factor-

1 (CSF-1), monocyte chemotactic protein-1 (MCP-1), CCL2, CCL3, CCL4, CCL5, CCL8, and VEGF [53]. Studies have demonstrated that the expression level of these proteins positively correlates with TIM numbers [54]. To further confirm the important role of macrophages in cancer progression, early studies were conducted using mice with a homozygous CSF-1 null mutation to deplete macrophages in a mouse model of breast cancer [55, 56]. Depletion of macrophages resulted in reduced progression of pre-invasive lesions to malignant lesions, as well as reduced lung metastases.

Of the many pro-tumoral functions that macrophages exhibit, angiogenesis is the most crucial for tumor growth since tumors require the development of new blood vessels for expansion [3, 57]. Via the production of many essential factors such as IL-1, TNF-α, IL-8, COX-2, MMP-9, and VEGF, TIM are able to contribute significantly to angiogenesis [58-64]. Within tumors, TIM cluster in areas of hypoxia, which up-regulates the transcription factor hypoxia-inducible factor-2α (HIF-2α). HIF-2α activation, in turn, induces the expression of VEGF [65]. VEGF expression is also able to up-regulate the production of CSF-1, and together, both factors serve as chemoattractants for additional macrophage recruitment [66]. Studies have further demonstrated that the production of IL-8, IL-1, and TNF-α promotes proliferation and migration of endothelial cells, along with matrix remodeling and blood vessel formation induced by MMP-9 and VEGF, respectively.

In addition to angiogenic roles, TIM are also critical mediators in tumor cell invasion and metastasis [67, 68]. During the metastatic process, TIM infiltrate the

basement membrane where they secrete various MMPs that are able to degrade the basement membrane. This allows for the creation of an egress, whereby tumor cells can invade into surrounding tissues [8]. Multiphoton intravital imaging of the breast microenvironment showed that TIM also directly promote tumor cell intravasation into the blood stream [69, 70]. Using this technology, which consisted of transgenic mice expressing fluorescently tagged cancer cells, endothelial cells, and macrophages, allowed for the visualization of interactions between these cell types at the site of intravasation. Wyckoff and colleagues found that TIM increased the motility of tumor cells, which was amplified when tumor cells were in close proximity with perivascular TIM [69]. In fact, tumor cells were found to invade blood vessels only where perivascular TIM were located. The use of *Csf1*$^{op/op}$ PyMT mice, which have reduced macrophage infiltration and a decrease in circulating cancer cells, demonstrated the functional importance of this interaction for intravasation. Moreover, a macrophage EGF-CSF paracrine loop was found to be crucial for intravasation, as inhibition of this signaling pathway led to reduced numbers of cancer cells in the blood stream [69]. Additional studies demonstrated that TIM also regulate the density of collagen fibers, which serve as tram lines for tumor cells and macrophages to travel along within the tumor stroma. Many of these fibers are bound to blood vessels, resulting in the accumulation of tumor cells at the vessels [71].

The Role of Macrophages in Obesity and Metabolic Diseases

In addition to having very important roles in the development and progression of cancer, macrophages are also heavily involved in obesity-related inflammation and metabolic syndrome [5, 72]. In 2003, two studies illustrated that in an obese state, macrophages infiltrate the adipose tissue and are primarily responsible for the inflammatory environment [6, 7]. Expanding adipocytes and neighboring pre-adipocytes produce signals that induce the recruitment and accumulation of macrophages into the adipose tissue in both mice and humans. During late-stage obesity, adipocyte death serves as an additional mechanism by which macrophages infiltrate the adipose tissue [73]. In addition to differences in the adipocyte to macrophage ratio, adipose tissue macrophages also display functional differences between lean and obese mice [74]. Adipose tissue macrophages in lean mice exhibit an anti-inflammatory, alternatively activated-like phenotype. These macrophages have increased production of IL-10, which plays a role in maintaining insulin sensitivity [74, 75]. In contrast, adipose tissue macrophages from obese mice have a pro-inflammatory, classically activated-like phenotype and they are typically found in 'crown-like' structures around dying adipocytes [72, 74]. There are several published studies supporting the idea that these macrophages are the primary cell type contributing to the pathogenesis of obesity-induced diseases, including insulin resistance, type 2 diabetes, and atherosclerosis [22, 76-78]. First, there is protection from obesity-induced inflammation and insulin resistance in mice lacking CC-chemokine receptor 2 (CCR2), which is a receptor that is required for the recruitment of inflammatory

monocytes and macrophages into tissues [79]. CCR2 and its ligand CCL2 are postulated to have a dominant role in the trafficking of monocytes and macrophages to the adipose tissue [79, 80]. Second, CD11c-DTR mice, which are used to selectively deplete $CD11c^+$ classically activated macrophages, also show reduced inflammation in the adipose tissue along with improved insulin sensitivity [81]. Third, by genetically deleting IKKβ in myeloid cells, Arkan and colleagues were able to reduce myeloid cell-mediated inflammation in the adipose tissue [82]. Additionally, reconstituting mice with JNK-deficient bone marrow conferred similar results [83]. Lastly, ablation of a G protein-coupled receptor GPR120, which mediates the anti-inflammatory actions of omega-3 unsaturated fatty acids, exacerbates inflammation in the adipose tissue and insulin resistance [84].

It has been suggested that saturated, but not unsaturated, fatty acids prompt the activation of M1-like macrophages in the adipose tissue of obese mice. This phenomenon is mediated by ligation of TLR-4 and subsequent signaling pathways [85-87]. For example, the infusion of lipids adequately induces adipose tissue inflammation and insulin resistance in wild-type but not TLR-4-deficient mice [88]. Moreover, studies have shown that high fat diet-induced insulin resistance requires TLR-4 expression by hematopoietic cells in the adipose tissue and liver [89, 90]. In addition to TLR-4 studies, others have shown that MyD88 signaling may link inflammation and metabolism. Deletion of MyD88 in mice confers more severe metabolic disease in response to a high fat

diet when compared to wild-type mice, suggesting that MyD88 may have a protective role [87].

Another macrophage-expressing factor that links metabolism and inflammation is adipocyte/macrophage fatty acid binding protein (A-FABP). Ongoing studies in our lab and others have demonstrated the influence of this protein on macrophage inflammatory function under conditions of metabolic stress.

Obesity and Cancer

Clinical and epidemiological studies have linked obesity and obesity-associated diseases to increased cancer risk, incidence, and mortality. Overweight and obesity led to an almost 2-fold increase in cancer risk in both men and women with a body mass index (BMI) of >25 kg/m^2 [91]. This increase does, however, comply with the type of cancer. For example, in men with a BMI of 35-40 kg/m^2, there is about a 5-fold increase in hepatocellular carcinoma, 2-3 fold increase in oesophageal cancer [92], and even the slightest increase in BMI can put individuals at risk for cancers of the pancreas, kidney, gastrointestinal tract, and liver [93]. In fact, several studies focusing on kidney cancer reported a dose-response correlation between increasing weight or BMI and kidney cancer incidence [94-96]. Obesity and breast cancer incidence have consistently mirrored one another, with a 30-50% increase in breast cancer rates amongst obese women, and mortality and survival studies have shown that very obese

women, with a BMI of ≥40 kg/m^2 have a 3-fold increase in breast cancer death rates compared to lean women (BMI <20.5 kg/m^2) [97]. Cancer of the endometrium was the first to be recognized as being associated with obesity. Studies show a linear increase in endometrial cancer risk and increasing BMI [98, 99]. In regards to prostate cancer, evidence shows that although there is only a slightly higher risk of prostate cancer in obese men compared to lean men, prostate tumors in obese men are significantly more aggressive and there is a higher chance of recurrence after radical prostatectomy [100, 101]. In contrast, studies have shown that maintaining healthier weights can actually lower the risk for many cancers including colon, breast, endometrium, kidney, and oesophageal cancer [99]. In 2009, it was reported that 15-20% of cancer deaths was associated with obesity, however in 2010, the American Association for Cancer Research annual report showed that 30% of all cancer deaths in the United States were associated with obesity. Thus, this relationship is becoming an increasing problem, however the mechanism(s) behind the obesity and cancer association is poorly understood.

Currently, the strongest evidence to mechanistically link obesity to cancer are hormonal effects. For example, increased estrogen production in the adipose tissue of obese women is associated with increased breast cancer risk. This is partly due to increased expression and activity of aromatase, an enzyme that is responsible for the biosynthesis of estrogen, in both the adipose tissue and mammary gland [102]. Additionally, it is well established that obesity leads to the development of insulin resistance and chronic hyperinsulinmaemia. Higher levels

of circulating insulin leads to a reduction of insulin-like growth factor binding protein 1 (IGFBP1), which in turn, increases the availability of insulin-like growth factor 1 (IGF1). Insulin and IGF1 both signal through insulin receptors to promote cellular proliferation and inhibit apoptosis in many cell types, thus contributing to tumorigenesis. Elevated levels in circulating insulin has also been identified as a risk factor for many cancers and is associated with late-stage disease and poor prognosis [103].

Excess adiposity is also associated with an increase in free fatty acids in the environment, and numerous studies have looked at fatty acid metabolism as a potential mechanism to link obesity to cancer. For example, under obese conditions, polyunsaturated fatty acids are chemically oxidized to generate high amounts of reactive oxygen species (ROS). ROS, in turn, promote cancer development via DNA damage. ROS generated from lipids can also lead to cyclooxygenase-2 (COX2) up-regulation, which in turn, promotes colorectal, breast, and prostate carcinogenesis [104, 105]. Hyperglycemia and elevated triglycerides, which are hallmarks of obesity, can also induce the generation of ROS and lead to tumorigenesis [104]. Fatty acid synthase (FAS), which is an enzyme that catalyzes fatty acid synthesis, is also associated with poor prognosis in breast and prostate cancer patients.

Park and colleagues showed that obesity is indeed a tumor promoter via the administration of a liver chemical carcinogen, diethylnitrosamine (DEN). DEN failed to induce hepatocellular carcinoma (HCC) on its own, however HCC did develop only in genetic or diet-induced obese mice, and the effect was just as

strong as the more common liver tumor promoter, phenobarbital [2]. Moreover, they found that the tumor-promoting effects of obesity in HCC were due to the low-grade chronic inflammatory state that it promotes, including elevated levels of TNF and IL-6 [2, 106]. It is well established that obesity promotes systemic low-grade chronic inflammation, but the influence of obesity-induced chronic inflammation in cancer development and progression hasn't been well explored. Adipose tissue is an organ of many functions that secretes a variety of pro-inflammatory adipokines and cytokines, including adiponectin, leptin, IL-6, MCP-1, IL-8, VEGF, and TNF-α, all of which have been implicated in metabolic diseases and cancer. Visceral adipose tissue, which has been described as being more metabolically active, correlates with circulating levels of leptin, which promotes proliferation, angiogenesis, and MMP expression in oesophageal and colon cancer [1]. Additionally, TNF-mediated activation of NF-κB has been shown to increase nitric oxide (NO) production. NO serves as a substrate for the generation of ROS, and ROS in combination with other inflammatory cytokines, leads to insulin resistance and glucose intolerance. It has been proposed that insulin resistance, ROS, and inflammatory cytokines lead to a vicious cycle since these factors facilitate continuous NF-κB activation, and this may be a potential hallmark in obesity-induced chronic inflammation and cancer [107]. Because macrophages are huge contributors to the inflammatory milieu in both adipose tissue and tumors, we decided to focus our work on this cell type in the context of obesity and cancer.

Fatty Acid Binding Proteins

Fatty acids are well known for their role in maintenance of cell structure and energy metabolism, but they also function as metabolic signaling molecules, thus regulating vital cellular and physiological processes. Fatty acids are capable of modulating the activity of G protein-coupled receptors (GPCRs) by serving as ligands for several GPCRs, including GPR40, GPR43, GPR84, and GPR120. In macrophages, GPR120 binds omega 3 fatty acids resulting in anti-inflammation and insulin sensitization [108]. Fatty acids can also activate and inhibit kinases, such as I kappa kinase β (IKKβ), and serve as ligands for transcription factors, including peroxisome proliferator-activated receptors (PPARs) [109]. In order to carry out these tasks, fatty acid binding proteins (FABPs) are required for the trafficking and targeting of intracellular fatty acids and other bioactive lipids throughout the cell [110].

FABPs constitute a family of small (14-15 kDa), highly homologous intracellular lipid chaperones that coordinate lipid trafficking to various cellular compartments including lipid droplets for storage; the mitochondria for oxidation; the nucleus for regulation of gene transcription; and outside of the cell for autocrine and paracrine signaling. Most importantly, FABPs are strongly linked to the regulation of both metabolic and inflammatory pathways [110, 111]. Thus far, nine FABPs have been identified and include adipocyte (A-), heart (H-), intestinal (I-), brain (B-), epidermal (E-), ileal (Il-), testis (T-), and myelin (M-) FABPs.

However, the nomenclature can be misleading since no FABP is exclusive for a given tissue or cell type. In fact, there are tissues and cells that express more than one type of FABP. Extensive work has been conducted to elucidate the structure and ligand binding characteristics of FABPs. The structural characteristics of FABPs consist of β-barrels arranged in such a way that it forms an interior water-filled cavity where the ligand binds. All FABPs reversibly bind hydrophobic long chain fatty acids, with the specificity and affinity of ligand binding varying amongst the isoforms due to small structural differences. In general, FABPs bind saturated long-chain fatty acids with the highest affinity, followed by unsaturated fatty acids, and then polyunsaturated fatty acids, as well as eicosanoids [112-114].. Tissue distribution of FABP expression varies, ranging from fairly widespread, as is the case for H-FABP which has been shown to be expressed in a variety of tissues, to narrow, as is the case for A-FABP [110]. Generally, FABPs are abundantly expressed in tissues that are actively involved in lipid metabolism [115]. For example, FABPs make up 1-5% of all soluble cytosolic proteins in adipocytes, hepatocytes, and cardiac myocytes, which all undergo high rates of lipolysis. **Table 1** summarizes the type and locations of FABPs that have been discovered to date.

Our work focuses on adipocyte/macrophage fatty acid binding protein (A-FABP) (Gene: *FABP4*), also known aP2 [116, 117]. It is best-characterized member in the FABP family. Initially, A-FABP expression had been thought to be restricted to adipocytes, where it is associated with adipocyte differentiation. However we, and others, have previously demonstrated that A-FABP is also

expressed in macrophages and our lab has shown strong expression of A-FABP in dendritic cells. A-FABP expression in macrophages becomes evident upon differentiation from monocytes, as well as by treatment with phorbol 12-myristate 13-acetate, lipopolysaccharide (LPS), peroxisome proliferator-activated receptor γ (PPAR-γ) agonists, insulin, oxidized low-density lipoprotein (ox-LDL), and tissue injury [118-121]. In contrast, atorvastatin, a cholesterol-lowering statin, reduces A-FABP expression in macrophages *in vitro* [122]. Treatment of macrophages with unsaturated fatty acids also results in A-FABP suppression in macrophages. Coleman et al., conducted a study wherein RAW 264.7 macrophages were pre-treated with various unsaturated fatty acids followed by stimulation with LPS, and despite being stimulated by a highly inflammatory factor, these macrophages maintained lower levels of A-FABP when compared to control-treated macrophages [123].

Macrophage A-FABP binds arachidonic acids and metabolites of the cyclooxygenase (COX) and lipoxygenase (LOX) pathways, including compounds that act as ligands for PPARs [124]. In fact, several A-FABP ligands have a high affinity for PPARs. PPARs are nuclear receptors consisting of three primary subtypes, α, β, and γ. PPARs regulate vital cellular processes that impact lipid homeostasis, inflammation, reproduction, adipogenesis, wound healing, and tumorigenesis. Previous studies from our lab have shown that in macrophages, A-FABP prevents cholesterol efflux via the inhibition of the PPAR-γ – liver X receptor-α (LXR-α) – ATP-binding cassette A1 (ABCA1) pathway, and thus contributes to macrophage foam cell formation in the context of atherosclerosis

[109, 125]. When A-FABP is present, it limits the availability of PPAR-γ ligands, thus preventing PPAR-γ activation and subsequent up-regulation of the genes involved in cholesterol efflux, LXR-α and ABCA1 **(Figure 5)**. Conversely, in A-FABP$^{-/-}$ macrophages, fatty acids are free to bind to PPAR-γ, resulting in LXR-α and ABCA1 expression, and the shuttling of cholesterol out of the cell **(Figure 6)** [109]. In parallel, A-FABP regulates the inflammatory response in macrophages (**Figures 5 and 6)**. In A-FABP deficient macrophages, elevated PPAR-γ activity allows for suppression of NF-κB activity, a mechanism which may be via ligand-dependent transrepression of NF-κB, although this has yet to be proven. Here, PPAR-γ undergoes sumoylation in the ligand-binding domain upon activation, which targets PPAR-γ to nuclear co-repressor (NCoR)/histone deacetylase-3 (HDAC) complexes on inflammatory gene promoters. Because of this, the ubiquitylation/19S proteasome machinery, which indirectly mediates removal of co-repressor complexes required for gene transcription, is not recruited. Thus, NCoR complexes remain bound to promoters, and inflammatory genes are maintained in a repressed state [126]. Additionally, some fatty acids can also directly inhibit IKK activity. A-FABP$^{-/-}$ macrophages also display impaired adaptor protein 1 (AP1) activity via inhibition of the JNK pathway [110, 127]. Relative to wild-type macrophages, A-FABP deficient macrophages show reduced expression of several pro-inflammatory mediators in response to LPS and CD40 ligand stimulation, including prostaglandin E_2, iNOS, IL-1α, IL-1β, IL-6, IL-12, MCP-1/CCL2, and TNFα. However in the presence of A-FABP, restriction of fatty acid nuclear localization allows for components of the NF-κB pathway to be

activated, which is also a contributing factor to foam cell formation and inflammation in atherosclerosis as well as in a model of EAE [109, 126, 128].

Table 1. Family of fatty acid binding proteins.

Gene	Name	Alternate Names	Tissue/Cell Expression
Fabp1	Liver FABP	L-FABP	Liver, intestine, pancreas, kidney, lung, stomach
Fabp2	Intestinal FABP	I-FABP	Intestine, liver
Fabp3	Heart FABP	H-FABP, MDGI	Heart, skeletal muscle, brain, kidney, lung, stomach, testis, aorta, adrenal gland, mammary gland, placenta, ovary, brown adipose tissue
Fabp4	Adipocyte FABP	A-FABP, aP2	Adipocyte, macrophage, dendritic cell
Fabp5	Epidermal FABP	E-FABP, PA-FABP, mal1	Skin, tongue, adipocyte, macrophage, dendritic cell, mammary gland, brain, intestine, kidney, liver, lung, heart, skeletal muscle, testis, retina, lens, spleen
Fabp6	Ileal FABP	IL-FABP, I-BABP, gastrotropin	Ileum, ovary, adrenal gland, stomach
Fabp7	Brain FABP	B-FABP, MRG	Brain, glia cell, retina, mammary gland
Fabp8	Myelin FABP	M-FABP, PMP2	Peripheral nervous system, Schwann cell
Fabp9	Testis FABP	T-FABP	Testis, salivary gland, mammary gland

The Relationship Between PPARs and FABP

A-FABP and PPAR-γ share several ligands indicating a functional link between the two proteins (**Figure 3**). Two possible scenarios are 1) FABPs target ligands to PPARs, resulting in PPAR activation, or 2) FABPs sequester PPAR ligands, resulting in decreased ligand availability and PPAR inactivation. In 2001, Wolfrum and colleagues sought to understand how signaling molecules reached nuclear receptors such as PPARs. Using laser-scanning microscopy, they first showed that liver-FABP (L-FABP) and PPAR-α colocalized in the nucleus of mouse hepatocytes. Pull-down assays and immunocoprecipitation proved that L-FABP and PPAR-α interacted directly, and further transactivation assays showed that PPAR α and γ transactivation correlated with intracellular L-FABP concentrations, suggesting that L-FABP transported PPAR ligands to the nucleus [129, 130]. Using CV-1 cells transfected with A-FABP, Helledie and colleagues demonstrated that A-FABP, when expressed at high levels, resulted in negative regulation of PPAR activation and PPAR-mediated gene transcription [131]. Our lab previously conducted a study where wild-type and A-FABP$^{-/-}$ macrophage cell lines were treated with 13-HODE, a high affinity ligand that binds both A-FABP and PPARγ (**Figure 4**). Using confocal microscopy, we were able to show that in wild-type macrophages, nuclear localization of 13-HODE was inhibited, whereas 13-HODE was free to enter the nucleus and bind to PPARγ in the absence of A-FABP, suggesting that in macrophages, FABPs bind and sequester PPAR ligands, acting as negative regulators of PPAR activity. We have also demonstrated that in the absence of FABPs, PPAR activity is

enhanced [109], resulting in the up-regulation of LXRα and ABCA1 expression, which leads to enhanced cholesterol efflux and protection from atherosclerosis. The finding that A-FABP deficiency protects against atherosclerosis in the apoE-deficient murine model complements our data showing that FABPs act to sequester PPAR ligands.

In addition to a model of atherosclerosis, we have also shown that elevated PPAR activity due to A-FABP deficiency protects mice from developing EAE [128]. In this report, A-FABP deficient mice had lower levels of pro-inflammatory cytokine expression, including impaired IFN and IL-12 production, in the central nervous system tissue compared to wild-type mice, resulting in decreased disease incidence and clinical symptoms [128]. This evidence is also supported by findings demonstrating that PPAR agonists exert a protective effect against EAE development [132]. Moreover, FABPs have high affinity for retinoind-binding receptors, including retinoid X receptor (RXR), which is a heterodimeric partner of PPARs [133]. Retinoic acid influences macrophage inflammatory phenotype by down-regulating IL-12 production via NF-κB inhibition [134]. Because A-FABP binds to retinoic acid, it may function as a negative regulator of RXRs as well. To complement this, we have shown that A-FABP deficient macrophages display elevated responses to agonists of RXR, and further published studies have shown that when combined with PPAR agonists, RXR agonists confer additional protection from EAE [135]. Thus, our data fit very well with literature supporting an anti-inflammatory role of PPARs in metabolic

and inflammatory diseases along with the functional link between FABPs and nuclear receptors.

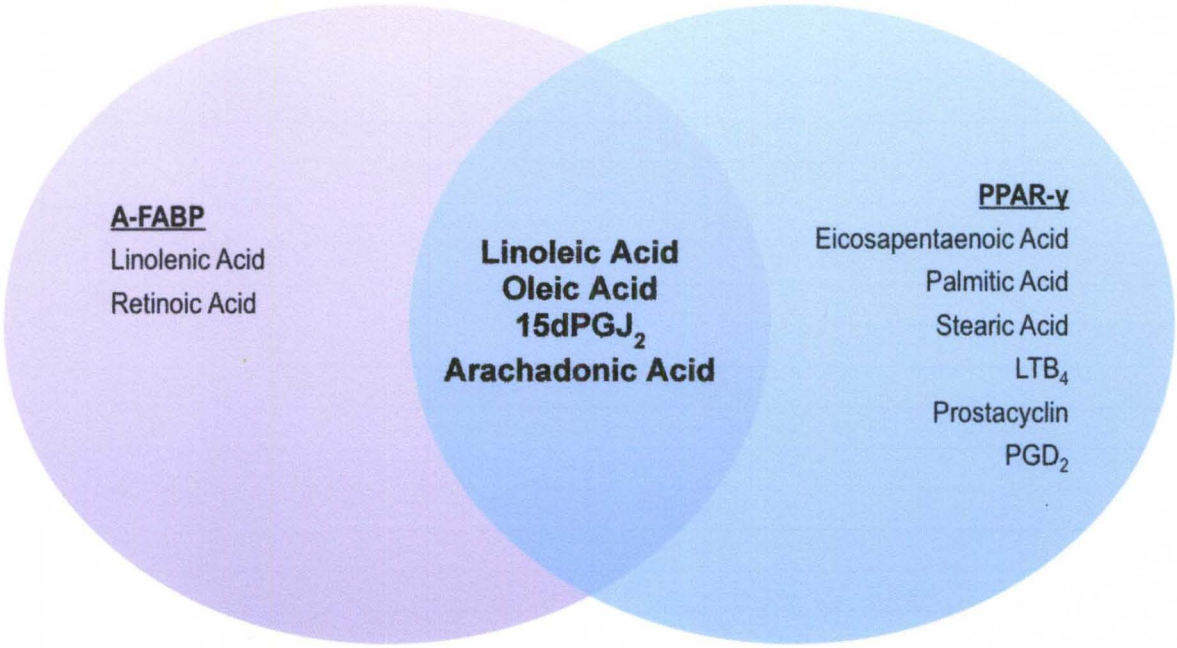

Figure 3. A-FABP and PPARγ ligands. Both A-FABP and PPARγ share several ligands, thus creating a functional link between these two proteins.

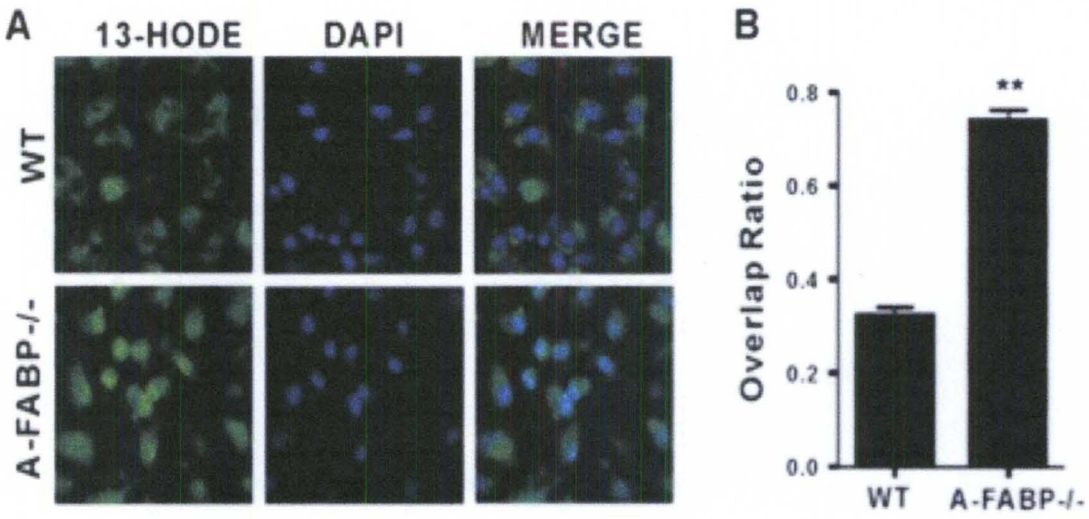

Figure 4. A-FABP expression restricts 13-HODE, a PPARγ ligand, to the cytoplasm. A, 13-HODE (10μM) (green) was restricted in the cytoplasm of macrophages in the presence of A-FABP, but able to enter the nucleus in A-FABP$^{-/-}$ macrophages (turquoise). B, Quantitation of co-localization of 13-HODE in nucleus (**p<0.001).

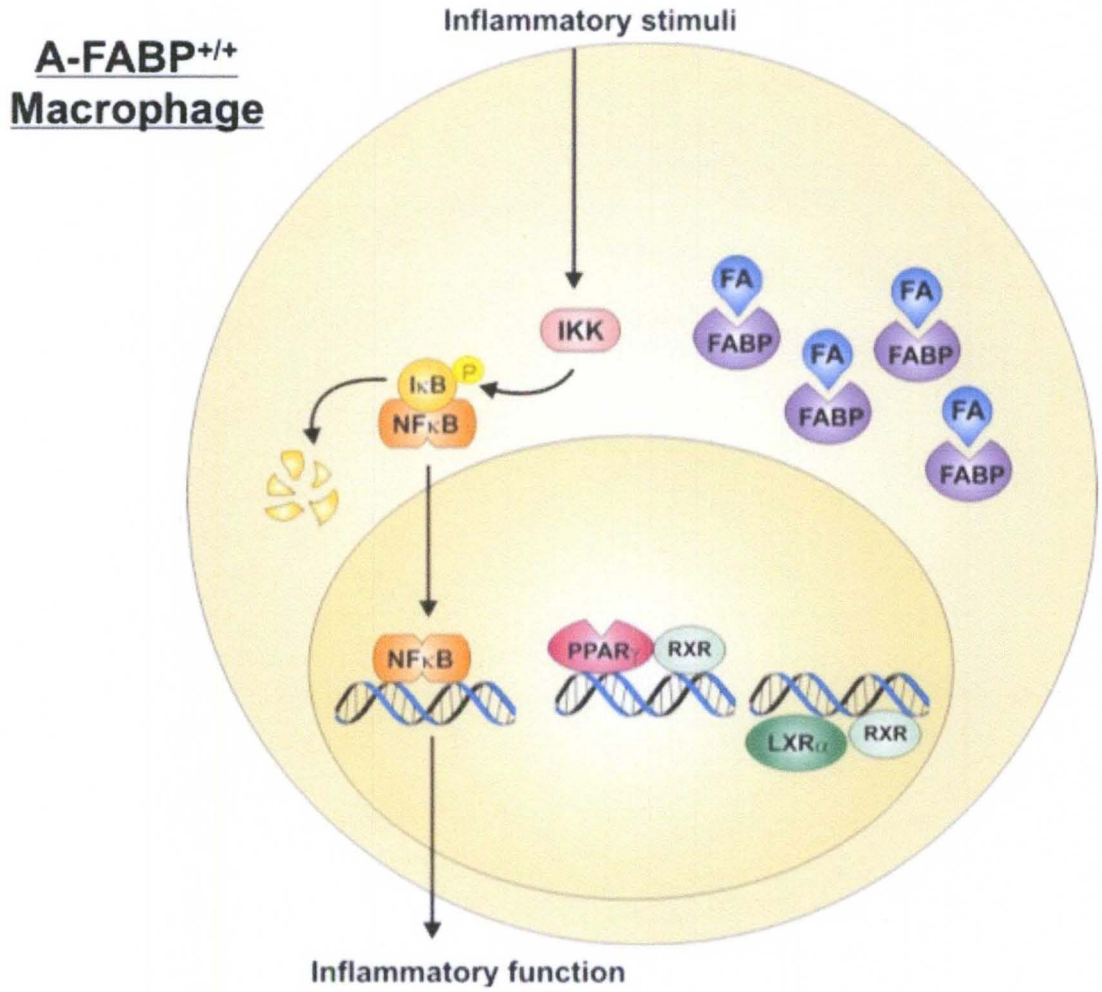

Figure 5. A-FABP promotes inflammation in wild-type macrophages. A-FABP expression in macrophages limits the availability of PPARγ ligands, and in doing so, inhibits PPARγ activity while allowing components of the NF-κB pathway to be activated.

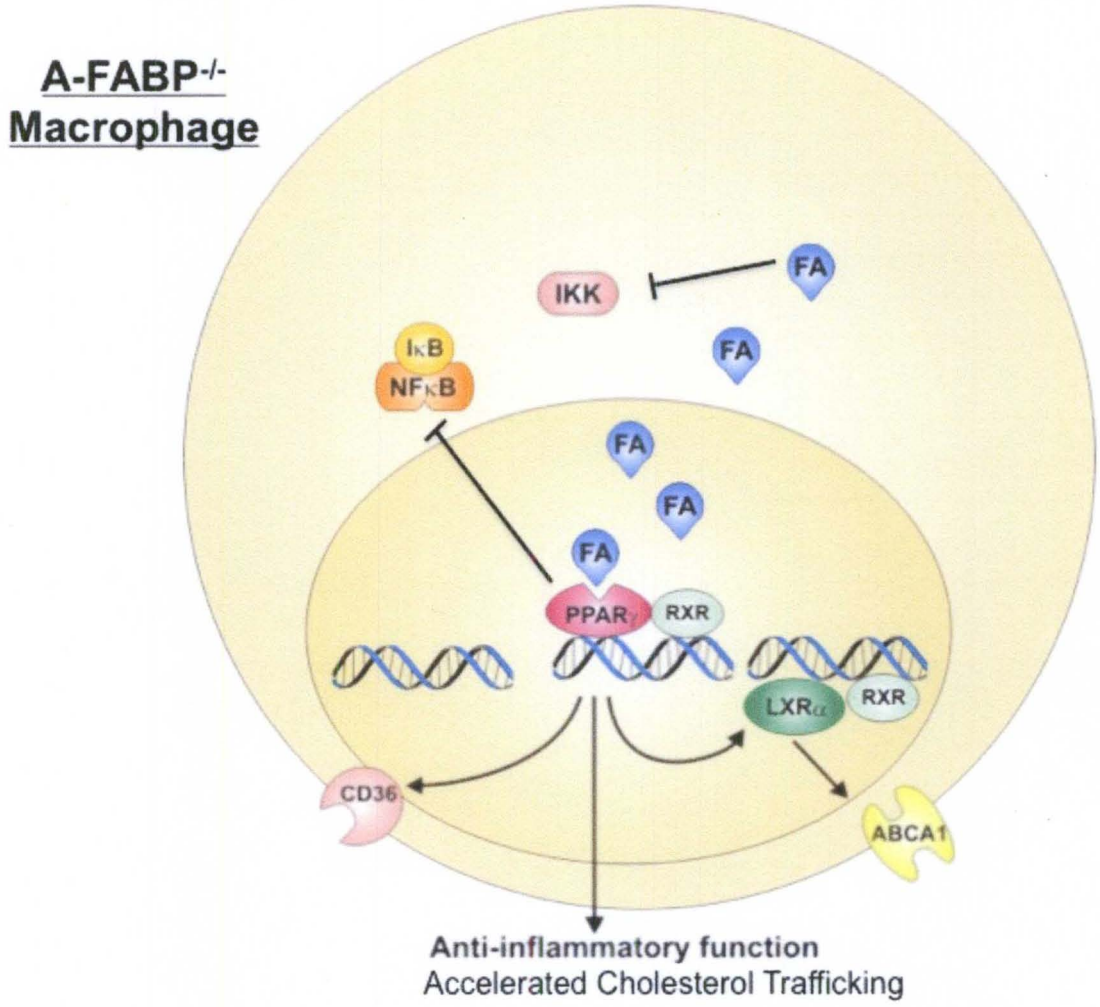

Figure 6. A-FABP deficiency promotes an anti-inflammatory response and cholesterol efflux. In the absence of A-FABP, fatty acids are free to enter the nucleus, bind to and activate PPARγ. This results in up-regulation of genes involved in cholesterol trafficking, including LXRα, ABCA1, and CD36. Enhanced PPARγ activity also inhibits activation of the NF-κB pathway via ligand-dependent transrepression. Additionally, some fatty acids can directly inhibit IKK activity.

The Role of A-FABP in Obesity and Other Disease Models

Despite the extensive work done on the structure and ligand binding properties of FABPs, there are still many questions regarding the primary functional role of these proteins. The elucidation of A-FABP function was facilitated by the production of A-FABP deficient animals generated by Dr. Gokhan Hotaslimigil at Harvard University School of Public Health. Mice deficient in A-FABP are healthy with no defects in metabolism or reproduction and development, however the effects of A-FABP deletion can be observed when mice are subjected to systemic stress such as diet-induced obesity or disease. When subjected to genetic or diet-induced obesity, A-FABP deficient mice, despite weighing slightly more than wild-type controls, are protected from development of insulin resistance and diabetes [124, 136, 137]. Hotamisligil and colleagues showed that free fatty acids from a high-fat diet induce the expression of TNF-α, a molecule which has been highly connected to obesity-related insulin resistance. This study further showed that by binding and shuttling free fatty acids to specific intracellular compartments, A-FABP regulates the expression of TNF-α. In the A-FABP deficient model, mice failed to express adipose tissue TNF-α and showed improved insulin resistance and glucose metabolism compared to wild-type mice [136, 137].

Numerous observations, including the documented role of PPARγ in foam cell formation and the ability of PPARγ to up-regulate A-FABP expression, spurred an examination of the influence of A-FABP deficiency in the development

and progression of atherosclerosis. In these studies, A-FABP deficient mice were cross-bred with the apoE-deficient background such that the apoE-deficient model of atherosclerosis could be exploited. The apoE$^{-/-}$, A-FABP$^{-/-}$ mice displayed significant protection from the development of atherosclerosis when compared to apoE$^{-/-}$, A-FABP$^{+/+}$ animals. Moreover, apoE$^{-/-}$, A-FABP$^{-/-}$ mice fed a normal diet developed 88% less atherosclerosis while apoE$^{-/-}$, A-FABP$^{-/-}$ mice fed a high fat, Western diet developed 91% less atherosclerosis when compared to apoE$^{-/-}$, A-FABP$^{+/+}$ mice fed either diet, suggesting that A-FABP provides an increased protective effect under conditions of metabolic excess. Further bone marrow transplantation studies where A-FABP$^{-/-}$, ApoE$^{-/-}$ bone marrow was transplanted into A-FABP$^{+/+}$,ApoE$^{-/-}$ revealed that the atheroprotective effect of A-FABP deficiency was primarily due to its expression in macrophages [138].

The fact that mice under normal physiologic conditions do not have a compromised phenotype when A-FABP is deleted, but they benefit when faced with systemic inflammatory or metabolic stress, gives rise to the question of why A-FABP exists if it seems to promote dysfunction. Acutely activated signaling systems, such as in acute inflammation, display regulatory mechanisms to amplify and/or attenuate a particular response. A-FABP seems to be required to induce a strong inflammatory response. However too strong of a response can be damaging. In an obese state, macrophages are constantly exposed to excess free fatty acids and pro-inflammatory cytokines released from adipocytes. This may result in an increased and continuous expression of A-FABP in macrophages, leading to a sustained polarized pro-inflammatory phenotype and

systemic low-grade chronic inflammation. This scenario has been demonstrated in the promotion and progression of atherosclerosis, but could this also be the case in cancer?

Hypothesis and Significance

Published studies have suggested a link between FABP expression and cancer progression, but the focus has been on FABP expression in cancer cells themselves as opposed to primary cells of the host, and the results are quite mixed. For example, reduced levels of L-FABP, I-FABP, and A-FABP are associated with the progression of liver, colon, and bladder cancers [139-141], whereas increased levels of B-FABP and E-FABP have been found in the advancement of astrocytoma tumors and prostate cancer, respectively [142, 143]. Neiman and colleagues demonstrated that A-FABP expression in adipocytes promoted ovarian cancer cell metastasis to the omentum, an organ primarily composed of adipocytes, while A-FABP deficiency led to a significant reduction in metastatic tumor growth in mice [144]. However, many tumors display heavy immune cell infiltration, and the role of FABP expression in leukocytes in the regulation of tumor growth and progression has not been explored.

Furthermore, A-FABP expression levels in macrophages is up-regulated in response to a high fat diet, particularly saturated fatty acids found in a Western

diet, and contribute to a polarized pro-inflammatory phenotype in macrophages as well as the development and progression of a cluster of diseases that make up metabolic syndrome. One study conducted by Hancke et al., focused on A-FABP expression in the serum of breast cancer patients, and the results showed higher A-FABP levels in breast cancer patients compared to healthy patients, and this expression was increased even further in obese breast cancer patients [145]. Additionally, the A-FABP expression was found to be associated with increased breast cancer risk and positively correlated with tumor size and lymph node involvement [145], suggesting that A-FABP not only has a putative role in cancer progression, but may also exacerbate this disease under obese conditions.

Over the past few years, studies in our lab have focused on macrophage plasticity and how this can be used in the treatment of a variety of diseases. Our work has provided evidence that macrophages possess a spectrum of activation states rather than existing as distinct subsets. In response to changes in the microenvironment, macrophages can reversibly shift their phenotype through a multitude of patterns. For example, by treating macrophages with different cytokines, distinct functional phenotypes can be observed, and sequentially treating macrophages with multiple cytokines results in a succession through several functional phenotypes [18]. This can also be seen *in vivo* whereby distinct macrophage phenotypes that are established in aged or tumor-bearing mice can be altered by inducing changes in the microenvironment [24, 37]. As mentioned previously, A-FABP mediates both metabolic and inflammatory

pathways, thus regulating the outcome of innate and adaptive immune responses and making A-FABP a potential therapeutic target for several inflammatory and metabolic diseases, as well as cancer [110]. Moreover, it has been reported that a genetic polymorphism in the A-FABP locus in humans [146], which results in reduced A-FABP expression, produces a similar phenotype as in mice, suggesting that the biological roles of A-FABP are similar between species and inhibiting this protein in humans is feasible. The identification of small molecule inhibitors of A-FABP suggests that targeting this protein for therapeutic purposes is attainable, however this also requires a more in-depth understanding of how A-FABP functions on both a cellular and whole animal level. Herein, we address a more complete understanding of how A-FABP regulates cancer progression. Given our previous findings regarding the role of A-FABP in macrophage inflammatory function, we hypothesize that macrophage expression of A-FABP may influence the functional outcome of tumor-macrophage interactions in the tumor microenvironment. Moreover, because a high fat diet induces A-FABP expression in macrophages, we also hypothesize that A-FABP may serve as a link between high fat consumption and cancer progression.

MATERIALS AND METHODS

Mice and macrophage cell lines.

A-FABP$^{-/-}$ mice were generated as previously described [137, 147], and back-crossed >10 generations onto a C57BL/6J background. A-FABP$^{-/-}$ mice are bred and maintained at the University of Louisville Research Resources Facility. All animal care and experimental procedures used in this study were approved by the University of Louisville's Institutional Animal Care and Use Committee. A-FABP$^{-/-}$ macrophage cell lines used in this study were generated by J2 retroviral transformation of bone marrow progenitors as previously described (CITATION).

Normal and High Fat Diet

High fat diet (60% kcal from fat) food and its matched low fat diet control (10% kcal from fat) was purchased from Research Diets, Inc. After being weaned, WT and A-FABP$^{-/-}$ mice were placed on either diet for 120 days. Tumor studies were then performed. The formula of both the normal and high fat diet is below.

Table 2. Formula of Normal Diet

Formula **Normal (10%) Diet**

Product #		D12450B	
		gm%	kcal%
Protein		19.2	20
Carbohydrate		67.3	70
Fat		4.3	10
	Total		100
	kcal/gm	3.85	

Ingredient	gm	kcal
Casein, 80 Mesh	200	800
L-Cystine	3	12
Corn Starch	315	1260
Maltodextrin 10	35	140
Sucrose	350	1400
Cellulose, BW200	50	0
Soybean Oil	25	225
Lard*	20	180
Mineral Mix S10026	10	0
DiCalcium Phosphate	13	0
Calcium Carbonate	5.5	0
Potassium Citrate, 1 H2O	16.5	0
Vitamin Mix V10001	10	40
Choline Bitartrate	2	0
FD&C Yellow Dye #5	0.05	0
Total	1055.05	4057

Formulated by E. A. Ulman, Ph.D., Research Diets, Inc., 8/26/98 and 3/11/99.

*Typical analysis of cholesterol in lard = 0.95 mg/gram.
Cholesterol (mg)/4057 kcal = 19
Cholesterol (mg)/kg = 18

Table 3. Formula of High Fat Diet

Formula High Fat (60%) Diet

Product #		D12492	
		gm%	kcal%
Protein		26.2	20
Carbohydrate		26.3	20
Fat		34.9	60
	Total		100
	kcal/gm	5.24	

Ingredient	gm	kcal
Casein, 80 Mesh	200	800
L-Cystine	3	12
Corn Starch	0	0
Maltodextrin 10	125	500
Sucrose	68.8	275.2
Cellulose, BW200	50	0
Soybean Oil	25	225
Lard*	245	2205
Mineral Mix, S10026	10	0
DiCalcium Phosphate	13	0
Calcium Carbonate	5.5	0
Potassium Citrate, 1 H2O	16.5	0
Vitamin Mix, V10001	10	40
Choline Bitartrate	2	0
FD&C Blue Dye #1	0.05	0
Total	**773.85**	**4057**

Formulated by E. A. Ulman, Ph.D., Research Diets, Inc., 8/26/98 and 3/11/99.

*Typical analysis of cholesterol in lard = 0.95 mg/gram.
Cholesterol (mg)/4057 kcal = 232.8
Cholesterol (mg)/kg = 300.8

Tumor models.

LL/2 Lewis Lung Carcinoma cells were obtained from the late Dr. Gordon Ross of the James Graham Brown Cancer Center in Louisville, KY. Tumors were maintained by *in vivo* passage with limited intermittent culture *in vitro*. Tumor cells were injected subcutaneously into the left flank of wild-type C57BL/6J (Jackson Laboratories), A-FABP$^{+/-}$, or A-FABP$^{-/-}$ mice. Five to seven mice were used in each group (10% WT, 10% A-FABP$^{-/-}$, 60% WT, 60% A-FABP$^{-/-}$) during in vivo studies and an in vitro tumor invasion study. Tumors were measured at 3-day intervals with calipers at two bisecting diameters and an approximate volume was calculated by the formula {(0.4) x (large diameter) x (small diameter)^2}. Mice were sacrificed after 26 days. To prepare single cell suspensions, resected tumors were digested in a digestion mixture consisting of 5% FBS in RPMI 1640, 0.5mg/ml collagenase A (Roche Diagnostic), 0.2mg/ml hyaluronidase type V (Sigma-Aldrich), and 0.02mg/ml DNase I (Sigma-Aldrich) at 37ºC for 30 minutes on a rotating platform. The resulting cell suspensions were filtered through 70µm cell strainers (BD Biosciences) and washed with 5% FBS in RMPI 1640. Cell debris/dead cells were removed by centrifugation with Lymphocyte-M (Cedarlane Laboratories) as recommended by the manufacturer. Tumor-infiltrating macrophages were purified by positive selection with anti-CD11b (Mac-1) magnetic beads (Miltenyi Biotec). A purity of >95% CD11b$^+$ cells was confirmed by flow cytometry.

Generation of bone marrow-derived macrophages.

Bone marrow-derived macrophages were generated from wild-type and A-FABP$^{-/-}$ mice. Briefly, femurs and tibias from 8-10 week old mice were flushed with DPBS and bone marrow was filtered and washed. Bone marrow was plated overnight in RPMI 1640 supplemented with 10ng/ml M-CSF (R&D Systems). After overnight incubation, non-adherent cells were plated in RPMI 1640 containing 25% filtered L929 fibroblast supernatants (ATCC) and 10ng/ml M-CSF in 6-well ultra low cluster plates (Corning). Cells were harvested on day 7 and purified by centrifugation on 35% Ficoll (Atlanta Biologicals). Macrophages were analyzed for CD11c, CD11b, CD80, CD86, CD40, and I-A^b expression by flow cytometry. Macrophages were consistently >98% CD11b$^+$.

Flow Cytometric Analysis.

For cell composition of tumor, 10^6 single cells were treated with Fc block (BD Biosciences) for 15 minutes prior to incubation with fluorochrome-conjugated anti-CD4, anti-CD8, anti-CD19, anti-CD11c, anti-CD11b, anti-F4/80, anti-NK1.1, and anti-Ly6G (all from BD Biosciences) for 30 minutes at 4°C. Samples were analyzed using a FACS Calibur flow cytometer and FlowJo Software (Tree Star Inc.).

Quantitative Real-time RT-PCR analysis. mRNA was isolated from 10^6 purified tumor-infiltrating macrophages and converted to cDNA using MACS One-Step cDNA Columns (Miltenyi Biotec). Real-time RT-PCR was performed with a DNA-

Opticon 3 Monitor (Bio-Rad) using SYBR Green (Qiagen). MIF, MCP-1, CSF-1, VEGF, EGF, MMP-12, MMP-9, TNFα, TGFβ, IL-6, IL-10, IL-12, IL-15, IL-23, FABP4, and β-actin expression was analyzed by Quantitect Primer Assays (Qiagen). Results were normalized to β-actin. Relative expression of RNA transcripts were quantified using the Relative Expression Software Tool, REST.

Western blot analysis.

10^6 macrophages were lysed in buffer containing 25mM Tris-HCl, 1% deoxycholate, 0.35M NaCl, phosphatase inhibitor solution (Cayman Chemical), and 1% Triton X-100 (Fisher Scientific). Protein quantity was assayed by bicinchoninic acid (Pierce) and 12μg of protein was loaded per well on a 10% or 15% Tris-HCl gel (Bio-Rad). The contents of the gel were transferred onto nitrocellulose membranes (Amersham Biosciences) using a Trans-Blot SemiDry Transfer Cell (Bio-Rad). The membranes incubated with different antibodies. Antibody-bound proteins were detected using an ECL Western Blotting Analysis System (Amersham Biosciences), and the membranes were exposed to Kodak Biomax XL X-ray Film (Eastman Kodak).

Immunohistochemistry.

Tumors were excised and snap frozen in tissue freezing medium (OCT). Tissue blocks were cut and fixed with cold acetone. To detect intratumoral MMP-9 expression or tumor vascularization, the sections were first blocked with 2.5% BSA in 10X PBS, and then stained with biotinylated anti-MMP-9, anti-CD31 (R&D

Systems), or anti-MMP-12 (Santa Cruz) for 1 hour at room temperature. After washing, the sections were incubated with VECTASTAIN Elite ABC Reagent (Vector Laboratories) for 30 minutes. Sections were developed with peroxidase substrate solution DAB to desired stain intensity and counterstained with hematoxylin to provide morphologic detail. The images were taken and analyzed with Aperio Imagescope Software (Aperio Technologies).

Tumor Invasion Assay

CD11b+ TIMs were isolated from WT and A-FABP-/- mice on a normal or high fat diet. 1x10^6 TIMs were seeded into the bottom chamber of the Fluoroblok Tumor Invasion System (BD Biosciences) in serum-free media. Serum-free media and media alone were used as negative and positive controls, respectively. 2.5x10^4 LL/2 cells were seeded in the top chamber in serum-free media. Tumor cells were allowed to invade through the 0.8μm Matrigel matrix for 20 hours at 37°C. Invaded tumor cells were then stained with calcien for 1 hour at 37°C. Fluorescence of invaded tumor cells was read at 494/517nm (Ex/Em) using a fluorescent plate reader (SoftMax Pro). Pictures of the fluorescent invaded tumor cells were taken with a fluorescent microscope (Evos). Magnification 20X.

Oil Red O Staining

Tumors were excised and snap frozen in tissue freezing medium (OCT). Tissue blocks were cut and fixed with 10% neutral buffered formalin for 10 minutes and then with propylene glycol for 2 minutes. To look at adipocyte infiltration, tumors

were stained with filtered Oil Red O 0.05% in propylene glycol (Poly Scientific) for 1 hour. After washing in distilled water, tumors were counterstained with Mayer's Modified Hematoxylin (Poly Scientific) for 30 seconds. Tumor stainings were preserved with Faramount Aqueous Mounting Medium (DAKO).

RESULTS

The Role of A-FABP in Tumor Growth and Metastasis

The tumor microenvironment enhances A-FABP expression in macrophages.

To determine the expression level of A-FABP in macrophages in response to tumor burden or the tumor microenvironment, WT mice were challenged with a subcutaneous injection of Lewis Lung Carcinoma (LL/2) cells, and after 26 days, $CD11b^+$ macrophages were isolated from the spleen. WT mice without tumors were used as a control. RT-PCR analysis showed that splenic macrophages isolated from tumor-bearing mice had about a 20-fold increase in A-FABP mRNA levels compared to splenic macrophages taken from mice without a tumor (**Figure 7A**). Additionally, bone marrow-derived macrophages generated from WT mice were treated with LL/2 supernatant or media alone. These results also showed a significant increase in A-FABP mRNA levels in macrophages exposed to tumor supernatant (**Figure 7B**) when compared to unstimulated macrophages, suggesting that the tumor microenvironment is sufficient to induce A-FABP expression in macrophages.

A-FABP deficiency suppresses metastasis, but not tumor growth.

To elucidate the function of A-FABP in tumor growth and metastasis, mice with different levels of A-FABP expression were used (A-FABP$^{+/+}$, A-FABP$^{+/-}$, and A-FABP$^{-/-}$). Tumors grew at a similar rate between these groups of mice, and there was no difference in the tumor weight (**Figure 8**). However, there was a significant difference in metastasis, with WT mice averaging about 15 metastatic nodules in the liver and about 8 nodules in the lung, while little to no spots of metastasis were observed in A-FABP$^{-/-}$ mice (**Figure 8**). A-FABP$^{+/-}$ mice showed about a 50% reduction in metastatic nodules in both liver and lung as compared to WT mice (**Figure 8**). Taken together, this data shows that A-FABP deficiency protects mice from metastasis even at the same tumor growth rate.

A-FABP deficiency reduces the production of pro-inflammatory and metastasis-promoting proteins in tumor-infiltrating macrophages.

Metastasis is a multi-step process that involves proteins of the extracellular matrix, cytokines, chemokines, and growth factors. Many of these factors, including MMPs, EGF, CSF-1, and MCP-1, are secreted by tumor-infiltrating macrophages (TIMs), and are critical for in the induction of metastasis. To further confirm the role of A-FABP in metastasis, we observed the expression levels of various molecules involved in the metastatic process in CD11b^{+} TIMs isolated from WT and A-FABP$^{-/-}$ mice. As shown in **Figure 9**, TIMs isolated from A-FABP$^{-/-}$ mice had a 30-fold decrease in MMP-9 expression, 20-fold decrease in MMP-12 expression, and about a 10-fold decrease in MCP-1, CSF-1, and EGF

expression as compared to TIMs isolated from WT mice. Moreover, compared to WT TIMs, A-FABP$^{-/-}$ TIMs showed reduced expression of TNF-α, IL-6, and IL-23, while exhibiting higher levels of anti-inflammatory proteins, TGF-β and IL-10 (**Figure 9B**). Bone marrow-derived macrophages form WT and A-FABP$^{-/-}$ mice also exhibited similar gene expression levels after stimulation with LL/2 supernatant *in vitro* (data not shown). Additionally, staining of frozen tumor sections confirmed more MMP-9 and MMP-12 production in the tumor tissue from WT mice than that of A-FABP$^{-/-}$ mice (**Figure 9C, 8D**). However, when tumor sections were stained with CD31 to observe blood vessel formation in LL/2 tumors, there were no statistical differences between WT and A-FABP$^{-/-}$ mice (data not shown).

Use of a small molecule inhibitor of A-FABP also suppresses metastasis. To further evaluate the function of A-FABP in suppressing metastasis, we employed a small molecule inhibitor of A-FABP, HTS01037 [148]. Oral feeding of HTS01037 did not affect tumor growth (**Figure 10B**), but it did suppress tumor metastasis to the lung (**Figure 10C, 10D**). Some inhibitor-treated mice exhibited similar numbers of metastatic nodules as compared to non-treated mice, but the size and intensity of the nodules were much less than control groups (**Figure 10C, 10D**). More importantly, TIMs isolated from inhibitor-treated mice showed a significant decrease in MMP-9 and MMP-12 gene expression levels compared to control mice (**Figure 10E**). When HTS01037 was added to *in vitro* cultured bone marrow-derived macrophages pre-stimulated with LL/2 supernatant, MMP-9 and

MMP-12 production was also reduced in a dose-dependent manner (**Figure 10A**). These data further demonstrate that A-FABP suppresses metastasis via impacting the production of metastasis-promoting proteins by TIMs.

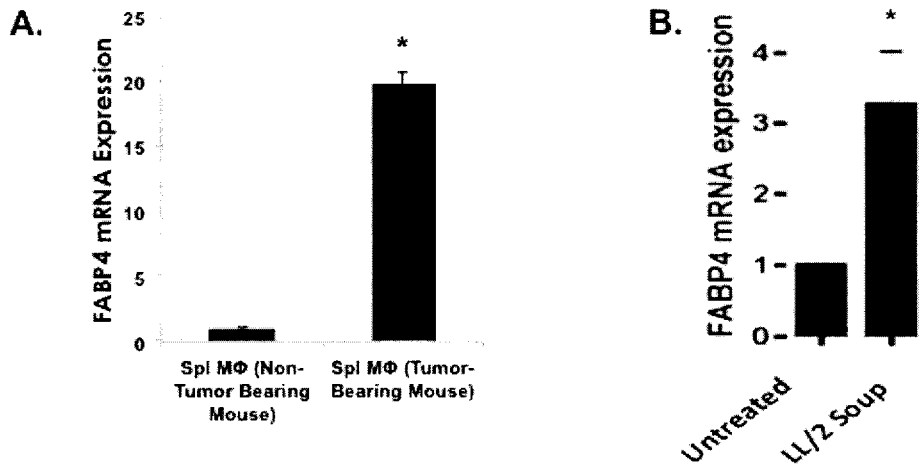

Figure 7. Increased A-FABP expression in macrophages in response to the tumor microenvironment. *A*, Splenic macrophages isolated from tumor-bearing mice have increased mRNA levels of A-FABP when compared to splenic macrophages isolated from mice without a tumor. *B*, bone marrow-derived macrophages treated with tumor supernatant show higher mRNA levels of A-FABP compared to the untreated group (* $p = 0.001$).

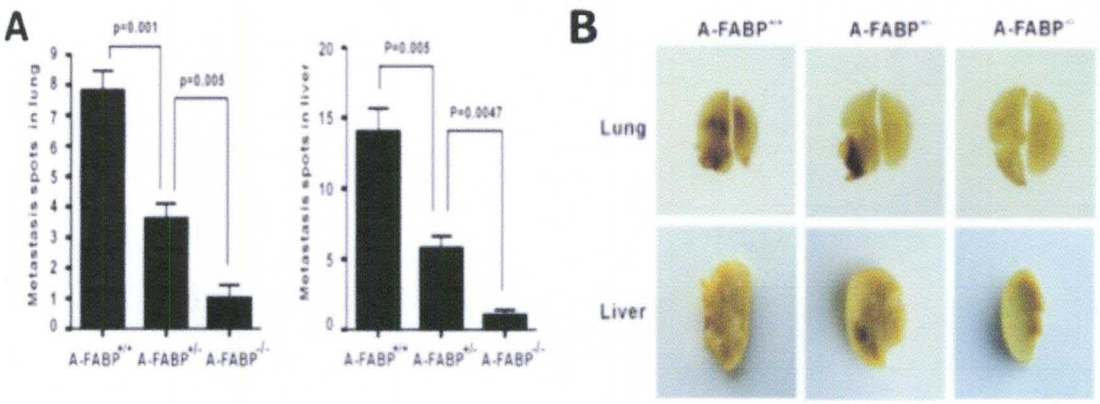

Figure 8. A-FABP deficiency suppresses tumor metastasis. Compared to A-FABP$^{+/+}$ mice, A-FABP heterozygous knockouts showed about a 50% reduction in lung and liver metastasis, which was reduced even further in A-FABP homozygous knockouts. *A* is graphical representation of *B*. (Conducted by Bing Li).

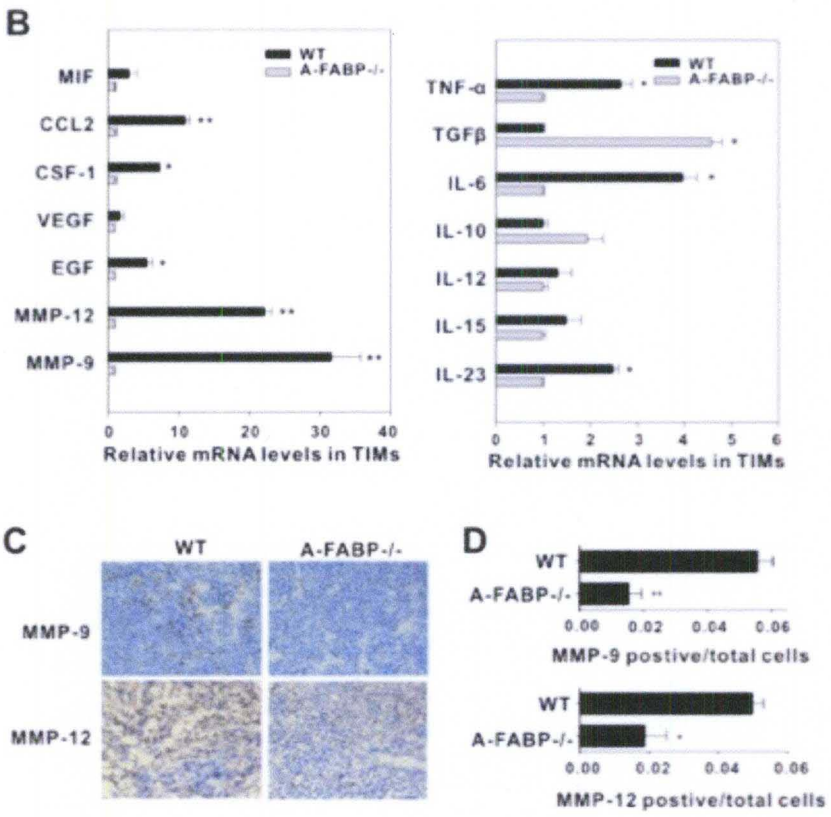

Figure 9. A-FABP deficient macrophages show decreased production of pro-inflammatory and metastasis-promoting proteins. *A*, RT-PCR analysis of TIM isolated from WT and A-FABP$^{-/-}$ mice. Compared to WT mice, A-FABP$^{-/-}$ show reduced expression of proteins involved in metastasis, including MMP-9, MMP-12, EGF, and CCL2; along with reduced expression of pro-inflammatory cytokines such as TNF-α and IL-6, and increased of expression of anti-inflammatory cytokines TGF-β and IL-10. *B* and *C*, Immunohistochemical analysis shows reduced expression of MMP-9 and MMP-12 in the tumor tissue of A-FABP$^{-/-}$ mice compared to that of WT mice (* $p = 0.001$) (Conducted by Bing Li).

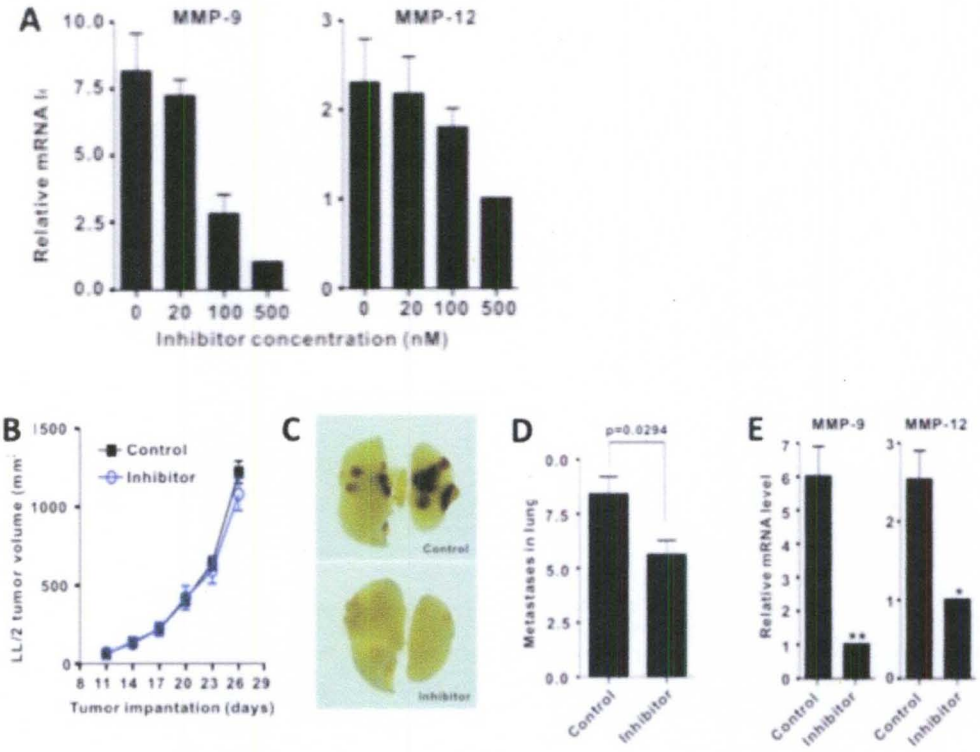

Figure 10. HTS01037, a small molecule inhibitor of A-FABP, reduces lung metastasis and MMP production, but has no effect on tumor growth. *A*, Bone marrow-derived macrophages from WT mice were treated with increasing concentrations of HTS01037. RT-PCR analysis shows that MMP-9 and MMP-12 mRNA levels decrease as the concentration of the inhibitor increases. *B through E*, WT mice were treated with HTS01037 or vehicle. *B*, Inhibition of A-FABP had no effect on tumor growth, however there was reduced lung metastasis in inhibitor-treated mice as compared to the control group (*C, D*). *E*, TIMs isolated from inhibitor-treated mice had reduced MMP-9 and MMP-12 production (Conducted by Bind Li).

The Role of A-FABP in Tumor Growth and Metastasis in Diet-Induced Obese Mice

A-FABP is not only increased in response to the tumor microenvironment, but it is also increased in response to high fat consumption.

Previous studies have demonstrated higher macrophage A-FABP levels in diet-induced obese mice compared to lean mice, as well increased expression in atherosclerotic lesions of obese mice compared to lean or healthy mice [137]. We have already shown significantly higher levels of A-FABP in splenic macrophages in response to the tumor microenvironment, but is this expression increased even more in obese tumor-bearing mice? Here, WT mice were fed a normal (10%) diet or high fat (60%) diet for 120 days prior to implantation of LL/2 cells. **Figure 11** shows that WT and A-FABP$^{-/-}$ mice fed a high fat diet have similar weights. CD11b^{+} splenic macrophages and TIM taken from high fat-fed WT mice showed a significant increase in A-FABP expression when compared to splenic macrophages and TIMs isolated from mice fed a normal diet, respectively (**Figure 12**). These data along with results from **Figure 7** suggest that A-FABP expression level increases in response to both the tumor microenvironment and high fat consumption in macrophages.

A-FABP deficiency confers protection against tumor growth and metastasis in both lean and diet-induced obese mice.

Next, we observed the effect of A-FABP deficiency on tumor growth and metastasis under conditions of high fat feeding. There was no difference in tumor growth between WT and A-FABP$^{-/-}$ mice fed a regular chow diet. There was also no observed difference in tumor growth in WT mice treated with an inhibitor of A-FABP when compared to the untreated group (**Figure 10**). This same trend was also observed in WT and A-FABP$^{-/-}$ mice fed a normal (10%) diet, however when mice were placed on a high fat (60%) diet, WT mice showed a significant increase in tumor growth, while A-FABP$^{-/-}$ mice fed a high fat diet displayed reduced tumor growth rates that were similar to those of lean mice (**Figure 13A)**. The tumor weights correlated with tumor growth rates between each group of mice (**Figure 13B**).

We also observed lung metastasis in normal (10%) and high fat (60%) diet fed WT and A-FABP$^{-/-}$ mice. WT mice on a normal diet averaged about 7 metastatic nodules, while WT mice on a high fat diet averaged about 12 metastatic nodules in the lung. A-FABP$^{-/-}$ mice on normal or high fat diets showed a significant reduction in lung metastasis with an average of 2 and 4 metastatic nodules in the lung, respectively (**Figure 14**). To further determine the role of A-FABP in high fat consumption and metastasis, we assessed TIM-mediated tumor cell invasion using an *in vitro* tumor invasion assay. CD11b^{+} TIM isolated from WT and A-FABP$^{-/-}$ mice on normal and high fat diets were seeded in the bottom chamber of the invasion system with LL/2 cells seeded in the top insert of the

invasion system. Tumor cells were allowed to invade the Matrigel matrix for 20 hours. As shown in **Figure 15**, TIMs isolated from high fat fed WT mice induced the highest level of tumor cell invasion, followed by TIM from normal diet fed mice. TIMs taken from A-FABP$^{-/-}$ mice fed either diet showed a reduction in the ability to induce tumor cell invasion. These data are also consistent with the *in vivo* lung metastasis data (**Figure 15**).

Tumors from high fat fed WT mice show an increase in macrophage infiltration.

To determine the immune cell composition of WT and A-FABP$^{-/-}$ tumors, single cell suspensions were generated and used in flow cytometric analysis. Compared to tumors from normal diet fed WT mice, there was no significant difference in CD8^{+} T cell, B cell, or NK cell infiltrates in each group of mice (**Figure 16 C-E)**. However, there was significant increase in macrophage infiltration in tumors from high fat fed WT mice (**Figure 16A**) along with an increase in CD4^{+} T cells (**Figure 16B**). Surprisingly, there was also a significant increase in CD4^{+} T cells in high fat fed A-FABP$^{-/-}$ mice (**Figure 16B**). Whether these CD4^{+} cells found in high fat fed WT and A-FABP$^{-/-}$ tumors were CD25^{+}Foxp3^{+} or CD25^{+}Foxp3^{-} was not determined.

A-FABP deficiency, despite a normal or high fat diet, decreases the production of pro-inflammatory cytokines and proteins involved in metastasis.

Next we determined the cytokine profile of TIM isolated from WT and A-FABP$^{-/-}$ mice fed a high fat diet. Relative to TIM isolated from normal diet fed WT mice, TIM from high fat fed WT mice showed an increase in several proteins involved in metastasis, including MIF, VEGF, and MMP-9, which may explain why more metastatic nodules were observed in these mice. Conversely, TIM taken from A-FABP$^{-/-}$ mice fed a high fat diet showed a reduction in metastasis-promoting proteins and pro-inflammatory cytokines, while increased mRNA levels of anti-inflammatory cytokines, TGF-β and IL-10 (**Figure 17**). These data are in conjunction with the cytokine profile shown in Figure 9, suggesting that A-FABP deficiency can promote an anti-tumoral phenotype in macrophages despite dietary conditions. In the diet-induced obese models, there was a drastic increase in CCL2 mRNA levels. This chemokine has been implicated in the recruitment of macrophages to the adipose tissue in obese mice and humans [7]. Compared to WT mice on a normal diet, TIM isolated from high fat fed WT mice had about a 7000-fold increase in CCL2 followed by a 2000-fold increase in TIM from high fat-fed A-FABP$^{-/-}$ (**Figure 17B**). These data suggest that despite the increase in CCL2 levels, which seems to correlate with increased macrophage recruitment in tumors of high fat fed mice (will be discussed later in Figure 16), A-FABP$^{-/-}$ TIM still display an anti-tumoral phenotype, indicating that it is the activation state rather than quantity that influences the LL/2 tumor outcome.

We also looked at the expression of the metastasis-promoting protein, MMP-9, in the tumor tissue as well as TIM by Western blot (**Figure 19 and 18, respectively**). When compared to normal diet or high fat diet fed WT mice, A-

FABP$^{-/-}$ mice showed reduced MMP expression on either diet. Moreover, CD31, an endothelial cell marker, was also observed in the tumor tissue. Although there was no difference in the expression in normal diet fed WT and A-FABP$^{-/-}$ mice (data not shown), there was a significant reduction in CD31 expression in A-FABP$^{-/-}$ mice on a high fat diet relative to high fat fed WT mice (**Figure 19**). Using an Oil Red O stain, we were able to look at lipid deposition in the tumor. As expected, it appears to be lipid deposits in the tumors of high fat fed WT and A-FABP$^{-/-}$ mice compared to normal diet fed mice (**Figure 20**). However, the role of these cells in our model has not been explored.

Several studies have demonstrated the requirement of NF-κB activation in macrophages for cancer progression [149, 150]. NF-κB activation in macrophages has been shown to trigger the release of a variety of cytokines including TNF-α and IL-6, which induce pro-survival signals in tumor cells. Additionally, these macrophages also release metastasis-promoting proteins including VEGF and CSF-1, which support malignant progression and tumor growth. Here, we evaluated the activation status of NF-κB in TIM. Compared to TIM isolated from high fat fed WT mice, A-FABP$^{-/-}$ on the same diet displayed a drastic reduction in the phosphorylation of the p65 subunit of NF-κB (**Figure 21**). These data complement those in **Figure 17**, as this may be why there is reduced expression of metastasis-promoting proteins and pro-inflammatory cytokines in TIM from A-FABP$^{-/-}$ mice.

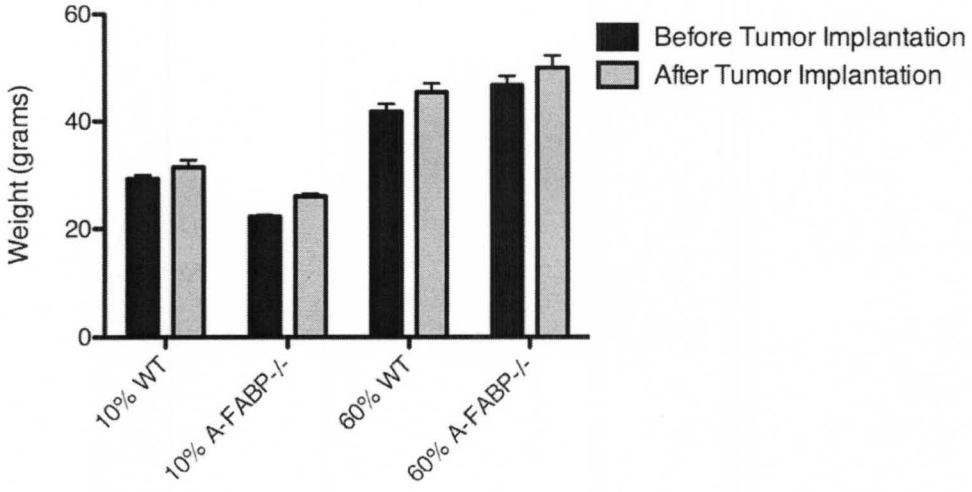

Figure 11. Weight of WT and A-FABP$^{-/-}$ mice fed a normal or high fat diet.

Weight of WT and A-FABP$^{-/-}$ mice fed a normal or high fat diet was taken before and after tumor implantation. High fat fed A-FABP$^{-/-}$ mice have weights that are similar to WT mice on the same diet.

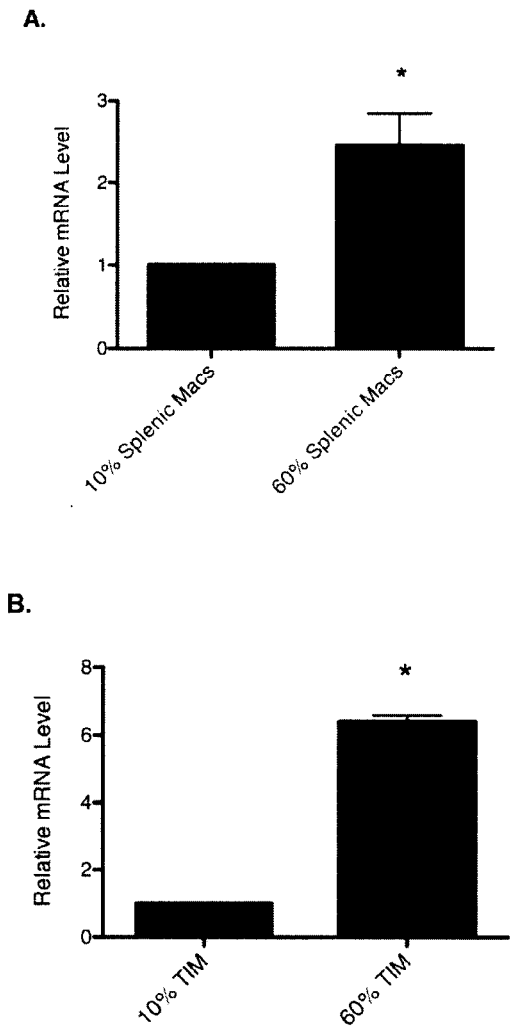

Figure 12. A-FABP expression in lean versus diet-induced obese mice.

Splenic macrophages *(A)* and TIM *(B)* isolated from high fat fed WT mice show higher mRNA levels of A-FABP compared to lean counterparts (* $p = 0.001$).

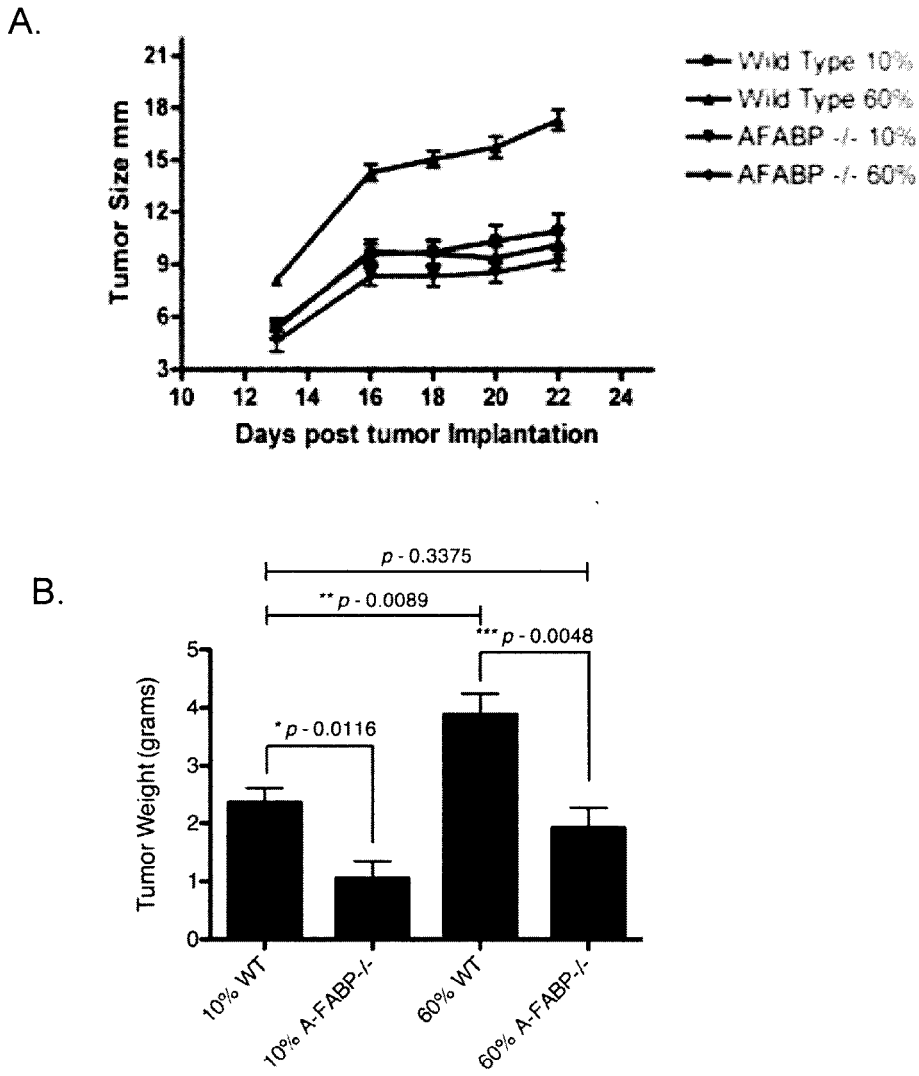

Figure 13. A-FABP deficiency protects against tumor growth in mice fed a high fat diet. High fat fed wild-type mice had a significant increase in tumor growth (A) and weight (B), whereas A-FABP$^{-/-}$ mice on the same diet had tumor growth rates and weight that were very similar to that of lean mice.

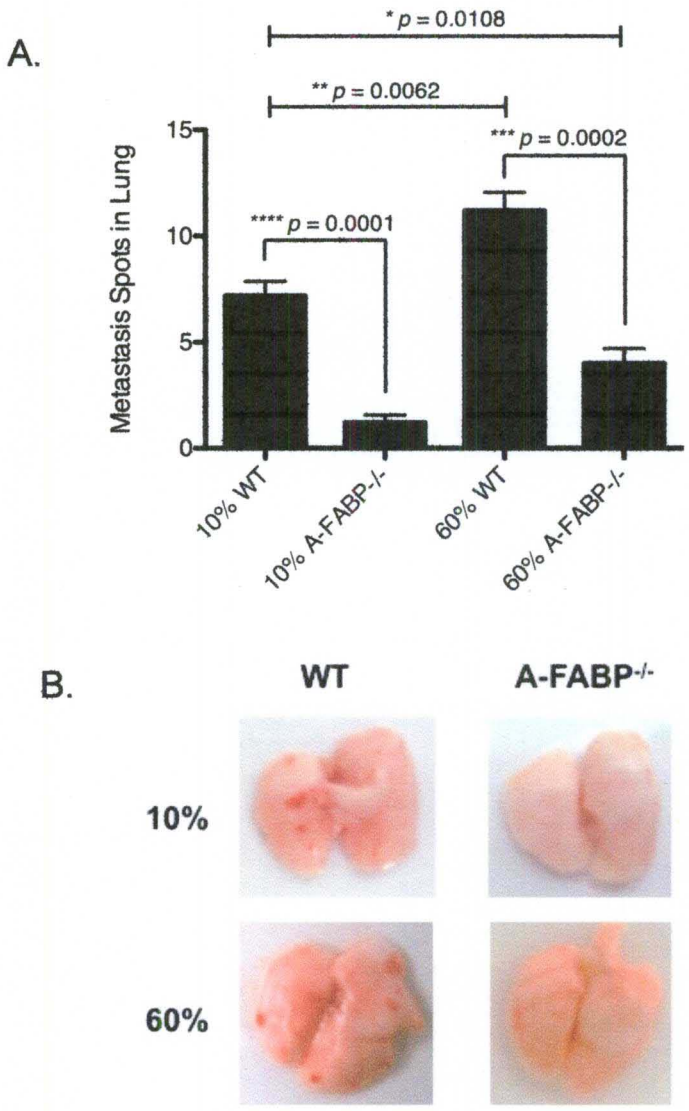

Figure 14. Lung metastasis in WT and A-FABP$^{-/-}$ mice fed a normal or high fat diet. Relative to normal diet fed WT mice, high fat fed WT mice showed a significant increase in metastasis to the lung, which can reduced in A-FABP deficient mice fed either diet.

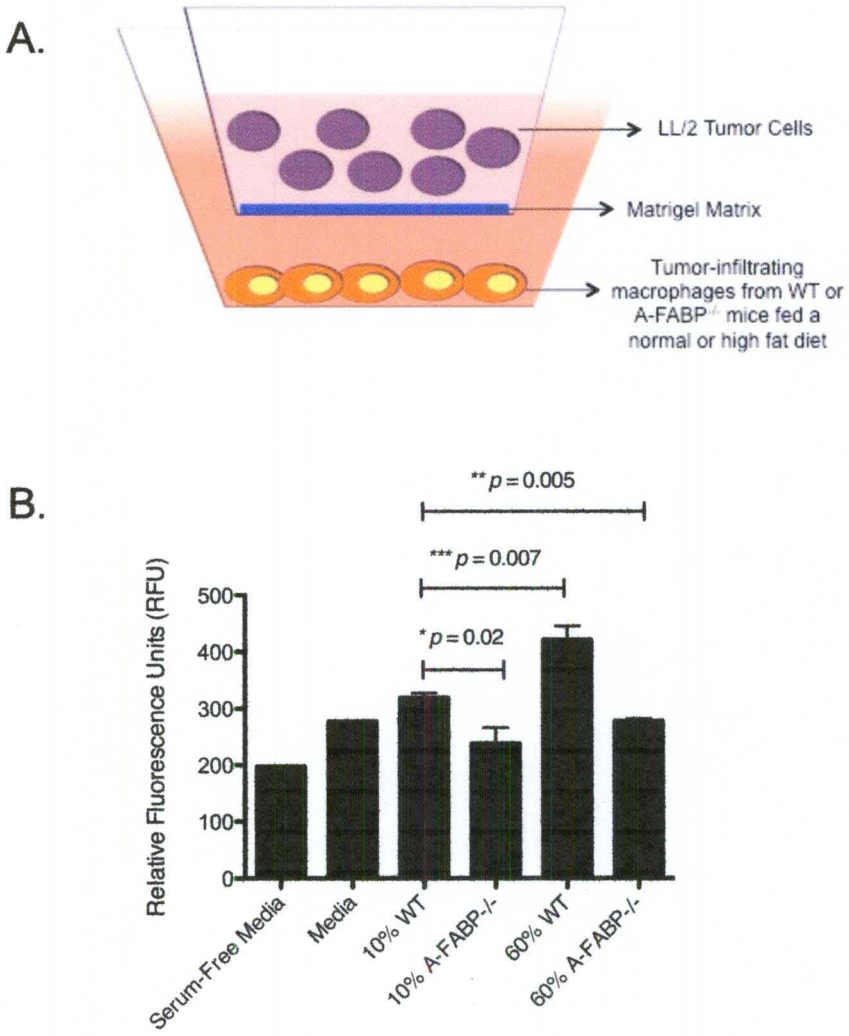

Figure 15. TIM-induced invasion of LL/2 cells. *A,* an *in vitro* tumor invasion assay was performed by seeding TIM from WT and A-FABP$^{-/-}$ mice on a normal or high fat diet in the bottom chamber of the invasion system and LL/2 tumor cells in the top chamber. Tumor cells were allowed to invade a Matrigel matrix for 20 hours. Tumor cells that did invade were stained with a fluorescent dye, calcien. *B,* quantification of calcien-stained tumor cells that invaded through the Matrigel matrix mediated by tumor-infiltrating macrophages.

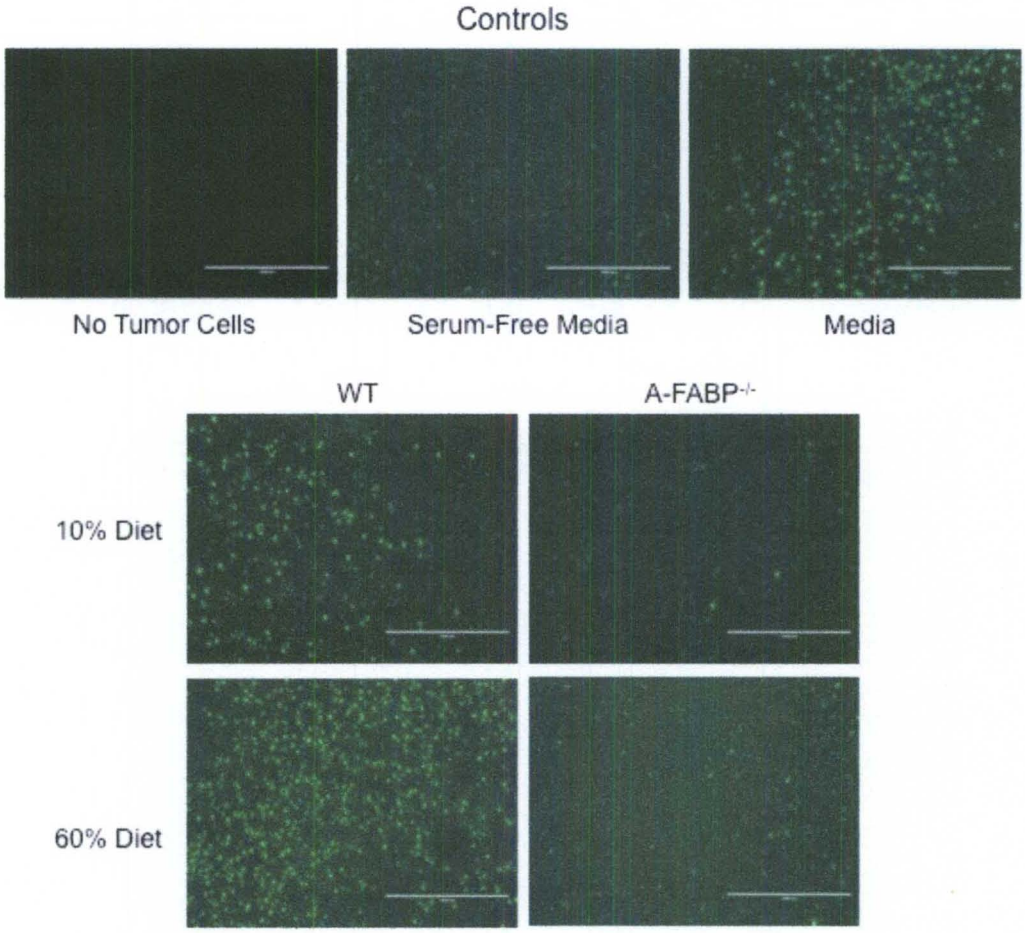

Figure 15. *C*, Fluorescent microscopy images of LL/2 tumor cells that invaded through the Matrigel matrix mediated by tumor-infiltrating macrophages.

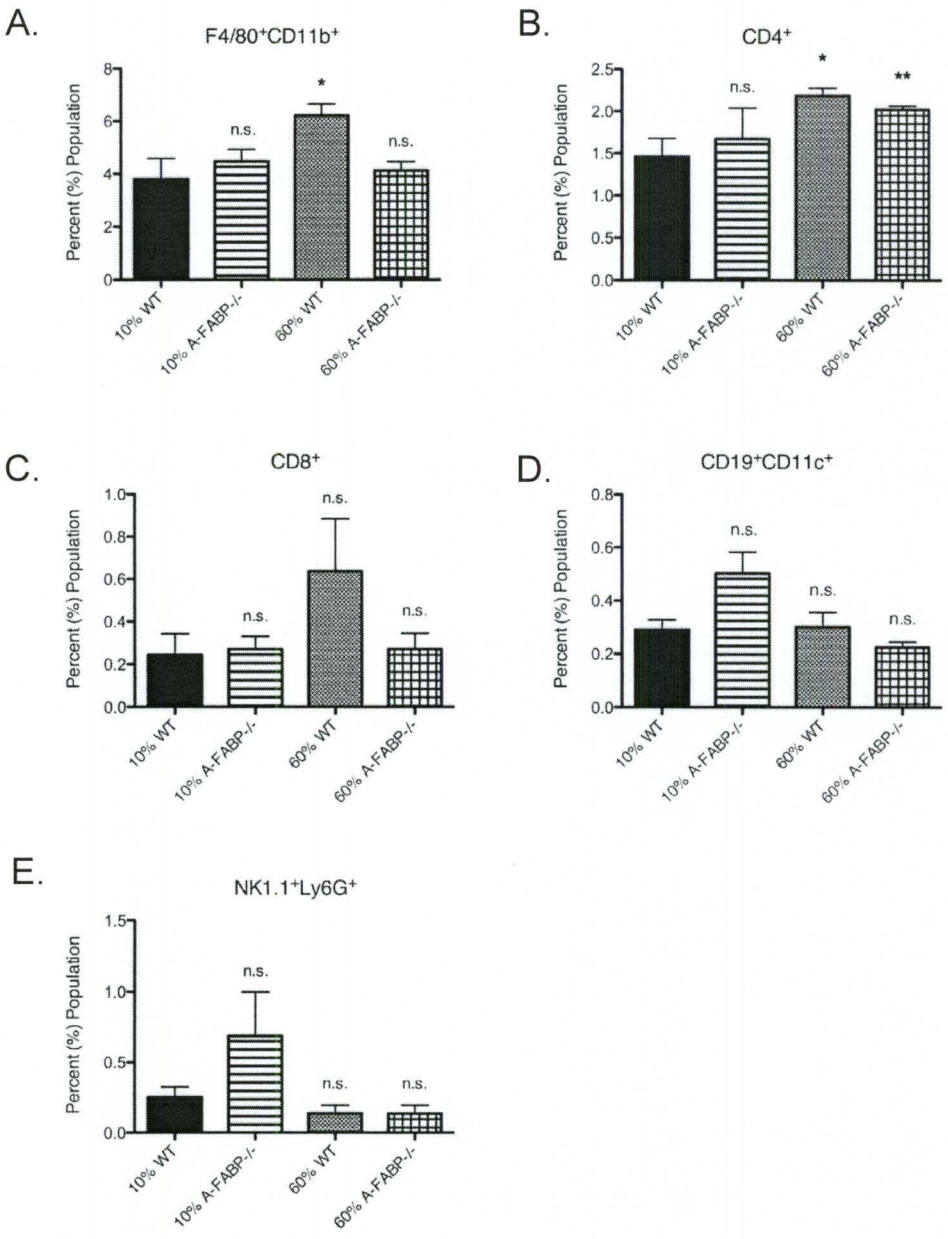

Figure 16. Composition of immune cell infiltration in tumors from WT and A-FABP$^{-/-}$ mice on a normal or high fat diet. Single cell suspensions of tumors from WT and A-FABP$^{-/-}$ mice fed a normal or high fat diet were used to elucidate the composition of the immune cell infiltrate by flow cytometry. There was no significant difference in CD8^{+} T cell, B cell, and NK cell infiltrates between each

group of mice, however, there was a significant increase in$CD4^+$ T cells in tumors from high fat fed WT and A-$FABP^{-/-}$ mice, as well as $F4/80^+CD11b^+$ macrophages from high fat fed WT mice relative to tumors from normal diet fed WT mice.

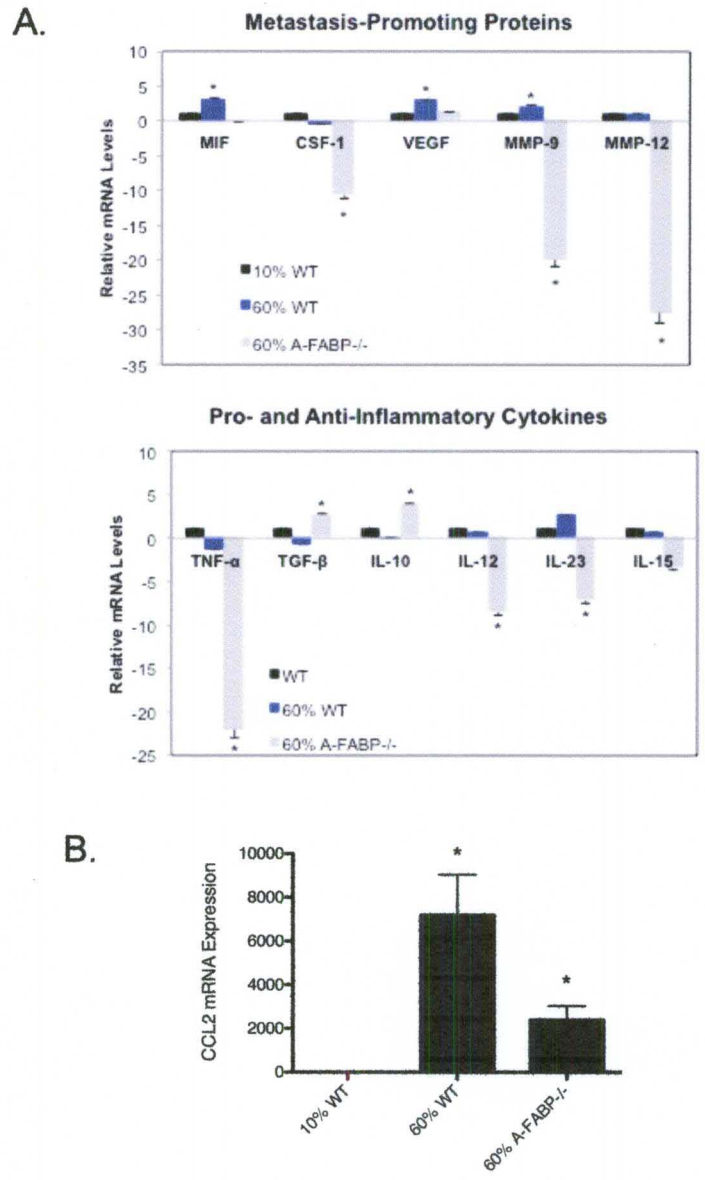

Figure 17. TIM from normal and high fat diet fed A-FABP deficient mice show decreased production of pro-inflammatory and metastasis-promoting proteins. RT-PCR analysis of TIM isolated from WT and A-FABP$^{-/-}$ mice fed a normal or high fat diet. *A*, compared to normal diet fed WT mice, TIM isolated from A-FABP$^{-/-}$ mice show reduced expression of proteins involved in metastasis and pro-inflammatory cytokines while displaying increased of expression of anti-

inflammatory cytokines TGF-β and IL-10. In contrast, TIM from high fat fed WT mice display increased mRNA levels of MMP-9, VEGF, and MIF (*p = 0.001). *B*, moreover, compared to TIM from normal diet fed WT mice, TIM from both high fat fed WT and A-FABP$^{-/-}$ mice show a dramatic increase in CCL2 expression (* p = 0.001).

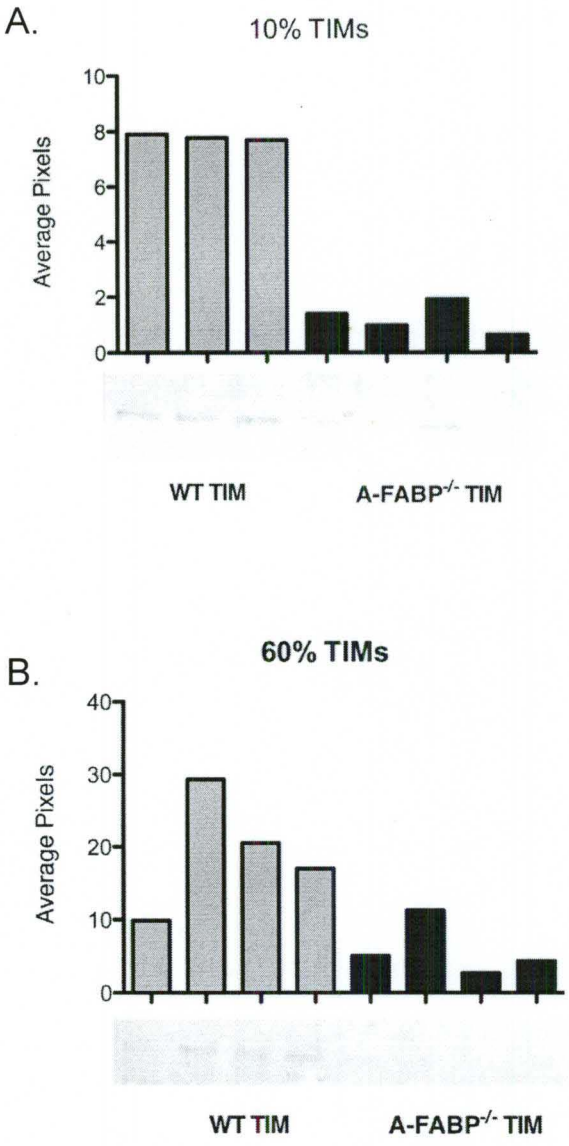

Figure 18. Decreased protein expression of MMP-9 in TIM from A-FABP$^{-/-}$ mice. Protein lysates taken from WT and A-FABP$^{-/-}$ mice fed a normal (*A*) or high fat diet (*B*) were used to examine at MMP-9 expression by Western blot. Compared to WT TIM, TIM isolated from A-FABP$^{-/-}$ mice display reduced protein expression of MMP-9.

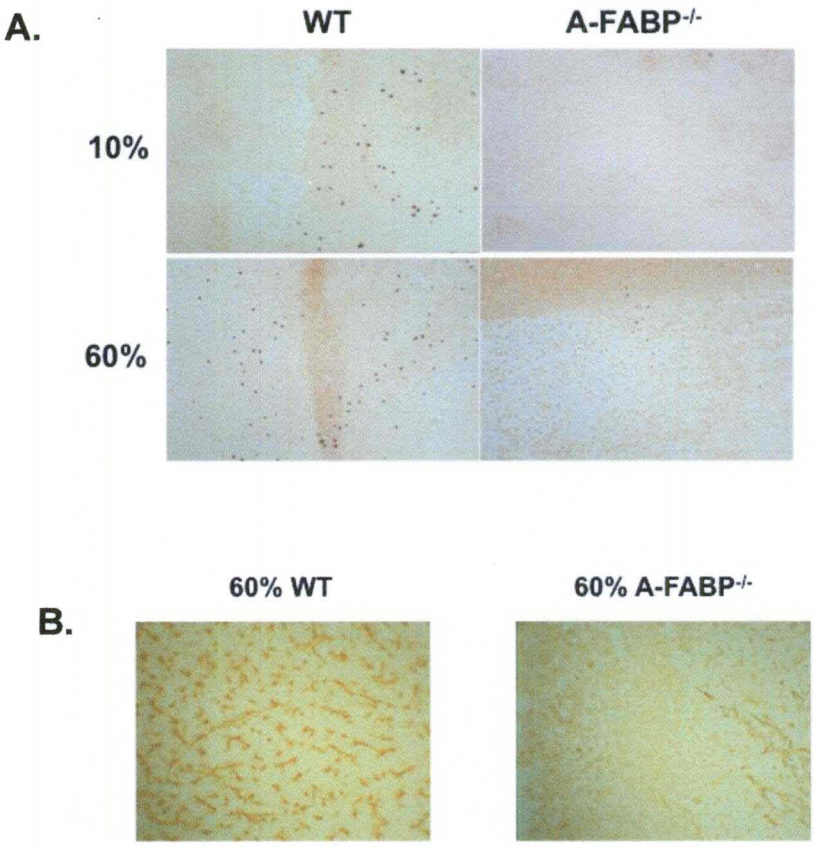

Figure 19. Immunohistochemical staining in WT and A-FABP$^{-/-}$ tumors. *A*, MMP-9 expression in tumor tissue. Compared to WT on a normal of high fat diet, A-FABP$^{-/-}$ tumors showed a significant reduction of in MMP-9 expression on either diet. *B*, CD31 expression in tumor tissue. Tumors from high fat fed A-FABP$^{-/-}$ mice showed reduced CD31 expression and vascularization than tumor from WT mice.

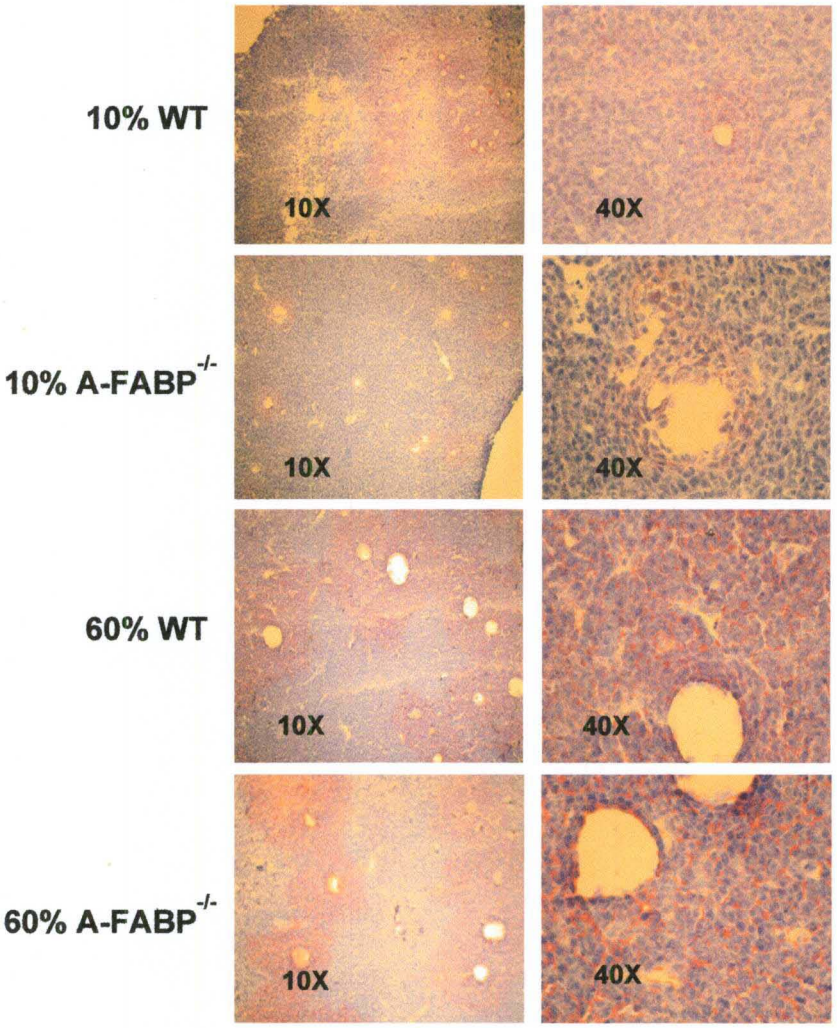

Figure 20. Lipid deposition in WT and A-FABP$^{-/-}$ tumors from normal and high fat diet fed mice. Using an Oil Red O stain, lipid deposition was observed in the tumors displaying a more intense stain in tumors from high fat fed WT and A-FABP$^{-/-}$ mice when compared to mice fed a normal diet.

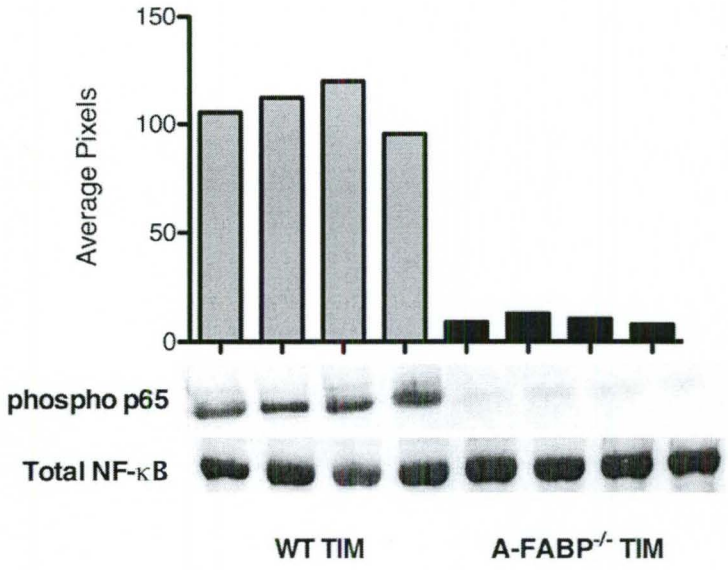

Figure 21. NF-κB activation in TIM taken from WT and A-FABP$^{-/-}$ fed a high fat diet. Protein lysates taken from WT and A-FABP$^{-/-}$ mice fed a high fat diet were used to look at NF-κB expression by Western blot TIM isolated from diet-induced obese A-FABP$^{-/-}$ mice show significant phosphorylation of p65 when compared to TIM from WT mice.

DISCUSSION

The obesity epidemic in the United States is steadily increasing. Currently, about two thirds of the adult population in the U.S. is considered to be obese [151], and parallel to this are increasing rates of obesity-related cancer deaths [93]. These statistics demonstrate the urgent need to understand the mechanisms of obesity-associated cancer progression. In the current study, we identify a factor that appears to serve as a link between high fat consumption and cancer progression, A-FABP.

Herein, we demonstrate a contribution of host A-FABP expression towards tumor metastasis and present evidence that A-FABP expression in macrophages facilitates the acquisition of tumor-promoting functions induced via macrophage-tumor interactions. Moreover, our findings suggest that under conditions of high fat feeding, A-FABP exacerbates the acquired tumor-promoting functions of macrophages. In the normal (10%) diet LL/2 model, tumor metastasis was consistently lower in A-FABP$^{-/-}$ mice despite equivalent tumor growth kinetics, indicating that the effect on metastasis was unrelated to tumor burden. However, in the high fat (60%) diet model, lower metastasis appeared to be related to reduced tumor load and vascularization, suggesting that A-FABP deficiency may have an enhanced protective effect in diet-induced obese mice.

Since the expression of A-FABP is largely limited to adipocytes and mature myeloid cells, including macrophages and dendritic cells, we focused the study on macrophages as likely contributors to the observed phenomenon. We found that TIM isolated from both normal and high fat diet fed A-FABP$^{-/-}$ mice displayed reduced expression levels of proteins involved in inflammation and metastasis while up-regulating genes that are anti-inflammatory. Many studies suggest that the tumor-supporting functions of macrophages are due to an alternatively activated, M2-like phenotype. It is this phenotype that promotes cancer progression via remodeling of the extracellular matrix and angiogenesis [152, 153]. In our normal and high fat diet LL/2 model, A-FABP$^{-/-}$ TIM did display up-regulated M2-like genes, including TGF-β and IL-10. However, several other genes that contribute to the alternatively activated phenotype, including MMP-9, MMP-12, and VEGF were significantly down-regulated, conducive to tumor regression, and further demonstrating that macrophages employ a spectrum of activation phenotypes rather than distinct subsets. Additionally, many studies have focused on the immunosuppressive effects of IL-10 in the tumor microenvironment, however IL-10 also exerts some immunostimulatory effects, such as activation of B cells, induction of cytotoxic T lymphocytes, and up-regulation of genes in TLR-activated dendritic cells and macrophages [154-157]. IL-10 secretion from A-FABP deficient TIM may also be a contributing factor in reduced tumor growth and metastasis by influencing several other infiltrating immune cells. The effects of IL-10 in the A-FABP$^{-/-}$ tumor model is an ongoing effort in our lab.

As mentioned previously, A-FABP deficiency is associated with increased PPAR-γ activity [109]. PPAR-γ has been shown to induce cell growth arrest, apoptosis, and differentiation in many cancer types including breast, lung, colon,

gastric, and prostate cancers [158, 159]. In immune cells, PPAR-γ regulates inflammatory gene transcription. Several cytokines including IL-6 and TNF-α have been implicated in the progression of several types of cancer, and studies show that PPAR-γ agonists, which have been shown to have therapeutic potential in the treatment of inflammatory and metabolic disease as well as cancer, can suppress the expression of these cytokines via inhibition of NF-κB or C/EBPβ [159, 160]. In our model, A-FABP deficiency down-regulates several inflammatory genes, which is likely due to PPAR-γ-mediated transrepression of NF-κB. Activators of PPAR-γ have also been shown to inhibit MMP-9 expression in human bronchial epithelial cells as well as MMP-12 in macrophages [161]. Furthermore, activation of PPAR-γ represses VEGF expression in human endometrial cells and reduce serum VEGF levels in diet-induced insulin resistant mice [162, 163]. Thus, enhanced PPAR-γ activation in TIMs from A-FABP deficient mice may also suppress MMP and VEGF expression in addition to other pro-inflammatory mediators, thereby contributing to reduced lung and liver metastasis and to tumor regression. Additionally, we have previously shown that PPAR-γ up-regulates genes involved in cholesterol trafficking. Several published studies have demonstrated that tumor-infiltrating macrophages can take on a foam cell phenotype as seen in atherosclerosis [164]. In the diet-induced obese A-FABP deficient model, PPAR-γ activation may allow for the transport of cholesterol out of the cell, thus contributing to anti-inflammation.

We have also found that macrophages isolated from healthy A-FABP$^{-/-}$ mice have increased levels of phosphorylated AMP-activated kinase α1, AMPK-α1 (data not shown). Additionally, we, and others, have found that in an obese state, AMPK, which also promotes anti-inflammatory gene expression, is down-regulated while A-FABP expression levels are increased [165]. This phenomenon

may contribute to the polarized pro-inflammatory phenotype found in macrophages bathed in an environment of metabolic excess. AMPK is a metabolic sensor that, upon activation, inhibits energy-consuming processes and promote ATP-producing pathways [166]. AMPK activation has been shown to provide protection against tumor growth and metastasis in both mice and diabetic humans [167, 168]. Moreover, the anti-diabetic drug, metformin, which has gained recent interest in the treatment of cancer, is a potent activator of AMPK. Because AMPK has been shown to suppress various proteins involved in metastasis, including MMP-9 [167, 169], it may also serve as an additional mechanism by which A-FABP deficiency suppresses tumor growth and metastasis in both lean and obese mice. Furthermore, both A-FABP deficiency and AMPK activation improves insulin sensitivity and reduces circulating levels of insulin, which may also contribute to tumor regression, particularly in diet-induced obese mice.

Several studies have shown that tumor-infiltrating adipocytes display an activated phenotype and are capable of inducing cancer cell proliferation, migration, and invasion [170-172]. Neiman and colleagues further showed that the tumor-promoting functions of adipocytes could be attributed to A-FABP expression [144]. Because A-FABP is expressed in both adipocytes and macrophages, it is possible that the effect of A-FABP knockdown on tumor progression can be attributed to functions in both cell types, especially since we are using a whole animal knockout. In a model of atherosclerosis, Boord and colleagues conducted a bone marrow transplantation study to determine if the effects of A-FABP expression was due to its function in macrophages or adipocytes. When bone marrow from A-FABP$^{-/-}$ApoE$^{-/-}$ mice were transferred into ApoE$^{-/-}$ mice, less atherosclerosis developed, suggesting that it was the

expression of A-FABP in macrophages and not adipocytes that contributed to disease progression [138]. This may also be the case in our LL/2 tumor model. With the recent development of macrophage-specific A-FABP conditional knockout mice available at the Jackson Laboratory, this question can soon be addressed.

In addition to adipocytes, dendritic cells, and macrophages, A-FABP has recently been shown to be expressed in endothelial cells of capillaries and small veins in both mouse and human tissue [173]. Studies illustrate that A-FABP expression is induced by treatment with VEGF-A and basic fibroblast growth factor (bFGF) via VEGF receptor-2 (VEGFR2). A-FABP knockdown in endothelial cells resulted in decreased proliferation of these cells in both untreated and VEGF-A- and bFGF-treated cells, suggesting that A-FABP induces endothelial cell proliferation via the VEGF-A/VEGFR2 pathway [173]. Further studies demonstrated that chronic administration of an A-FABP inhibitor, BMS309403, improved endothelial cells dysfunction in ApoE deficient mice significantly [174]. Although no one has shown A-FABP expression in tumor-associated endothelial cells, it very well may be the case since protein expression in tumors are dysregulated. This is another avenue of the role of A-FABP in cancer progression to be explored. Inhibition of A-FABP would not only suppress the tumor-promoting functions of macrophages and adipocytes, but it could possibly lead to a significant reduction in angiogenesis via inhibition of endothelial cell proliferation.

In this study, we show a role of A-FABP in cancer progression, while deletion of this protein protects against tumor growth and metastasis. We also demonstrate the importance of A-FABP in the link between high fat consumption and cancer progression. Moreover, by using a lung cancer model, (past and

recent data suggests that obesity does not affect the development and advancement of lung cancer [175]) we were able to show that dietary conditions can impact the progression of this type of cancer. Our data, along with the finding that A-FABP deficiency is protective against atherosclerosis, insulin resistance, and type 2 diabetes, indicates that inhibition of A-FABP may be utilized as a novel approach in treating inflammatory and metabolic disorders, as well as cancer that is associated and unassociated with obesity. Moreover, because agonists of AMPK and PPAR-γ have been implicated in cancer therapy, it may be more useful to inhibit A-FABP to activate AMPK and PPAR-γ simultaneously, which may induce an enhanced therapeutic effect.

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EDUCATION

2012	Ph.D., Immunology University of Louisville, Louisville KY
2009	M.S., Immunology University of Louisville, Louisville KY
2007	B.S., Biology Major, Chemistry Minor Tennessee State University, Nashville TN

RESEARCH AND PROFESSIONAL EXPERIENCE

2012-Present	Postdoctoral Fellow, Laboratory of Molecular Immunoregulation, Tumor Immunity and Tolerance Section, Cente r for Cancer Research, National Cancer Institute, Frederick, MD.
2007 – 2012	Graduate Research Student, Department of Microbiology and Immunology, University of Louisville, Louisville KY
Fall 2006	Undergraduate Research Student, Microbiology Tennessee State University, Nashville TN
Summer 2006	Undergraduate Research Student, Microbiology University of Minnesota, Minneapolis MN
2004 – 2006	Undergraduate Research Student, Pharmacology Tennessee State University, Nashville TN

AWARDS

2012	Cancer Research Training Award, NCI
2011	FASEB/MARC Program Poster/Oral Presentation Travel Award
2011	University of Louisville, School of Interdisciplinary and Graduate Studies Minority Doctoral Dissertation Award
2010	FASEB/MARC Program Poster/Oral Presentation Travel Award
2010	American Association of Immunologists Trainee Abstract Award
2009	American Association of Immunologists Minority Scientist Travel Award
2008	Graduate Student Council Travel Award, University of Louisville
2007	University of Louisville, Integrated Programs in Biomedical Sciences Fellowship
2006	FASEB/MARC Undergraduate Student Training in Academic Research Fellowship
2005	Sigma Xi Scientific Research Society Award

MEMBERSHIPS

Society for Leukocyte Biology

American Association for Cancer Research

American Association of Immunologists

Microbiology and Immunology Student Organization

Black Biomedical Graduate Student Organization

Beta Kappa Chi Scientific Honor Society

POSTER PRESENTATIONS

1. **Triplett, A.**, Gralnick, J. *Identification of Antibiotic Resistant Genes in Escherichia coli and Citrobacter.* Tennessee Lois-Stokes Alliance for Minority Programs Research Conference, 2006.

2. **Triplett, A.**, Gralnick, J. *Identification of Antibiotic Resistant Genes in Escherichia coli and Citrobacter.* Life Sciences Summer Undergraduate Research Symposium, University of Minnesota, 2006.

3. Azeem, A., **Triplett, A.**, Alard, A., Kosiwicz, M. *IL-6 may interfere with the development of functional adaptive Tregs in NOD mice*. American Association of Immunologists Annual Meeting, 2009.

4. **Triplett, A.**, Singh, R., Sharma, P., Lillard, J., Singh, S. *CXCR6-Mediated Cellular and Molecular Events Involved in Breast Cancer Cell Migration and Invasion.* American Association of Immunologists Annual Meeting, 2010.

5. **Triplett, A.,** Li, B., Vanchinathan, M., Hansen, R., Suttles, J. *A-FABP Deficiency in Mice Provides Protection from Tumor Growth and Metastasis.* Society for Leukocyte Biology Annual Meeting, 2010.

ORAL PRESENTATIONS

1. **Triplett, A.**, Li, B., Suttles, J. *Expression of Adipocyte/Macrophage-Fatty Acid Binding Protein Promotes Tumor Growth and Metastasis.* Society for Leukocyte Biology Annual Meeting, 2011.

PUBLICATIONS

Published Abstracts

Azeem, A., **Triplett, A.S.**, Parnell, S, Zhao, Y., Alard, P., Kosiewicz, M. 2009. *IL-6 may interfere with the development of functional adaptive Tregs in NOD mice.* J. Immunol. 182: 48.19.

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Manuscripts in Preparation

Triplett, A.S., Li, B., Stout, R.D., Bernlohr, D.A., Suttles, J. 2012. Expression of Adipocyte/Macrophage-Fatty Acid Binding Protein Promotes Tumor Growth and Metastasis.