The Effects of Obesity on Stem Cell Function and the Development of Osteoarthritis

by

Chia-Lung Wu

Department of Biomedical Engineering
Duke University

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Farshid Guilak, Supervisor

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Virginia B. Kraus

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Charles A. Gersbach

Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Biomedical Engineering in the Graduate School of Duke University

2015
ABSTRACT

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Abstract

Obesity due to a high-fat diet is characterized by accumulation of inflammatory macrophages in tissues, leading to chronic low-grade systemic inflammation. Obese individuals also exhibit impaired tissue healing. With a high-fat feeding, cells are exposed to the elevated levels of dietary fatty acids (FAs), and such a change of microenvironment may alter their properties. Stem cells are cells capable of multipotent differentiation, and this potential allows them to play a promising role in healing and regenerative medicine. However, the effect of obesity, particularly various types of dietary FAs, on the function of stem cells remains largely unknown. Furthermore, obesity is a primary risk factor of osteoarthritis (OA), a disease of entire joint involving degradation of cartilage, synovitis, and subchondral bone changes. Yet, the mechanisms linking obesity and OA are not fully understood. Furthermore, although macrophages are well recognized for their inflammatory role in obesity, little is known regarding functionality of these cells in regulating the effect of obesity on OA. This dissertation develops fundamental stem cell isolation and culture techniques, and utilizes animal models to investigate (1) the influences of high-fat diet induced-obesity on function of adult stem cells, (2) examine the effect of obesity and dietary FAs on OA, and (3) evaluate the role of macrophages in obesity-associated OA by depleting macrophages using a transgenic mouse model.
A variety of adult stem cell populations including bone marrow-derived mesenchymal stem cells (MSCs), subcutaneous adipose-derived stem cells (sqASCs), and infrapatellar-derived stem cells (IFP cells) were successfully isolated from lean and obese mice and expanded in vitro. Obese stem cells demonstrated altered multilineage differentiation potential and distinct immunophenotypes as compared to lean stem cells. Furthermore, FA treatment of lean stem cells significantly changed their multipotency but did not completely recapitulate the properties of obese stem cells.

Supplementation of ω-3 polyunsaturated fatty acids (PUFAs) in a high-fat diet was capable to mitigate injury-induced OA and decrease serum inflammatory cytokine levels. ω-3 PUFAs also significantly enhanced wound repair, while saturated FAs and ω-6 PUFAs act as a detrimental factor in OA, synovitis, and wound healing. Spontaneous locomotion of the mice was independent of OA development. Furthermore, using mathematical models and weight-matched mice, we found that OA was significantly associated with dietary FA content but not with body weight and mouse activity. These results suggest that metabolic factor plays a more significant role in obesity-associated OA than mechanical factor.

Despite their temporary improved metabolic parameters and reduced osteophyte formation, obese mice receiving short-term, systemic macrophage depletion did not mitigate cartilage degeneration following joint injury. Instead, macrophage depletion significantly enhanced joint synovitis in the surgery-operated joint. Macrophage-
depleted mice also exhibited up-regulated expression of inflammatory cytokines in synovial fluid. These findings indicate that despite their recognized pro-inflammatory role, macrophages are vital in regulating the homeostasis of immune cells in the joint following injury.

Taken together, this research further elucidates the relationships among obesity, stem cells, and OA. The results from our study may provide a framework to develop stem cell therapy for obese patients and intervention program for obese OA patients in the future.
Dedication

To my parents, Tien-Szu Wu and Ching-Su Lo, and my lovely wife, Irene.
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### List of Abbreviations

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<th>Description</th>
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<tbody>
<tr>
<td>Adipokines</td>
<td>adipose-tissue cytokines</td>
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<td>ANOVA</td>
<td>analysis of variance</td>
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<tr>
<td>ASCs</td>
<td>adipose tissue-derived stem cells</td>
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<tr>
<td>AUC</td>
<td>area under curve</td>
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<tr>
<td>BMI</td>
<td>body mass index</td>
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<td>bone morphogenetic protein</td>
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<td>bovine serum albumin</td>
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<td>eGFP</td>
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<td>lymphocyte antigen 6G</td>
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<td>ITS+</td>
<td>insulin transferring selenous acid premix</td>
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<td>MAFIA</td>
<td>Macrophage Fas-Induced Apoptosis</td>
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<td>MSCs</td>
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<td>OA</td>
<td>osteoarthritis</td>
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<td>PDGFRα</td>
<td>platelet derived growth factor receptor α</td>
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<td>P/S</td>
<td>penicillin/streptomycin</td>
</tr>
<tr>
<td>P/S/F</td>
<td>penicillin/streptomycin/fungizone</td>
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<tr>
<td>PUFAs</td>
<td>polyunsaturated fatty acids</td>
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<tr>
<td>Acronym</td>
<td>Full Form</td>
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<tr>
<td>PGE₂</td>
<td>prostaglandin E₂</td>
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<td>RT-PCR</td>
<td>reverse-transcription polymerase chain reaction</td>
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<tr>
<td>SFAs</td>
<td>saturated fatty acids</td>
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<td>Sca-1</td>
<td>stem cell antigen-1</td>
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<tr>
<td>SEM</td>
<td>standard error of the mean</td>
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<td>sqASCs</td>
<td>subcutaneous fat-derived stem cells</td>
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<td>TLRs</td>
<td>toll-like receptors</td>
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<tr>
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<td>transforming growth factor-β₁</td>
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<td>TGF-β₃</td>
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<td>tumor necrosis factor-α</td>
</tr>
<tr>
<td>TP</td>
<td>tibial epiphysis</td>
</tr>
<tr>
<td>TV</td>
<td>total volume</td>
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</table>
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1. Background and Significance

1.1 Obesity and dietary fatty acids

Obesity is defined when body mass index (BMI) calculated as weight in kilograms divided by height in meter squared is equal or larger than 30 (de Lange-Brokaar et al., 2012). In 2009-2010, the prevalence of age-adjusted obesity was about 35% among adults in the US, and the trend of such high-prevalence does not significantly change over the past decade (Flegal et al., 2012).

Obese individuals have increased tissue adiposity found not only in adipose tissues but also in heart, liver and bone marrow (Bechmann et al., 2012; Bredella et al., 2011; Thanassoulis et al., 2010). These accumulated adipocytes are capable of producing various types of adipose-tissue cytokines (i.e. adipokines) such as leptin which is involved in joint and bone diseases (Scotece et al., 2013). In addition, free fatty acids (FFAs) released from the apoptosis and lipolysis of adipocytes due to the cell stress caused by obesity may also contribute inflammation (Furukawa et al., 2004). FFAs and their lipid mediators can serve as either pro- or anti-inflammatory signaling molecules, depending on their types. Saturated fatty acids (SFAs) activate macrophages through Toll-like receptor 4, inducing macrophages to secrete pro-inflammatory cytokines (Shi et al., 2006; Suganami et al., 2007). ω-6 polyunsaturated fatty acids (PUFAs) such as arachidonic acid are substrate for cyclooxygenase-2 (COX-2) and lipoxygenase, and can be converted into prostaglandin E2 (PGE2) and 4-series leukotrienes that are both
involved in pain (Bagga et al., 2003; Funk, 2001). On the other hand, ω-3 PUFAs
including eicosapentaenoicacid (EPA) and docosahexaenoicacid (DHA) may provide a
protective role by inhibiting the synthesis of PGE₂ because they compete with ω-6 PUFA
for COX-2 enzymes (Wauquier et al., 2012). Obese individuals, due to these increased
levels of adipokines, circulating FFA, and inflammatory cytokines, are often associated
with insulin resistance, impaired tissue healing and several skeletal muscular diseases
such as osteoarthritis.

1.2 Obesity, inflammation and macrophages

In addition to the increased tissue adiposity, infiltration of macrophages into
adipose tissues, particularly into visceral fat depot, is also observed in obese patients
and animals (Kanda et al., 2006). Recent studies have found that obesity polarizes tissue-
resident macrophages from M2 anti-inflammatory phenotype to M1 pro-inflammatory
phenotype. M1 macrophages, also called classically activated macrophages, are often
CD11c positive, express nitric oxide synthase (iNOS), and secrete pro-inflammatory
cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-1 (Li et al., 2010),
while M2 macrophages, i.e. alternatively activated macrophages, express CD206 and are
responsible in releasing anti-inflammatory cytokines such as IL-10 (Aron-Wisnewsky et
al., 2009). In adipose tissue, TNF-α secreted by the inflammatory macrophages may
induce lipolysis of adipocytes, and the FFAs released from the adipocytes actually in
turn activate macrophage to secrete more TNF-α. Such a cross-talk between adipocytes
and macrophages constitutes a vicious cycle, aggravating local and systemic inflammatory levels in obese individuals (Suganami and Ogawa, 2010). The mechanisms underlying how obesity alters macrophages function in the adipose tissues is still debated. Some researchers suggest that obesity may change phenotype of M2 cells by altering surface marker expression (Kawanishi et al., 2010). However, the recent evidence indicates that elevated adipose tissue may maintain M2 macrophage number but recruit M1 macrophages or their precursor cells from peripheral blood (Lumeng et al., 2008).

1.3 Obesity and wound healing

It is well known that obese individuals are often associated with wound complications and infections post-operation. It has been reported that hypoxia in obese subcutaneous tissue may contribute the increase in infection risk, as oxygen is an important component in oxidative killing of pathogens by neutrophils in body’s defense mechanism (Kabon et al., 2004). Furthermore, increased pressure at wound site due to excess weight may also cause wound dehiscence (Sandy-Hodgetts et al., 2013). In addition, adipokines such as leptin and adiponectin produced by adipocytes may also play a significant role in modulating wound healing process (Frank et al., 2000; Shibata et al., 2012b). Four overlapping cascades of normal wound healing have been identified: Hemostasis, inflammation, proliferation and matrix remodeling (Schultz et al., 2011). Alterations in the functions of immune functions may perturb wound regeneration process. For instance, macrophages isolated from diabetic humans and obese transgenic
\textit{db/db} mice had prolonged inflammasome activity and IL-1 expression, extending inflammation phase and leading to chronic wounds (Sandy-Hodgetts et al., 2013).

Although the effect of dietary FAs on wound healing has been the field of interest, the results seem to be discrepancy. Some studies suggest that \(\omega-3\) enhance wound regeneration (Alexander and Supp, 2013), others indicate that healing is delayed when \(\omega-3\) is either supplement in the diet or applied topically (McDaniel et al., 2008; Turk et al., 2013). Indeed, most studies have used lean animal models in their investigations; therefore, it remains to be elucidated how different types of dietary FAs may influence wound healing in obesity.

1.4 Obesity and adult stem cells

Adult stem cells such as mesenchymal stem cells (MSCs) are multipotent cells able to differentiate into mesodermal lineages including adipocytes, osteoblasts and chondrocytes (Caplan, 2007). This potential allows MSCs to play a significant role in tissue repair and tissue engineering (Moyer et al., 2008; Pittenger et al., 1999; Prichard et al., 2007). In addition to bone marrow, similar but distinct populations of cells with multilineage potential have been recently identified in various tissues such as subcutaneous fat (sqASCs) and infrapatellar fat pad (IFP cells) (Toghraie et al., 2011a; Wickham et al., 2003). With high-fat diet induced obesity, these tissues are likely to be exposed to high concentrations of FFAs, and such a change of microenvironment may alter the characteristics of stem cells resident in these tissues. Indeed, stem cells
harvested from the omental fat (visceral adipose tissue) of obese patients exhibit impaired adipogenesis and osteogenesis (Roldan et al., 2011). In a simulated obese environment containing the conditioned medium from FFA-treated adipocytes, MSCs isolated from lean mice demonstrated decreased adipogenesis but enhanced osteogenesis (Shan Lv and Long Wang, 2010). However, the effects of obesity or FFAs on the intrinsic cellular properties of adult stem cells, such as in vivo frequency, self-renewal ability, or multilineage differentiation capacity (particularly, chondrogenic differentiation ability), is still largely unknown.

1.5 Isolation of murine adult stem cells

Isolation and expansion of mouse stem cells have been complicated by slow growth of stem cells and persistent outgrowth of contaminating cell populations such as fibroblasts and macrophages in vitro (Peister et al., 2004; Phinney et al., 1999). Morikawa et al. were able to prospectively isolate pure murine MSC population using a combination of specific surface markers (Sca-1+PDGFRα-CD45-TER119-) by flow cytometry cell sorting and expanded these cells with a hypoxia culture conditions (Morikawa et al., 2009a). Sca-1 (stem cell antigen-1) and PDGFRα (platelet derived growth factor receptor α) are both belong to stem cell markers, while CD45 and Ter119 are used as hematopoietic cell markers. In our recent report, we also successfully isolated pure MSCs from C57BL6/J and MRL/MpJ strains by adapting this method (Diekman et al., 2012).
Using a similar sorting strategy but a slightly different marker combination, adipose-derive stem cells (Sca-1$^{+}$CD34$^{+}$CD31$^{-}$CD45$^{-}$Ter119$^{-}$) have been purified from subcutaneous fat depot from mice by Rodeheffer et al. (Rodeheffer et al., 2008). Recent evidence shows that only freshly isolated ASCs express CD34 and the cells would lose this marker when expanded in vitro (Maumus et al., 2011). Numerous reports have demonstrated that sqASCs are resident at perivascular region by immunohistochemistry (Lin et al., 2008; Traktuev et al., 2008). Therefore, CD31 is used to distinguish ASCs from endothelial progenitor cells, in which it uniquely expresses (Liang et al., 2011).

Adult stem cells with mesodermal lineage differentiation capacity were also found in infrapatellar fat pad, also known as Hoffa’s fat pad, in humans, porcine and rabbits (Buckley et al., 2010; Toghraie et al., 2011b). It has been reported that stem cells derived from IFP (IFP cells) have chondrogenic differentiation capacity and thus may have potential application for OA treatment (Wickham et al., 2003). However, recent studies have reported that obesity and OA alter cytokine secretion and assumes pro-inflammatory phenotype (Distel et al., 2009). These findings imply that stem cells resident in IFP may also be altered by obesity. The most commonly used isolation protocol for IFP cells to directly plate the cells from collagenase-digested joint fat pads and allow stem cell colonies to emerge overtime. To date, a cell-sorting strategy to isolate IFP cells has not been reported yet. Furthermore, there have been no reports on the existence of this stem cell population in rodents.


1.6 Composition of articular cartilage

Articular cartilage is a thin layer of avascular, aneural connective tissue located at the end bone of synovial or diarthrodial joints such as knees, hips, and ankles. The dry weight of cartilage matrix contains around 70% collagen (predominately type II collagen) and 30% proteoglycans. According to the orientation of collagen fibers, four zones of cartilage are defined: superficial, intermediate, deep, and calcified. Generally, in the superficial zone, collagen fibers are thin and parallel to the cartilage surface plane. With increasing the depth of the cartilage, the diameter of collagen bundles increases and the orientation of collagen fibers gradually become orthogonal to the surface plane. The cross-linking and the network of the collagen fibers provide the tensile and shear strength of cartilage (Burstein et al., 2009).

Proteoglycans is another essential component in cartilage matrix. Among them, aggregcan is most prominent. Aggrecan consists of a core protein with several functional domains that are able to bind various sulfated glycosaminoglycans such as keratin sulfate and chondroitin sulfate. By the presence of link protein, aggrecan can interact with hyaluronic acid, forming macromolecular aggregates. An aggrecan aggregate can comprise of 10-100 of aggrecans on a single hyaluronic acid (HA) molecule (Malfait et al., 2002). Because of their negatively charged carboxyl and sulfate groups, proteoglycans are highly polar. To balance these negative charges, positive cations are attracted into cartilage, creating a high ion density environment and osmotic pressure in the
matrix (Matzat et al., 2014). The high osmotic pressure in cartilage makes cartilage absorb water easily. High-water content and negative charge, which can make up to 60-80% of wet weight of the tissue, also helps cartilage to generate swelling pressure that counteracts compressive loading stress (Roughley and Lee, 1994).

The cartilage matrix is maintained by a single cell population known as chondrocytes. Chondrocytes have round to oval shape and are sparsely distributed in cartilage (only account for 5% of cartilage tissue volume). Despite their low density in tissue, chondrocytes tightly regulate the hemostasis of the cartilage matrix. For example, chondrocytes express hyaluronan receptor, CD44, which can bind to HA component of proteoglycan aggregate in the matrix. The interactions between cell-matrix modulate chondrocyte metabolism and affect cartilage remodeling (Knudson and Knudson, 2004).

1.7 Osteoarthritis

Osteoarthritis (OA) is a disease of joint degeneration. The disruption of the hemostasis between anabolic and catabolic processes of chondrocytes results in an imbalance of the turnover of the cartilage matrix. However, in addition to cartilage degradation, cytokines involved in OA often lead to synovial inflammation, osteophyte formation, and subchondral bone changes. Therefore, OA has been recognized as a disease of the entire joint. OA patients may show symptoms of joint pain and stiffness. However, the disease could lead to disability of the joint and eventually total joint
replacement is required for severe OA patient. It is estimated that at least 27 million people suffer from OA in the U.S. in 2005 and the number could be doubled in 2030 (Hootman and Helmick, 2006). Furthermore, the job-related OA costs could be up to $13.2 billion annually (Buckwalter et al., 2004; Lawrence et al., 2008). Thus, OA is a severe socioeconomic burden of a country.

Two categories have been classified for OA: primary (idiopathic) and secondary OA. Generally, primary OA develops in the previously intact joint without clear causes for disease initiation, while secondary OA is resulted from anatomic joint abnormalities, and trauma. Indeed, OA has been well recognized its multifactorial nature. The risk factors for include age, genetic, occupation, and obesity.

Current treatments for OA mainly focus on helping patients manage pain either via medications or non-pharmacologic therapies such as pain control programs. However, these methods do not reverse the progressive loss of cartilage and therefore do not cure OA. Therefore, scientists have been developing novel methods that can “regenerate” the damaged cartilage such as delivery of stem cells capable of chondrogenic differentiation into OA joint in the attempt to restore cartilage matrix.

1.8 Obesity and osteoarthritis

Obesity is a major risk factor for OA. It has been reported that 21.4% of overweight and 31.1% of obese individuals have arthritis (The Centers for Disease Control). It was initially proposed that increased mechanical loading on the joints due to
weight gain was the primary contributor for accelerated OA progress in obese patients. However, mechanical factor alone does not account for why obese patients also have significantly higher OA incidence in the non-weight bearing joints, such as hands (Felson and Chaisson, 1997; Yusuf, 2012). Several current studies have suggested that elevated inflammation associated with obesity and high-fat diets may play a crucial role in catabolic process of joint tissues (Sowers and Karvonen-Gutierrez, 2010). Our recent study also supports this growing evidence by showing that morbid obese mice due to impaired leptin signaling did not develop OA when fed a standard low-fat diet (Griffin et al., 2009).

Furthermore, macrophages are also observed in the joint synovial tissue, namely synovial macrophages. Under normal conditions, synovial macrophages are relatively quiescent, however; when joints are inflamed, for example due to injury, these macrophages assume M1 pro-inflammatory phenotype and secrete TNF-α and IL-1 that may further drive destruction of cartilage and other joint tissues. It is known that SFAs from high-fat diet polarizes adipose tissue-resident macrophages into pro-inflammatory phenotype; however, it still remains unknown whether circulating free SFAs can also active synovial macrophages into inflammatory pathway and thus contribute high-incidence of OA in obese people. Furthermore, how different types of FFA such as ω-6 and ω-3 PUFAs modulate synovial macrophages and joint tissue is an important topic for understanding the pathogenesis of OA in obese patients and yet to be fully explored.
1.9 Surgically-induced model for osteoarthritis and destabilization of medial meniscus

Numerous small animal OA models have been developed in an attempt to mimic human OA (Vincent et al., 2012). For example, chemically-induced OA models are easy to implement and can provide rapid OA progressing, and thus quick OA assessment. However, despite of having these advantages, chemically-induced OA models do not correlate with the pathogenesis of any type of human OA (Lampropoulou-Adamidou et al., 2013). It has been demonstrated that joint instability due to damage of meniscus, anterior/posterior cruciate ligament, or anterior talofibular and calcaneofibular ligament may be linked to OA, particularly associated with altered cartilage contact regions, and cartilage strains (Bischof et al., 2010; Caputo et al., 2009; Van de Velde et al., 2009). Recently, a surgically induced-OA model involving destabilization of medial meniscus (DMM) has been developed (Glasson et al., 2007). DMM model is fairly reproducible and leads to OA within 8-10 weeks post-surgery in lean mice. OA induced by DMM has relatively mild-to-moderate severity compared with those induced with chemical and cruciate ligament transection.

1.10 Depletion of macrophages and transgenic Macrophage Fas-Induced Apoptosis (MAFIA) mouse.

Macrophages have been well recognized their key role in inflammation. Investigators have attempted to study whether ablation of macrophages is able to reduce inflammation and thus mitigate the progress of inflammation-associated
diseases (Duffield et al., 2005; Frantz et al., 2013; Li et al., 2012). Feng et al. reported that high-fat diet induced obese mice showed improved metabolic profile if depleting macrophages resident in visceral adipose tissue (Feng et al., 2011). Depleting synovial macrophages also ameliorates osteophyte formation in a murine collagenase-induced OA model (Blom et al., 2004). In a collagen-induced arthritis model in mice, it has been showed that removal synovial lining macrophages also decreases joint swelling (Van Lent et al., 1998). A recent clinic study demonstrated that clodronate treatment trends toward reducing pain of erosive hand OA (Saviola et al., 2012). However, up to date, it is unknown whether depletion of macrophages in high-fat diet-induced obese mice can prevent or delay development of injury-associated knee OA in both human and animal models.

Liposome-encapsulated clodronate are widely applied to ablate macrophages, in which liposomes are used as vehicles to deliver clodronic acid (Cl\textsubscript{2}MBP) to induce apoptosis of macrophages (Van Rooijen and Sanders, 1994). This model provides a useful platform to investigate the role of macrophages in a variety of skeletal muscular diseases like OA; however, a recent study shows that clodronate-laden liposomes only deplete synovial lining macrophages, while leaving macrophages in the deeper layer of the synovium intact (Van Lent et al., 1998). These remaining macrophages within the synovium may still affect OA development in obesity given that massive infiltration of macrophages into joint synovium has been previously reported (Wu et al., 2014).
Currently, a newly developed transgenic mouse strain called Macrophage Fas-Induced Apoptosis (MAFIA) strain (strain name: C57BL/6-Tg(Csf1r-EGFP-NGFR/FKBP1A/TNFRSF6)2Bck/J) are able to systemically ablate macrophages upon delivery of depleting agent (Burnett et al., 2004). MAFIA mice have an inducible Fas apoptotic system driven by the colony-stimulating factor 1 receptor (csf1r) promoter, a promoter known to promote expression of csf1r and is predominantly active in phagocytic cells in mouse (Sasmono et al., 2003). In addition to the cytoplasmic Fas receptor, the inserted transgene also contains membrane insertion protein, enhanced green fluorescent protein (eGFP) as a reporter, and a mutant human FK506 binding protein (FKBP) that preferentially binds the dimerizing protein AP20187. Upon on the administration of AP20187, cross-linking of Fas receptor drives macrophages into caspase 8-dependent apoptosis pathway (Pope, 2002).

Although it remains unknown why the macrophages in the synovium are not completely removed by clodronate-laden liposomes, the limitation of liposomes to cross the vascular endothelium of capillaries could be a potential explanation (Galeazzi et al., 2000). AP20187, on the other hand, is a small-size cell membrane permeable protein (only 1428.8 Da); therefore, it may provide a better opportunity to reach and ablate macrophages within the synovium.
1.11 Research goals and significance

The overall goals of this research is to develop fundamental techniques and strategies to:

1. investigate the influences of high-fat diet induced-obesity on function of various adult stem cells populations,
2. study the effect of obesity and different types of dietary FAs on development of injury-induced OA, and
3. evaluate the role of macrophages in progress of obesity-associated OA by transient, systemic depletion of macrophages in obese mice.

Adult stem cells with the capacity of mesodermal lineage differentiation have been proposed as a promising candidate for tissue regeneration. However, the effect of obesity on the intrinsic properties of adult stem cells is still largely unknown. The goal of the first research is to identify how obesity may alter stem cell function, which is vital for the future application of stem cell therapy for obese patients.

Furthermore, obesity is known a major risk factor for OA; yet, little is known whether mechanical factor such as increased weight on the joint or metabolic factor such as dietary FAs plays a more significant role in obesity-associated OA following joint injury. The goal of the second research seeks to identify the primary factors linking obesity and OA.
Finally, macrophages have been shown to be involved in obesity-associated inflammation and OA. Studies have demonstrated that depletion of macrophages may reduce inflammation and improve metabolic profile of obese mice. The goal of third research seeks to elucidate functionality of macrophages in regulating the effect of obesity on injury-induced OA.

1.12 Hypotheses and aims

The research goals were addressed through three hypotheses and specific aims. Aim 1 was accomplished by developing novel methods for the isolation and expansion of murine MSCs, SqASCs and IFP cells. Stem cells were harvested from lean and obese mice, and intrinsic properties of the cells were compared through performing various in vitro experiments to examine how obesity alters their function. Aim 2 involved the establishment of reliable DMM OA model. Mice fed diets rich in different types of dietary FAs received surgery for DMM to induce OA, and ear punch to create ear wound. The progress of OA, ear wound healing, and mouse behavioral measurements were evaluated. Aim 3 utilized transgenic MAFIA mice that were capable of conditional macrophage ablation upon administration of the dimerizing agent. MAFIA mice were fed high-fat diet, and received DMM surgery. Macrophages of these mice were depleted to investigate the role of macrophages in OA development and synovitis in obesity.

Hypothesis 1: High-fat diet induced-obesity will alter cellular properties of various types of adult stem cells in mice.
Specific Aim 1: Isolate defined population of adult stem cells including MSCs, sqASCs and IFP cells from lean and high-fat diet induced-obese mice. In vivo frequency and in vitro characterization including proliferation capacity, multipotency and immunophenotypes of the stem cells from lean and obese mice were compared. To further examine one potential mechanism by which a high-fat diet affects multipotency of stem cells, the lean stem cells were differentiated in an in vitro environment rich in FFAs.

Hypothesis 2: High-fat diets supplemented with pro-inflammatory FAs, such as SFA and ω-6 PUFA, would accelerate injury-induced OA, while anti-inflammatory FA such as ω-3 PUFA would mitigate joint degeneration.

Specific Aim 2: C57BL/6J mice were fed a control low-fat diet (10% kcal fat) or one of the three high-fat diets (60% kcal fat): SFA-rich, ω-6 rich, or ω-3 rich diet. Mice received surgery for DMM to induce knee OA. Serum and synovial fluid biomarkers were collected to determine systemic and local inflammatory levels. The extent of bone changes, OA severity, and synovitis due to high-fat feeding were evaluated. Biomechanical and neurobehavioral measurements were evaluated at various time points to investigate the effect of dietary FA on mouse activity levels.

Hypothesis 3: High-fat diet induced obese mice receiving transient, systemic depletion of macrophages will have decreased OA severity compared to the obese mice without macrophage depletion.
Specific Aim 3: Macrophage Fas-Induced Apoptosis (MAFIA) transgenic mice were fed a high-fat diet rich in SFA and contains high ω-6 and ω-3 ratio. Mice received DMM surgery to induce knee OA. Macrophages were depleted at various time points by intravenous administration of AP20187, a dimerizing protein. Cytokines and biomarkers from serum and synovial fluid were collected to determine systemic and local inflammatory levels. The effect of macrophage ablation on bone changes, OA development and joint synovitis were evaluated.
2. Diet-induced obesity alters the differentiation potential of stem cells isolated from bone marrow, adipose tissue and infrapatellar fat pad: the effects of free fatty acids

A version of this chapter was published as the following:

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2.1 Introduction

Obesity is characterized by chronic low-grade systemic inflammation, which in addition to insulin resistance (Greenberg et al., 2011) is believed to contribute to several musculoskeletal diseases, such as osteoarthritis (OA) (Griffin et al., 2010) and impaired tissue healing (Kuo et al., 2011). Obesity due to a high-fat diet is associated with increased lipid deposits found not only in adipose tissue but also bone marrow (Bredella et al., 2011), liver (Bechmann et al., 2012), and heart (Thanassoulis et al., 2010). Increased tissue adiposity is associated with elevation of several adipose-derived cytokines (adipokines), while apoptosis and lipolysis of adipocytes promote levels of circulating FFAs in the body. Importantly, altered cell functions have also been reported in obese individuals. For example, reduced numbers of endothelial cell have been observed in the bone marrow of obese patients (McGuire et al., 2011). Wang et al. also found that contribution of bone marrow cells for tissue homeostasis was affected by diabetes and
obesity (Wang et al., 2009). Results of these studies suggest altered tissue repair potential in obese patients. Furthermore, adipose tissue-resident macrophages in obese individuals appear to switch from an anti-inflammatory M2 phenotype to an inflammatory M1 phenotype, increasing inflammatory levels in obesity (Lumeng et al., 2008).

The mechanisms by which high fat diet-induced obesity alters cell function are not fully understood but may involve the chronic exposure to FFAs. FFAs can activate macrophages through JNK-dependent inflammatory pathways (Nguyen et al., 2007b). Rat skeletal muscle cells cultured with FFAs have been reported to show impaired mitochondrial function (Hirabara et al., 2010). For osteoblasts and osteoclasts, FFAs have been suggested to modulate bone formation and resorption (Kruger et al., 2010). Although it is still unclear whether FFAs have an impact on chondrocyte function, accumulation of lipids in the chondrocytes has been shown to correlate positively with the degree of OA in patients, implying possible involvement of FFAs in cartilage degeneration (Lippiello et al., 1991).

Mesenchymal stem cells (MSCs) are multipotent cells capable of differentiating into specific lineages including adipocytes, osteoblasts and chondrocytes (Noth et al., 2002). This potential allows MSCs to play a significant role in tissue repair and remodeling, particularly within the marrow itself (Rosen and Bouxsein, 2006). In addition to their presence in bone marrow, similar but distinct populations of cells with
multilineage potential have been recently identified in various tissues such as subcutaneous fat (sqASCs) (Fujisaka et al., 2009) and infrapatellar fat pad (IFP cells) (Wickham et al., 2003). With high-fat diet induced obesity, these tissues are likely to be exposed to high concentrations of FFAs, and such a change of microenvironment may alter the characteristics of stem cells resident in these tissues. Indeed, stem cells harvested from the omental fat (visceral adipose tissue) of obese patients exhibit impaired multipotency (Roldan et al., 2011). In a simulated obese environment containing the conditioned medium from FFA-treated adipocytes, MSCs isolated from lean mice demonstrated decreased adipogenesis but enhanced osteogenesis (Shan Lv and Long Wang, 2010). However, the effects of obesity or FFAs on the intrinsic cellular properties of adult stem cells, such as in vivo frequency, self-renewal ability, or multilineage differentiation capacity, is still largely unknown.

In the present study, we investigated the effects of diet-induced obesity on the properties and function of several adult stem cell populations. We first isolated MSCs, sqASCs, and IFP cells from lean and high-fat diet induced obese mice and then compared their in vivo frequency, proliferation capacity, multipotency, and immunophenotype. To examine one potential mechanism by which a lard-enriched high-fat diet affects stem cell multipotency, we further differentiated lean stem cells in an in vitro environment rich in FFAs. We used a combination of palmitic acid, stearic acid (both saturated FA), and oleic acid (monounsaturated FA), as recent studies have
shown that lard-enriched high-fat diet promotes levels of these FFAs in blood and adipose tissues (Kleemann et al., 2010; Tallman and Taylor, 2003).

2.2 Materials and Methods

2.2.1 Animals

Male C57BL/6J mice fed either a high-fat diet (D12492, 60% energy from fat, Research Diets, Inc.) or a low-fat diet (D12450B, 10% energy from fat, Research Diets, Inc.) for 14 weeks were obtained from The Jackson Laboratory. Mice were sacrificed at 20 weeks of age in accordance with an Institutional Animal Care and Use Committee (IACUC) approved protocol at Duke University.

2.2.2 Cell isolation and expansion

Bones (femurs and tibias), subcutaneous adipose tissue (inguinal fat pad), and the IFP were collected from lean and obese mice and digested at 37 °C with 0.2% collagenase type I (Worthington) for 1-1.5 hours (Estes et al., 2010). MSCs were purified for Sca-1+PDGFRα+CD45−Ter119− from the bone fragments as previously described (Diekman et al.; Morikawa et al., 2009b) and sqASCs were purified for Sca-1+CD34+CD31−CD45−Ter119− from the digested inguinal fat by the method described by Rodeheffer et al. with a slight modification (Rodeheffer et al., 2008). In preliminary studies, the same marker combination as sqASCs was used to isolate a similar cell population from the epididymal fat pad (visASCs). However, due to their inability to differentiation into the chondrogenic or adipogenic lineages, these cells were not
included in the overall analysis and are reported in Appendix A, Figure A-1. A Cytomation MoFlo® sorter (Beckman Coulter) with 100 µm nozzle was used to sort cells with designated markers (all antibodies from Biolegend). Due to the small size of the joint fat pad, stem cells were directly derived as the adherent cell fraction of the IFP after collagenase digestion (Wickham et al., 2003).

Freshly sorted MSCs and sqASCs were plated at 100 cells/cm² and 3,000 cells/cm², respectively. IFP cells were seeded at 1,500 cells/cm² for the primary passage. All the cells were cultured in expansion medium consisting of α-Modified Eagle’s Medium (αMEM, Invitrogen), 20% lot-selected fetal bovine serum (FBS, Sigma), and 1% penicillin/streptomycin/fungizone (P/S/F, Invitrogen) in hypoxic conditions (37 °C, 2% O₂, 5% CO₂, remaining gas N₂). In previous studies, we have shown that these culture conditions allow for rapid expansion of mouse stem cells while maintaining their multipotency (Diekman et al.). After 8 days with media changes every 3 days, cells were trypsinized using 0.25% trypsin-EDTA (Sigma) and plated at 3,000 cells/cm². Cells were passaged every 5-6 days upon 90% confluence.

2.2.3 Multilineage differentiation

Passage three cells were pooled from 2 sets of isolations (n = 6 mice per isolation) and differentiated into adipogenic, osteogenic, and chondrogenic lineages to evaluate their multipotency. For adipogenesis, 10,000 cells were cultured in wells of 48 well plates for 2 days in expansion medium. Media was then switched to control medium consisting of
Dulbecco’s Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12, Lonza) with 10% FBS and 1% P/S/F or adipogenic differentiation medium consisting of control medium supplemented with (all from Sigma) 33 μM biotin, 17 μM pantothenate, 1 μM bovine insulin, 1 μM dexamethasone, and for the first three days 250 μM isobutylmethylxanthine and 2 μM rosiglitazone (Avandia™, GlaxoSmithKline). Cells were cultured either with control or adipogenic medium for 14 days with media changes every 3 days. Lipid droplets were stained by 0.5% Oil Red O (Sigma), which was released and quantified by absorbance at 535 nm and normalized to DNA content measured by Quant-iT™ PicoGreen® (Invitrogen). For osteogenesis, 10,000 cells were plated in wells of 48 well plates for 2 days in expansion medium. Media was then switched to control medium consisting of DMEM (4.5 g/L glucose, Invitrogen) with 10% FBS and 1% P/S/F or osteogenic differentiation medium consisting of control medium plus 10 mM β-glycerophosphate (Sigma), 250 μM ascorbate (Sigma), 2.5 μM retinoic acid (Sigma), and 50 ng/ml human bone morphogenetic protein-2 (BMP-2, R&D systems) for 21 days with media changes every 3 days. Mineral deposits were stained by 2% Alizarin Red S (Electron Microscopy Sciences). The stain was then released by heated acid extraction (Gregory et al., 2004) and normalized to DNA. For chondrogenesis, 250,000 cells were centrifuged in 15 ml polypropylene tubes at 300 g for 5 minutes to form pellets. After 2 days, media were switched to control medium consisting of DMEM (4.5 g/L glucose, Invitrogen), 1% insulin-transferrin-selenous acid (ITS+, BD), 50 μg/ml
ascorbate (Sigma), 40 µg/ml proline (Sigma), and 1% P/S (Sigma) or chondrogenic
differentiation medium consisting of control medium supplemented with 10 ng/ml
human transforming growth factor-β3 (TGF-β3, R&D systems) and 500 ng/ml mouse
BMP-6 (R&D systems). For MSCs and IFP cells, serum free control and chondrogenic
medium were used but for sqASCs both media were supplemented with 10% FBS. After
28 days, pellets were analyzed for their glycosaminoglycan (GAG) and DNA content by
1,9-dimethylmethylene blue (DMB) and PicoGreen assay, respectively. Some pellets
were also processed for histochemical staining for sulfated GAGs by 1% Alcian Blue (pH
= 1, Acros) and immunohistochemical labeling for collagen type II (Hybridoma Bank).
For FFA treated groups, cells were performed as the same differentiation methods as
described above but with the supplement of FFA mixture or vehicle control as
appropriate in their differentiation medium.

### 2.2.4 FFA preparation and treatment

To simulate an obese environment rich in saturated fatty acid (SFA) and
monounsaturated fatty acid (MUFA), a FFA mixture (SA/MUFA) containing palmitic,
stearic and oleic acids (NuChek Prep) was used. 3.3 mM stock FFA mixture was
prepared by a method described by Nguyen et al. (Nguyen et al., 2007b). Briefly, FFA
was dissolved in ethanol and mixed with DMEM (4.5 g/L glucose, Invitrogen)
supplemented with fatty acid-free bovine serum albumin (BSA, Sigma). A ratio of 5:1
FFA:BSA was used to mimic elevated FFA levels. The FFA-BSA solution was then
conjugated at 37°C for 1.5 hr until homogeneous. A vehicle control (Control) that contained BSA with the same volume of ethanol but no FFA was also prepared. The FFA mixture was aliquoted and stored at -20 °C until use. In preliminary studies, we examined effects of several different concentrations (150 µM, 250 µM and 500 µM) of FFA to test the toxicity of FFA on stem cells. We did not observe any cell death under these conditions. These cells also maintained their spindle-shaped cell morphology and were able to reach confluence at a similar rate, independent of FFA concentration (Appendix A, Figure A-2). The final concentration of individual FFA used in the differentiation culture medium was 500 µM.

**2.2.5 Immunophenotype analysis**

Passage three cells were divided into aliquots of 100,000 cells, treated with Fc block (CD16/32) for 10 minutes at 4 °C to reduce unspecific binding, then incubated for 30 minutes at 4 °C with antibodies against following cell surface markers or appropriate isotype controls (all from Biolegend): CD45, CD31, Ter119, CD44, CD11b, platelet-derived growth factor receptor α (PDGFRα), CD34, CD105, stem cell antigen-1 (Sca-1). A C6 benchtop flow cytometer (Accuri Cytometers) was used for analysis and percentages obtained by subtracting the value of isotype controls.

**2.2.6 Statistical analysis**

Statistical analysis was carried out using a 2-tailed Student’s t-test for comparison of two groups ($\alpha = 0.05$). Values are expressed as mean ± SEM.
2.3 Results

2.3.1 Obesity alters stem cell percentage in bone marrow and adipose tissues

Obese mice weighed significantly more than lean mice (40.25 ± 1.17g obese vs. 31.14 ± 0.35g lean, p < 0.001). Inguinal fat pads from obese mice were a larger percentage of total body weight as compared to lean mice (2.43% ± 0.17% obese vs. 0.66% ± 0.03% lean, p < 0.05). Results were averaged from ≥ 15 mice per group with mean ± SEM displayed.

A highly purified population of MSCs was isolated based on a specific combination of cell surface markers (Morikawa et al., 2009b). MSCs were identified as cells that are double-negative for CD45 and Ter119 (hematopoietic cell markers) and double-positive for Sca-1 and PDGFRα (stem cell markers) (Figure 2-1A). Obesity showed a trend toward increased in vivo frequency of MSCs in obese mice (p = 0.07; Figure 2-1B). There was no significant difference in the percentage of CD45 Ter119- population in bone marrow cells between lean and obese mice (Figure 2-1C). Interestingly, among this double-negative cell population, obese mice had a significantly higher percentage of Sca-1+ PDGFRα+ cells. Results were averaged from 5 independent isolations with mean ± SEM displayed (n ≥ 4 mice per isolation).

To harvest a pure stem cell population from the inguinal fat, a similar sorting strategy was used but a slightly different combination of cell markers. CD31 has been used to distinguish endothelial progenitor cells from stem cells (Liang et al., 2011), while
CD34 has been reported to be expressed on freshly isolated adipose-derived stem cells (Maumus et al., 2011). Therefore, we defined sqASCs as cells triple-negative for CD31, CD45 and Ter119 but double-positive for Sca-1 and CD34 (Figure 2-1D). In inguinal fat, obese mice showed a moderated increase in sqASCs although not significant (Figure 2-1E). Obesity also had a trend toward an increased percentage of CD45, CD31, Ter119- cells in the inguinal fat compared to lean mice (p = 0.09; Figure 2-1F) but among this triple-negative cell population, no significant difference was observed in CD34 and Sca-1 double-positive cells between lean and obese mice. Results were averaged from 3 independent isolations with mean ± SEM displayed (n ≥ 4 mice per isolation).
Figure 2-1: Representative stem cell sorting result. Representative stem cell sorting result of (A-C) bone marrow and (D-F) inguinal fat from lean mice. In the bone marrow of lean mice, approximately 1% of stromal cells were Ter119/CD45 double-negative (green dots). Only 5% of these cells were Sca-1/PDGFRα double-positive (red dots) and the cells that were Sca-1+ PDGFRα+ Ter119- CD45- were identified as MSCs. In inguinal fat of lean mice, around 24% of adipose-tissue stromal cells were CD31/Ter119/CD45 triple-negative (green dots). Among this population, 63% were Sca-1/CD34 double-positive (red dots) and sqASCs were designated as the cells that were Sca-1+ CD34+ CD31- Ter119- CD45- . Obese mice showed a trend toward increased in vivo frequency of (B) MSCs (n = 5 isolations) and (E) sqASCs (n = 3 isolations). n ≥ 4 mice per isolation. In the stem cell population, obesity significantly increased (C) the percentage of Sca-1+ PDGFRα+ cells (red bar) among Ter119- CD45- cell population (green bar) in the bone marrow (# p < 0.05 vs. corresponding lean cell population). (F) Obese mice showed a trend toward increased CD45- CD31- Ter119- cells (green bar) in the inguinal fat.

2.3.2 MSCs, sqASCs and IFP cells exhibit similar rates of proliferation in vitro

The overall morphology of stem cells was similar between lean and obese mice. All stem cells exhibited fibroblastic-like morphologies, although sqASCs displayed larger cell protrusions than MSCs and IFP cells (Figure 2-2A-F). Cells were cultured through five passages to investigate their in vitro proliferation (n = 3 independent experiments). All the cell types proliferated robustly under hypoxic conditions. Lean MSCs exhibited greater expansion, but it was not significantly different from obese MSCs (Figure 2-2G). Both obese sqASCs and IFP cells showed a trend toward increased proliferation as compared to their corresponding lean cell types (Figure 2-2H-I).
Figure 2-2: Stem cell morphology and proliferation. Morphology of (A, C, E) lean and (B, D, F) obese stem cells at passage 3. The cumulative fold increase during expansion under hypoxic conditions through passage 5 of (G) MSCs (H) sqASCs and (I) IFP cells harvested from lean and obese mice. Obese sqASCs and IFP cells showed a trend toward increased proliferation, while obese MSCs had a trend toward decreased cell growth. Results averaged from 3 independent isolations with mean ± SEM displayed (n ≥ 3 mice per isolation). Scale bar is 100 µm.

2.3.3 MSCs, sqASCs and IFP cells exhibit distinct levels of surface antigens

Immunophenotype analysis was performed at passage three for each cell type (Table 2-1).
Table 2-1: Immunophenotype analysis for passage 3 stem cells from lean and obese mice

<table>
<thead>
<tr>
<th>Surface Marker</th>
<th><strong>MSCs</strong></th>
<th><strong>sqASCs</strong></th>
<th><strong>IFP cells</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lean</td>
<td>Obese</td>
<td>Lean</td>
</tr>
<tr>
<td>CD11b</td>
<td>≤ 0.1%</td>
<td>≤ 0.1%</td>
<td>≤ 0.1%</td>
</tr>
<tr>
<td>CD45</td>
<td>≤ 0.1%</td>
<td>≤ 0.1%</td>
<td>≤ 0.1%</td>
</tr>
<tr>
<td>TER119</td>
<td>≤ 0.1%</td>
<td>≤ 0.1%</td>
<td>≤ 0.3%</td>
</tr>
<tr>
<td>CD31</td>
<td>≤ 0.1%</td>
<td>≤ 0.1%</td>
<td>≤ 0.1%</td>
</tr>
<tr>
<td>CD34</td>
<td>≤ 0.5%</td>
<td>≤ 0.5%</td>
<td>1.95%</td>
</tr>
<tr>
<td>Sca-1</td>
<td>≥ 99%</td>
<td>≥ 99%</td>
<td>≥ 99%</td>
</tr>
<tr>
<td>CD44</td>
<td>&gt; 95%</td>
<td>&gt; 95%</td>
<td>90%</td>
</tr>
<tr>
<td>PDGFRα</td>
<td>68.9±6%a</td>
<td>82.7±7%b</td>
<td>27.3%</td>
</tr>
<tr>
<td>CD105</td>
<td>68.9±11%a</td>
<td>31.3±10%a</td>
<td>64.2%</td>
</tr>
</tbody>
</table>

Abbreviations: PDGFRα, platelet-derived growth factor receptor α. In response to obesity, sqASCs and IFP cells did not significantly alter surface marker expression. Interestingly, however, obesity significantly increased percentage of the MSCs expressing PDGFRα but a trend toward to decrease CD105 level. Results are from three independent experiments for lean and obese MSCs with Mean± S.E.M. displayed (n = 3 mice per experiment). Values with different superscript letters are significantly different; P > 0.05). For other cell types, results are averaged from two independent experiments (n = 3 mice per experiment).

All the stem cells were negative for hematopoietic cell markers (CD11b, CD45 and Ter119; all < 1%) and endothelial progenitor cell marker (CD31; all < 1%) but positive for Sca-1 (all ≥ 99%). Most cell types were negative for CD34, although IFP cells showed some CD34 expression. Both MSCs and IFP cells showed high percentage of
cells expressing CD44 and PDGFRα, while sqASCs had fewer cells expressing these two markers. Obese MSCs showed a trend towards a lower percentage of cells expressing CD105 but a significantly higher percentage expressing PDGFRα as compared to lean MSCs (for CD105, p = 0.06; for PDGFRα, p < 0.05).

2.3.4 Obesity alters the multipotency of adult stem cells in a manner that depends on the tissue source of the cells

Passage three cells were differentiated into adipo-, osteo- and chondrogenic lineages (each cell type was pooled from two independent isolations, n = 6 mice per isolation). For MSCs, obese cells showed significantly reduced adipogenic (Figure 2-3A-B) and osteogenic potential (Figure 2-3C-D) compared to lean MSCs (for adipogenesis, Figure 2-3E; for osteogenesis, Figure 2-3F). When MSCs were differentiated into chondrocytes, obese MSCs showed a trend toward reduced GAG/DNA ratio, less Alcian Blue and collagen type II staining intensity compared to lean MSCs (p = 0.07; Figure 2-4A-E). When the GAG/DNA ratio was normalized to the GAG/DNA ratio of pellets under control conditions, this trend was significant (data not shown).

For sqASCs, obese cells showed significantly enhanced adipogenic (Figure 2-3G-H) and osteogenic differentiation (Figure 2-3I-J) compared to lean sqASCs (for adipogenesis, Figure 2-3K; for osteogenesis, Figure 2-3L). However, lean sqASCs exhibited greater chondrogenic potential than obese sqASCs (Figure 2-4F-J).

Similar to sqASCs, obese IFP cells demonstrated significantly increased adipogenesis (Figure 2-3M-N) and osteogenesis (Figure 2-3O-P) compared to lean IFP
cells (for adipogenesis, Figure 2-3Q; for osteogenesis, Figure 2-3R). When differentiated into the chondrogenic lineage, lean IFP cells showed significantly higher GAG/DNA ratio than obese IFP cells (Figure 2-4K-O).

Figure 2-3: Adipogenesis and osteogenesis of stem cells harvested from lean and obese mice. Lipid droplets accumulation in (A, G, M) lean and (B, H, N) obese stem cells after 14 days culture in adipogenic medium. Cells were stained with 0.5% Oil Red O. Stain was then released and normalized to DNA content to quantify adipogenic potential of (E) MSCs, (K) sqASCs and (Q) IFP cells. For osteogenesis, calcium mineral deposits stained with 2% Alizarin Red S in (C, I, O) lean and (D, J, P) obese stem cells after 21 days culture in osteogenic medium. Stain was then extracted and normalized to DNA content to determine osteogenic capacity of (F) MSCs, (L) sqASCs and (R) IFP cells. Results from ≥ 5 samples per group of the cells pooled from two independent isolations (n = 6 mice per isolation) with mean ± SEM displayed. # p
< 0.05 vs. corresponding lean cell type by t-test. Scale bar is 100 μm for adipo- and 5 mm for osteogenesis, respectively.

Figure 2-4: Sulfated GAGs and proteoglycans of chondrogenic pellets from (A, F, K) lean stem cells and (B, G, L) obese stem cells after 28 days pellet culture in chondrogenic medium was detected by 1% Alcian Blue staining (pH = 1). Collagen II immunohistochemical staining was also performed for the pellets from (C, H, M) lean stem cells and (D, I, N) obese stem cells. Quantification of GAG content was performed by DMB assay and the value was then further normalized to DNA to determine chondrogenic potential of (E) MSCs, (J) sqASCs and (O) IFP cells. Obese
MSCs exhibited a trend toward decreased chondrogenesis ($p = 0.07$ vs. lean MSCs), while obese sqASCs and IFP cells showed significantly decreased chondrogenic capacity. Results from $\geq 4$ pellets per group of the cells pooled from two independent isolations ($n = 6$ mice per isolation) with mean $\pm$ SEM displayed. # $p < 0.05$ vs. corresponding lean cell type by t-test. Scale bar is 500 $\mu$m.

While lean IFP cells had significantly higher GAG content per pellet compared to obese IFP cells, other lean cell types exhibited a trend toward higher GAG content per pellet compared to corresponding obese cell types (Appendix A, Figure A-3).

A table summarizing the multilineage differentiation capacity of lean and obese stem cells isolated from different tissues is provided in Appendix A, Table A-1.

2.3.5 FFA treatment alters the multipotency of lean adult stem cells

To examine the potential influence of increased FFAs, high concentrations of palmitic and stearic acid as well as oleic acid (SA/MUFA mixture) were supplemented into the differentiation media of lean stem cells. When treated with SA/MUFA, lean MSCs demonstrated significantly enhanced adipogenesis and osteogenesis compared to vehicle control (for adipogenesis, Figure 2-5A; for osteogenesis, Figure 2-5B). However, there was no significant difference in chondrogenesis between SA/MUFA treated and vehicle control (Figure 2-5C).

When treated with SA/MUFA, lean sqASCs demonstrated significantly enhanced adipogenesis compared to vehicle control (Figure 2-5D). However, SA/MUFA did not alter the osteogenesis of lean sqASCs (Figure 2-5E). Supplementation with SA/MUFA significantly decreased the chondrogenic capacity (Figure 2-5F).
Similar to lean sqASCs, lean IFP cells exhibited enhanced adipogenesis but maintained osteogenic capacity when treated with SA/MUFA (for adipogenesis, Figure 2-5G; for osteogenesis, Figure 2-5H). SA/MUFA treatment also decreased chondrogenesis of lean IFP cells (Figure 2-5I).

Figure 2-5: Multi-lineage differentiation of lean stem cells with supplement of SA/MUFA. (A, D, G) all the stem cells treated with SA/MUFA demonstrated increased adipogenesis. SA/MUFA also significantly enhanced (B) osteogenesis of MSCs but did not significantly affect (E, H) osteogenic potential of sqASCs and IFP cells. However, MSCs did not alter (C) chondrogenic potential in response to SA/MUFA but the treatment of SA/MUFA significantly decreased (F, I) chondrogenic capacity of sqASCs and IFP cells. Results from 5 samples (for adipogenesis and osteogenesis) or ≥ 4 pellets (for chondrogenesis) per group of the cells pooled from two independent
isolations (n = 6 mice per isolation) with mean ± SEM displayed. # p < 0.05 vs. vehicle control by t-test.

A table summarizing the multilineage differentiation capacity of FFA-treated lean stem cells and vehicle control is provided in Appendix A, Table A-2.
2.4 Discussion

The findings of this study show that high-fat diet induced obesity significantly alters a number of cellular properties of adult stem cells derived from bone marrow, subcutaneous fat, and the infrapatellar fat pad. Obesity appeared to increase the \textit{in vivo} frequency of stem cells and alter their multilineage potential in a manner that was dependent on the cell source. Some of these effects were reproduced by culture with FFAs, suggesting that the increase systemic levels of FFAs associated with a high-fat diet may be responsible in part for the observed effects.

Our finding of an increased sqASC population is consistent with a previous study showing that the percentage of proliferating CD34⁺/CD31⁻ adipose-tissue progenitors was increased in class I obese patients (BMI 30-34.9 kg/m²) (Maumus et al., 2008). Increased proliferation of CD34⁺/Sca-1⁺ adipose tissue progenitors was also observed in subcutaneous fat using an \textit{in vivo} bromodeoxyuridine labeling method after mice were exposed to a high-fat diet (Joe et al., 2009). Our obese sqASCs also showed a trend toward increased \textit{in vitro} proliferation capacity compared to lean sqASCs. This result is supported by a previous study showing adipose progenitor cells obtained from subcutaneous fat pad of high BMI individuals exhibited higher proliferation than those from low BMI individuals (Fernandez et al., 2010). The expanded stem cell populations may reflect increased adipogenic differentiation in the marrow and possibly a commitment into an endothelial lineage for adapting hypoxic conditions \textit{in vivo} (Baptista...
et al., 2009). There is also evidence that stem cells are associated with vasculature (Crisan et al., 2008), and increased tissue adiposity may increase vascularity and thus the stem cell pool.

Obesity had a consistent inhibitory effect on multilineage potential of MSCs. Our results are in agreement with a recent study showing that stem cells isolated from lean individuals demonstrated better multipotency in mesodermal lineages than those from obese individuals (Roldan et al., 2011). MSCs are the common precursor cells for both adipocytes and osteoblasts in bone marrow (Caplan, 2005). The relationships between marrow fat and bone density in response to obesity is not fully understood and is an area of intensive investigations. Increased body mass seems to encourage bone formation, but inflammation due to excessive fat tissues may be detrimental to osteogenesis (Cao, 2011; Chen et al., 2010). Our findings provide evidence that obesity results in reduced in vitro adipogenic and osteogenic capacity of MSCs.

Obese sqASCs and IFP cells both exhibited significantly enhanced adipogenesis and a trend toward higher proliferation as compared to their corresponding lean cell types. These findings imply that subcutaneous fat and infrapatellar fat pad may have increased fat-storing capacities during weight gain. Several studies have shown that subcutaneous adipose tissues expand fat mass by hyperplasia (increased adipocyte numbers) instead of hypertrophy (increased adipocyte size) which particularly occurs in visceral fat depot such as epididymal fat pad (Joe et al., 2009). Increased adipocytes can
arise from the proliferation and adipogenic differentiation of sqASCs, as adipocytes are terminally differentiated cells and incapable of proliferation (Majka et al., 2011). In our preliminary studies, we also observed that adult stem cells isolated from epididymal fat pad had lower adipogenesis compared to sqASCs (Appendix A, Figure A-1). Compared to large adipocytes, new smaller adipocytes have better capacity in up-taking excess FFAs and therefore protect adipocytes from apoptosis (Ibrahim, 2010), which may inhibit infiltration of inflammatory macrophages into adipose tissues (Otabe et al., 2007). To investigate whether macrophages infiltrate into obese joint fat pads, inguinal fat, and epididymal fat, these tissues harvested from lean and obese mice were stained with an antibody against epidermal growth factor seven transmembrane protein (F4/80) expressed on macrophages. Interestingly, in contrast to obese visceral fat, we did not observe massively infiltrated macrophages in the obese IFP and inguinal fat pad (Appendix A, Figure A-4).

In this study, we also observed that both sqASCs and IFP cells from obese mice exhibited significantly higher in vitro osteogenesis. Recent studies have suggested that osteogenesis is closely linked to Wnt/β-catenin signaling pathways (Ling et al., 2009) and microRNA expressions such as miR-26a, -133 and -135 (Li et al., 2008; Luzi et al., 2008). Whether obese sqASCs and IFP cells have dysregulated Wnt signaling or altered microRNA levels requires further investigation.
Several approaches for cartilage repair and regeneration rely on chondrogenesis of stem cells. For example, microfracture is a procedure to stimulate MSC migration directly from bone marrow into focal cartilage defects (Lutzner et al., 2009). Scaffolds seeded with culture-expanded autologous adult stem cells for cartilage repair are also currently undergoing intensive investigation (Guilak et al., 2010). However, the potential impact of obesity on the intrinsic chondrogenic ability of these cells is not well understood. Our data show that obese MSCs, sqASCs and IFP cells exhibit decreased production of GAGs and collagen type II, implying that obesity might interfere with cartilage repair during autologous stem cell therapy.

While our results indicate that obesity significantly affected the multipotency of stem cells, we did not observe changes in antigen expression levels in sqASCs and IFP cells after exposed to a high-fat diet. Nevertheless, we cannot exclude possible alterations in other antigens such as Toll-like receptors (TLRs), which have been shown to modulate stem cell functions (Hwa Cho et al., 2006). Interestingly, obese MSCs did show a trend toward decreased percentage of the cells expressing CD105. CD105, also known as endoglin, is an accessory protein in mediating signaling of TGF-β superfamily (Dallas et al., 2008), and it is well known that TGF-β up-regulates the key transcription factor Sox9, critical for the commitment of MSCs to the chondrogenic lineage (Quintana et al., 2009). It has been shown that downstream SMAD signaling of CD105 is required for onset of chondrogenesis of MSCs (Hellingman et al., 2011). Furthermore, we also
found that obese MSCs had a significantly increased percentage of cells expressing PDGFRα. Previous studies have suggested that PDGF-AA promotes early stages of cartilage development of chicken embryo but may inhibit chondrogenesis at later stages (Ataliotis, 2000). Recent studies have shown that hypoxia-conditioned human embryonic stem cells chondrogenesis was associated with a high CD105 but low PDGRFα expression profile (Koay and Athanasiou, 2008). Our results in accordance with above studies indicate that the decreased chondrogenic capacity of obese MSCs could be potentially due to down-regulated expression of CD105 but up-regulated expression of PDGFRα.

Our findings also demonstrated that SA/MUFA strongly affected the multilineage potential of lean stem cells. MSCs treated with SA/MUFA up-regulated both adipogenic and osteogenic potentials but showed no marked alteration in chondrogenic ability. Our result of enhanced osteogenesis by SA/MUFA is supported by previous findings that oleic acid significantly promoted osteogenesis of mouse mesenchymal cells in the presence of BMP-2 (Deshimaru et al., 2005). Saturated FFAs, acting as lipopolysaccharide (LPS), can activate TLR4 via triggering MyD88-dependent pathways (Yu et al., 2008), shifting cytokine secretion profile in MSCs (Pevsner-Fischer et al., 2007). Furthermore, human MSCs with prolonged LPS treatment have been shown to exhibit enhanced osteogenic capacity (Mo et al., 2008). It is therefore plausible that palmitic and stearic acids in the FFA mixture we used both contributed to promote
osteogenic differentiation of lean MSCs. However, murine MSCs showed enhanced osteogenesis but decreased adipogenesis when cultured in a simulated obese environment containing the conditioned medium of palmitic and oleic acid-treated adipocytes (Shan Lv and Long Wang, 2010). This discrepancy may be a result of the different culture methods. The conditioned medium secreted by FFA-treated adipocytes might contain other cytokines that modulate stem cell functions.

We also observed that lean sqASCs and IFP cells had a similar response to FFA treatment in differentiation into three mesodermal lineages, although these cells exhibit distinct phenotypes. To date, few studies have investigated how SFA and MUFA modulate functions of adipose tissue stem cells, despite the fact that these two types of FFAs constitute an important part of our diets (German and Dillard, 2004). Manickam et al. reported that no significant alteration in lipid accumulation was observed when 3T3-L1 cells were treated with either stearic or oleic acid (Manickam et al., 2010). However, pre-adipocyte cell lines might have different responses to obesity compared to multipotent stem cells that are higher in the stem cell hierarchy. Future investigations are necessary to elucidate the molecular mechanism(s) by which SFA and MUFA modulate multipotency of stem cells.

Another significant finding of this study is that FFA-altered multipotency of lean stem cells did not completely recapitulate the multipotency of stem cells directly harvested from obese mice. This result suggests that the exposure to FFAs alone cannot
explain the alterations of stem cell functions in the obese environment, and it also implies that other obesity-associated cytokines might act synergically with FFAs on stem cells. Indeed, leptin, an adipokine often up-regulated in obesity, has been shown to modulate the balance between adipogenesis and osteogenesis of mouse and human mesenchymal progenitor cells (Scheller et al., 2010). In addition to adipokines, a number of studies have also reported that inflammatory cytokines such as TNF-α can inhibit osteogenesis of stem cells (Zhao et al., 2011). Moreover, that IL-17A produced by CD4+ Th17 cells, a possible infiltrating immune cells during weight gain, significantly suppresses adipogenic differentiation of human MSCs via the COX-2/prostaglandin E2 pathway (Shin et al., 2009).

One potential limitation of the current study is that multipotency of the stem cells was evaluated only by using histological and biochemical assays. Although previous studies have shown that these assays are reflected by similar changes in gene expression (Noth et al., 2002), detailed gene expression analysis may provide insight into whether lean and obese stem cells have a temporal difference in response to differentiation-inducing signals. To examine the specific mechanism(s) underlying the alteration of stem cell functions caused by obesity or FFA treatment, future studies may wish to investigate the genetic and/or epigenetic profile of these cells following differentiation into various lineages.
2.5 Summary

Our results indicate that obesity significantly alters the characteristics of stem cells resident in various tissues. This study is significant for the development of autologous stem cell therapy for obese patients as obesity is highly prevalent in the US and continuously increasing in other countries. An improved understanding of the effects of obesity on the adult stem cell pool may help in optimizing the response of obese stem cells, which may be necessary to enhance their therapeutic capacity. Our finding of an increased stem cell pool with altered properties in various obese tissues extends our understanding of the mechanisms underlying the remodeling of musculoskeletal tissues in obesity.
3. Dietary fatty acid content regulates wound repair and the pathogenesis of osteoarthritis following joint injury

A version of this chapter was published as the following:

3.1 Introduction

Obesity is one of the primary risk factors for osteoarthritis (OA), although the mechanisms linking these conditions are not fully understood (Aspden, 2011). While it has been believed that increased loading on the joints due to weight gain is responsible for accelerated OA with obesity, mechanical factors alone do not account for the higher incidence of OA in non-weight bearing joints, such as the hands (Felson and Chaisson, 1997). Furthermore, studies have shown that morbidly obese mice do not develop OA when fed standard (low-fat) chow (Griffin et al., 2009). These findings suggest that factors other than adiposity or body weight – such as dietary content or the circulating levels of adipokines – must contribute to OA in obesity.

Cellular stress due to obesity induces lipolysis of adipocytes, (Furukawa et al., 2004) increasing the levels of circulating free fatty acids (FAs). These FAs can serve as pro- or anti-inflammatory molecules in metabolic signaling. Saturated FAs (SFAs) can activate macrophages to secrete tumor necrosis factor alpha (TNF-α) and interleukin 1 (IL-1) (Nguyen et al., 2007a). Furthermore, the derivatives of ω-6 polyunsaturated FAs
(PUFAs) are involved in joint pain (Bagga et al., 2003; Pincus et al., 2004). Conversely, ω-3 PUFAs have been reported to reduce spontaneous OA in animals fed a low-fat diet (Knott et al., 2011). These findings imply that dietary or metabolic factors may play a more direct role in joint degeneration. Furthermore, little is known regarding the effects of dietary FAs on motor function, pain perception and wound healing in obesity.

The goal of this study was to determine the effects of dietary FAs content in injury-induced OA and to identify the primary factors linking obesity and OA. Mice fed various high-fat diets rich in SFA, ω-6, or ω-3 PUFA underwent surgery to destabilize the medial meniscus (DMM) to induce OA (Glasson et al., 2007). We also investigated the effect of FAs on wound regeneration and behavioral activity.

3.2 Methods

3.2.1 Animals

All procedures were approved by the Duke University IACUC. Beginning at 4 weeks of age, mice were fed a control low-fat diet or one of the three high-fat diets: SFA-rich, ω-6 PUFA-rich, or ω-3 PUFA rich (Appendix B, Table B-1). At 16 weeks of age, mice underwent DMM surgery to induce knee OA in the left hind-limb (Glasson et al., 2007) and ears were punched using 1-mm (right) and 1.5-mm (left) diameter ear punches to examine wound healing responses. To determine how diet affected behavior and activity levels, mice were monitored at 6, 14 and 24 weeks of age. The study design is presented in (Appendix B, Figure B-1).
3.2.2 Measurements of body fat composition and whole body bone mineral density.

The body fat content and bone mineral density of the mice, excluding the head, were measured at 7, 16, and 28 weeks of age using a dual-energy x-ray absorptiometry (DEXA) scanner (GE Lunar PIXIImus, GE Healthcare, Madison, WI, USA).

3.2.2 Quantitative real-time PCR of visceral adipose tissue.

Total RNA was isolated from epididymal fat from the mice at 28 weeks of age using RNAeasy Plus Midi Kit (Qiagen, Germantown, MD, USA). cDNA was then synthesized using SuperScript VILO Master Mix (Invitrogen, Grand Island, NY, USA) and gene expression was analyzed with a Applied Biosystems StepOne Real-Time PCR System (Applied Biosystems, Grand Island, NY, USA) using Taqman Gene Expression Master Mix (Applied Biosystems) for F4/80 and CD11c or with a Power SYBR Master Mix (Applied Biosystems) for IL-6, TNF-α and MCP-1. Each gene was normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression. Relative fold differences were calculated by further normalizing expression levels of individual high-fat feeding groups to the values of Control low-fat group. Primer sequences for each gene are presented in Appendix B, Table B-2.

3.2.3 Induction of OA by destabilization of the medial meniscus.

At 16 weeks of age, mice underwent surgery for destabilization of the medial meniscus (DMM) to induce knee OA in the left hind-limb as previously described
(Glasson et al., 2007). Briefly, anesthetized mice were placed on a custom-designed device, which positioned their hind-limbs in 90-degree flexion. The medial side of the joint capsule was opened and the medial meniscotibial ligament, that attaches the medial meniscus to the tibial plateau, was transected with a #11 scalpel blade. Bleeding due to the incision was controlled by absorption spears soaked with epinephrine 1:1000 (Neogen, Lexington, KY, USA). The joint capsule and subcutaneous layer of the skin were closed with a continuous 8-0 suture with taper point (Polysorb™, Covidien, Mansfield, MA, USA).

### 3.2.4 Ear-punch model for wound regeneration capacity

To investigate how dietary FAs could influence the wound healing process, mouse ears were punched using 1-mm diameter (right ears) and 1.5-mm (left ears) diameter metal ear punches (Roboz Surgical Instruments, Gaithersburg, MD, USA) at the time of DMM surgery. The punch was made as a through-and-through hole at the center of the ear as previously described (Bartelt et al., 2010). Wound area was photographed at 1 week, 8 weeks and 12 weeks post-wounding using a digital camera. Wound size was quantified by ImageJ 1.42q (National Institutes of Health, Bethesda, MD, USA). Upon euthanasia, ears were dissected, bisected and embedded in paraffin. Ears were sectioned at a thickness of 5 µm. To reveal the cell distribution, cartilage islands, sebaceous glands, and epithelial thickness, samples were stained with hematoxylin and eosin (H&E) and Masson’s trichrome. Samples were also stained with
Picrosirius red according the manufacturer's instructions (Scytek Laboratory, Logan, Utah, USA) and were imaged under polarized light microscopy to identify collagen type I fibers.

**3.2.5 Evaluation of OA severity**

All mice were euthanized at 28 weeks of age. Knee OA severity was determined as previously described (Louer et al., 2012). Briefly, both left and right hind-limbs were harvested and fixed in 10% buffered formalin. Limbs were then decalcified in Cal-Ex solution (Fisher Scientific, Pittsburgh, PA, USA), dehydrated and embedded in paraffin. The joint was sectioned in the coronal plane at a thickness of 8 μm. Sections were stained with hematoxylin, fast green, and Safranin-O. Three independent, blinded graders then assessed sections for degenerative changes of joints using a modified Mankin scoring system. Scores were averaged between graders for individual joint quadrants as well as for the whole joint, resulting in scores between 0 and 30 for each quadrant (medial femoral condyle, medial tibial plateau, lateral femoral condyle, and lateral tibial plateau).

**3.2.6 Evaluation of osteophyte severity.**

To access the severity of osteophyte pathology, a semiquantitative grading scale was created based on a review by van der Kraan and co-workers with slight modification (van der Kraan and van den Berg, 2007). Osteophytes in the joint were graded under high-power field (×200 magnification): 0 point = normal periosteal
surface, 1 point = early stage (cells in the periosteum and synovial lining layer starting to proliferate), 2 points = middle stage (cells in the periosteum and synovial lining layer showing massive proliferation and hypertrophic chondrocytes can be observed), 3 points = mature stage (osteophyte integrated with the subchondral bone with the presence of bone marrow cavities) (Appendix B, Figure B-2).

3.2.7 Evaluation of knee synovitis.

Joint sections were stained with H&E to reveal infiltrated cells and synovial structure. Three blinded, independent graders assessed sections for synovial lining thickness (0-3 points) and synovial stroma density (0-3 points) in each joint quadrant using a previously described scoring scale (Louer et al., 2012). Scores were averaged between graders for individual quadrants as well as for the whole joint, resulting in a maximum score of 24 per joint.

3.2.8 Evaluation of bone tomography.

Bone tomography was analyzed using a desktop micro-computed tomography system (microCT 40, Scanco Medical AG, Bassersdorf, Switzerland). As previously described (Louer et al., 2012). Briefly, both hind-limbs were scanned by a desktop micro-computed tomography system (microCT 40, Scanco Medical AG, Bassersdorf, Switzerland). A hydroxyapatite calibration phantom was used to calibrate bone density values (mg/cm³). Morphometric bone parameters were investigated in the distal femoral condyles, the tibial epiphysis immediately distal to the subchondral bone, the tibial
metaphysis, and heterotrophic bone fragments in the joints. Parameters reported for the femoral condyles were bone mineral density (BMD; mg/cm³) and cancellous bone fraction (bone volume/total volume, BV/TV, excluding the cortex), while parameters reported for the tibial epiphysis and metaphysis were BMD and BV (mm³) and heterotrophic bone fragments. Unless otherwise indicated, to determine how diet, surgery, and their interaction (surgery x diet) affected bone remodeling, bone parameters from non-operated (right) and DMM-operated (left) joints were analyzed by two-factor repeated measures ANOVA. Due to the low incidence of heterotopic bone formation in the Control low-fat and ω-3 PUFA high-fat diets fed mice, their heterotopic BV data were not normally distributed. Thus, the data were expressed as medians with 25%-75% interquartile ranges and analyzed by Kruskal-Wallis H test for the main effect of the diet, followed by the Mann-Whitney U test with Holm-Bonferroni corrections for multiple comparisons to evaluate differences among diet groups.

### 3.2.9 Immunohistochemistry

Macrophages in the inflamed joints and adipose tissues were detected using an antibody against F4/80, a commonly used marker for murine mature macrophages. Four operated joints from each diet group were immunostained for macrophages. Joint sections were pretreated by 0.01% Proteinase K (Sigma-Aldrich, St. Louis, MO, USA) at 37°C for antigen retrieval. For adipose tissues, sections were treated with a citric-based buffer (pH 6.0; Vector Labs, Burlingame, CA, USA) at 95°C for 20 min for antigen
retrieval. Endogenous peroxidase was then quenched with 3% H$_2$O$_2$ for 1 hour. Next, 2.5% normal goat serum (Vector Labs) was applied to the sections to reduce nonspecific binding. Sections were then stained with F4/80 primary antibody (clone CILA3-1; AbD Serotec, Raleigh, NC, USA) at 1:150 dilution at room temperature for 1 hour followed by micro-polymer kit incubation (ImmPRESS Anto-Rat IgG Reagent; Vector Labs).

Chromogenic detection was performed with the DAB peroxidase substrate kit (Vector Labs). Hematoxylin was used to identify cell nuclei. F4/80-stained joint quadrants were evaluated by 2 independent, blinded graders using a semiquantitative grading scheme based on counting F4/80+ cells and the presence of follicle-like lymphocytic infiltrates per high-power field (×200 magnification): 0 point = 0 cells, 1 point = 1-5 cells, 2 points = 6-10 cells, 3 points > 10 cells, 4 points = presence of lymphocytic infiltrates containing macrophages. Scores were averaged between graders for medial and lateral sides of the joint as well as for the whole joint, resulting in a maximum score of 16 per joint.

To detect proliferating cells in the ear wound, sections of ear samples were treated with the citric-based buffer at 95°C for 20 min for antigen retrieval. Ki-67 (clone 16A8, Biolegend, San Diego, CA, USA), a nuclear antigen present only in proliferating cells, was used at a 1:100 dilution at room temperature for 1 hour followed by the immunohistochemical staining steps as described above.
3.2.10 Spontaneously locomotor activity

Voluntary locomotion was measured by placing mice into the open field (22 x 22 x 30 cm; Accuscan Instruments, Columbus, OH, USA). Mice were given free access to the chamber for 1 hour. Horizontal activity or distance traveled in 1 hour (cm) was measured with infrared diodes interfaced to a computer using the Accuscan analysis software (Accuscan Instruments, Columbus, OH).

3.2.11 Musculoskeletal function, and coordination testing

Forelimb and hind-limb grip strength were measured with a mouse grip-strength meter (San Diego Instruments, San Diego, CA). Three grip-strength trials for forelimb and whole body were tested for individual animals. The hind-limb grip strength was determined by subtracting mean forelimb data from the mean whole body data. It is known that body size may influence grip strength. Thus, to obtain body mass-adjusted grip-force, grip-strength was further normalized to body weight (Rayavarapu et al., 2010) and reported as grams of force per grams body mass [g/(g, body mass)]. Motor learning, coordination, and endurance were assessed using a rotarod (Med-Associates, St Albans, VT, USA) with accelerating speed (4 to 40 rpm over 5). Four rotarod trials with 30 minute intervals between trials for each mouse were performed and the latency to fall over the 4 trials were averaged to obtain one latency score (in sec).
3.2.12 Thermal hyperalgesia experiments

Thermal sensitivity was evaluated by hot plate and tail flick tests. For the hot plate test, an animal was placed on a hot plate (52 ± 1°C; Columbus Instruments, Columbus, OH, USA) and latency to the first paw flick was recorded. For the tail flick test, animals were gently restrained in a towel and the mid-portion of the tail was placed in-line with a radiant light source (Columbus Instruments). Heat was applied by focused light and the latency for tail withdrawal was recorded.

3.2.13 Serum cytokine and adipokine analyses

Concentrations of both leptin and insulin in serum samples collected over the course of the study were measured using the Mouse Metabolic Assay (#K15124C; MSD, Gaithersburg, MD, USA) as a multiplexed sandwich ELISA. Samples were run without dilution as suggested by the manufacturer. The lower limit of detection for each of the analytes was 43 pg/ml for leptin and 15 pg/ml for insulin. The intra- and inter-assay coefficients of variation (CVs) were 4.9% and 4.2%, respectively, for leptin and 5.1% and 3.3%, respectively, for insulin.

The Mouse Adiponectin singleplex ELISA (#K152BXC; MSD) was used to determine concentrations of adiponectin in serum samples collected over the course of the study. Samples were diluted 1000-fold as directed by the manufacturer. The lower limit of detection was 0.04 ng/ml and the intra- and inter-assay CVs were 4.3% and 1.8%, respectively.
Concentrations of resistin in serum samples collected over the time-course of the study were measured using the Mouse Resistin singleplex sandwich ELISA (#K152FNC; MSD). Samples were diluted 20-fold as suggested by the manufacturer. The lower limit of detection was 1.9 pg/ml and the intra- and inter-assay CVs were 4.7% and 1.6%, respectively.

A competitive enzyme immunoassay (#KGE004B; R&D Systems, Minneapolis, MN, USA) was used to measure PGE2 in mouse serum samples at 28 weeks of age. Samples were diluted a minimum of 1500-fold to obtain values within the linear range of the assay. Several samples required additional dilutions to obtain valid values. The minimum detectable concentration of PGE2 was 30.9 pg/ml and the intra- and inter-assay CVs were 6.7% and 10.6%, respectively.

Two different sandwich ELISAs were employed for the determination of Free Active TGF-β1 (#437707; BioLegend, San Diego, CA, USA) and Total TGF-β1 (#436707; BioLegend). Samples were run undiluted for Free Active TGF-β1 and diluted 1000-fold for Total TGF-β1 as recommended by the manufacturer. The minimal detectable concentration of Free Active TGF-β1 was 2.3 pg/ml and 3.5 pg/ml for Total TGF-β1. The intra- and inter-assay CVs were 9.1% and 8.0%, respectively, for Free Active TGF-β1 and 3.6% and 6.6%, respectively, for Total TGF-β1.
3.3 Results

3.3.1 Body weight

At 28 weeks of age, SFA and ω-6 mice were heavier than Control and ω-3 mice (Figure 3-1A). Although animals slightly lost weight at 8, 17, and 24 weeks, likely due to the mild stress of behavioral testing, they maintained the trend of gaining weight. To describe the influence of weight on joints over time, the area under the weight-versus-time curves (AUC) was calculated by using a trapezoidal rule (Figure 3-1B) (Martins et al., 2010). All high-fat diet fed mice had higher AUC\(_{4-28\text{wk}}\) versus Control mice.

![Figure 3-1 Body weight, DEXA results, and gene expression in visceral fat. (A) The influence of diets on body weight over time. (B) To precisely describe the effect of weight on knee joint over time, the areas under weight curve of different diets were calculated for the period from 4 to 28 weeks (AUC\(_{4-28\text{wk}}\)) and from 17 to 28 weeks (AUC\(_{17-28\text{wk}}\)), respectively. The Control mice had lower AUC\(_{4-28\text{wk}}\) values as compared to the mice fed high-fat diets. (C and D) SFA and ω-6 mice had increased percentages of body fat, but decreased body BMD relative to Control mice at 28 weeks of age. (E) The ω-6 mice had significantly increased percentages of fat in the inguinal](image-url)
and epididymal depots relative to body weight compared to Control mice. (F) SFA and ω-6 mice showed increased F4/80+ macrophage infiltration (red arrowheads) into adipose tissues (Sub: subcutaneous fat; Vis: visceral fat). Infiltrated macrophages into the visceral fat pads in SFA and ω-6 mice also showed “crown-like” structure (green square). Scale bar = 100 µm. (G) Analysis of F4/80, CD11c, IL-6, TNF-α, and MCP-1 gene expression in visceral adipose tissue in the mice at 28 weeks of age. For gene expression, n = 4 mice/diet. For other figures, n = 11-14 mice/diet. Different letters are significantly different, p < 0.05, from each other. (A, C and D) Statistical significance was determined by two-way repeated measures ANOVA using age and diet as factors. (B, E and G) Statistical significance was determined by one-way ANOVA using diet as factor. ANOVA was then followed by Tukey’s post-hoc test. All data are presented as mean ± SEM.

3.3.2 Body composition and gene expression

At 28 weeks of age, SFA and ω-6 mice had increased body fat percentage but decreased bone mineral density (BMD) versus Control mice. The ω-3 mice displayed lower BMD but no difference in body fat percentage versus Control mice (Figure 3-1C,D). The SFA and ω-6 mice had a higher percentage of epididymal fat relative to body weight, and only ω-6 mice had a significantly higher percentage of inguinal fat than Control mice (Figure 3-1E).

F4/80+ macrophages staining in adipose tissue indicated that SFA and ω-6 PUFA diets were associated with massive macrophage infiltration (Figure 3-1F). Gene expression of epididymal fat showed that SFA and ω-6 PUFA diets trended toward higher expression of IL-6 with no difference in MCP-1 expression versus those of the ω-3 and Control mice (Figure 3-1G). All the high-fat diet fed mice had higher expression of F4/80 and CD11c versus Control mice.
3.3.3 Bone structure

MicroCT imaging of the joints (Figure 3-2A) showed that the ω-3 mice had a lower bone fraction (bone volume/total volume, BV/TV) of the femoral condyle as compared to the other diets (Figure 3-2B). Surgery, but not diet, decreased the BMD of the femoral condyle (Figure 3-2C). For the tibial epiphysis, ω-3 mice had lower BV and BMD versus the mice treated with the other diets. For all mice, surgery increased BV but decreased the BMD of the tibial epiphysis (Figure 3-2C,D). Diet affected the incidence of heterotopic ossification in the DMM-operated joint (Appendix B, Table B-3). The Control and ω-3 mice had a lower incidence of heterotopic ossification. The SFA and ω-6 mice had high BV of heterotopic ossification, while Control mice had the lowest (Figure 3-2E).

![Figure 3-2 Morphological bone changes and MicroCT analyses.](image)

(A) 3D reconstruction of MicroCT of limbs at 28 weeks of age. Right (non-operated) joint
from SFA mice showed intact bone structure (F = femur, T = tibia, P = patella; black arrows = partially calcified menisci). Left (DMM) joints of ω-6 and SFA mice had increased heterotopic ossification (white arrows) relative to the other groups. (B) Cancellous bone fraction (bone volume / total volume, BV/TV) for femoral condyle (FC), and cancellous and cortical BV/TV for tibial epiphysis (TP). ω-3 mice had significantly lower BV/TV in FC and TP as compared to the mice fed other diets. (C) Bone mineral density (BMD) of FC and TP. All the mice showed significantly decreased BMD after DMM surgery and the ω-3 mice also showed relatively low BMD to the mice fed other diets. (D and E) BV of TP and heterotopic ossification of DMM joints. All the mice had significantly increased BV in TP after DMM. ω-3 mice exhibited low heterotopic BV among the mice fed other high-fat diets. n = 11-14 mice/diet. * p < 0.05 versus corresponding right (non-operated) joints. Different letters are significantly different, p < 0.05, from each other. (B-D) Statistical significance was determined by two-way repeated measures ANOVA using right (non-operated) joints as the contralateral control. (E) The line inside the box represents the median of each diet group and the length of the box indicates the interquartile range. Statistical significance was determined by Kruskal-Wallis test with Mann-Whitney U test and Holm-Bonferroni correction. Except for (E), all data are presented as mean ± SEM.

3.3.4 ω-3 PUFAs mitigate injury-induced OA in obese mice

A modified Mankin grading scheme was used to determine OA severity. Severe cartilage loss was observed in the DMM-operated joints of SFA and ω-6 mice; the operated joints of Control and ω-3 mice showed surface fibrillation and moderate loss of proteoglycan (Figure 3-3A). Three out of fourteen ω-6 mice had severe subchondral bone erosions (Appendix B, Figure B-3). All operated joints had higher OA scores than their corresponding non-operated joints. The operated joints of SFA and ω-6 mice exhibited the most severe OA versus the operated joints of Control and ω-3 mice. The non-operated joints did not differ among diet groups.
Figure 3-3 Evaluation of OA, osteophyte, and synovitis. (A) Safranin-O (glycosaminoglycans) and fast green (bone and tendon) histology for the DMM-operated joint (F = femur, M = meniscus, T = tibia). Severe cartilage loss (yellow arrowheads) was found in ω-6 and SFA mice. The DMM-operated joints from Control and ω-3 mice had significantly lower OA scores compared to those from ω-6 mice and SFA mice. (B) Accumulative counts of osteophyte diseases-stages of the DMM-operated joint. The ω-6 and SFA mice had more mature osteophytes relative to Control and ω-3 mice. (C) OA severity was positively correlated with osteophyte disease stages. (D) H&E histology of the medial femoral condyle of DMM-operated joints (S = synovium). Thickened synovium from ω-6 and SFA mice with high density of infiltrated cells was observed (black arrows). The DMM-operated joints from ω-3 mice had significantly lower synovial inflammation than those from ω-6 mice. (E) F4/80+ macrophage IHC (red arrowheads) of each quadrant of the DMM-operated joint and its quantification (medial femoral condyle, MF; medial tibial plateau, MT; lateral femoral condyle, LF; lateral tibial plateau, LT). The ω-6 mice also exhibited high macrophage scores at medial side of the joint. Levels of serum (F) insulin, (G) leptin, (H) adiponectin, and (I) resistin at various time-points; (J) PGE2 and (K) the Active/Total TGF-β1 ratio were measured at 28 weeks of age. n = 11-14 mice/diet. * p < 0.05 for regression analysis. # p < 0.05, versus all the other diets. Different letters are significantly different, p < 0.05, from each other. (A and D) Statistical significance was determined by two-way repeated measures ANOVA using right (non-operated) joints as the contralateral control. (B) Statistical significance was determined by Kruskal-Wallis H Test, p = 0.07. (F-I) Statistical significance was determined by two-way repeated measures ANOVA using age and diets as factors, while (E, J and K)
statistical significance was determined by one-way ANOVA using diet as the factor. ANOVA was then followed by Tukey’s post-hoc test. All data are presented as mean ± SEM.

3.3.5 Osteophyte formation

Osteophytes were present primarily in the operated joints (Appendix B, Table B-4). SFA and ω-6 mice trended toward greater osteophyte severity than Control and ω-3 mice (Appendix B, Table B-5 and Figure 3-3B). Osteophyte score also correlated positively with OA (Figure 3-3C).

3.3.6 ω-3 PUFAs decrease synovitis in obese mice

Synovitis was determined by previously established grading scheme consisting of assessment of stromal cell density and lining layer thickness. (Diekman and Guilak, 2013) Compared to the non-operated joints, the DMM-operated joints of all diet groups had increased synovial lining hyperplasia (Figure 3-3D). Operated joints of SFA and ω-6 mice exhibited a thicker synovium with a higher number of infiltrating cells than those of mice fed Control or ω-3 diets. The joints of ω-3 mice had less synovitis versus those of ω-6 mice.

3.3.7 Macrophage distribution in synovial tissue

At 12 weeks post-surgery, macrophages were still present within the synovium of the operated joints from all groups (Figure 3-3E). However, for Control and ω-3 mice, macrophages appeared mostly in the synovial lining layer, while for SFA and ω-6 mice,
macrophages were either distributed throughout the synovial stroma or were contained in the follicle-like lymphocytic infiltrates. The ω-6 mice exhibited increased frequency of macrophages in the synovium (Appendix B, Table B-6). The ω-6 mice had the highest macrophage score in the medial side of the joint versus Control and ω-3 mice (Figure 3-3E).

3.3.8 Serum cytokines and mediators

The SFA and ω-6 mice had the highest insulin concentrations versus Control and ω-3 mice at 23 weeks of age. Although the insulin levels of all mice decreased at 28 weeks of age (potentially due to the time of serum collection on the day of euthanasia was different from that of other time-points), ω-6 mice still exhibited higher insulin concentrations than Control mice (Figure 3-4A). At 23 and 28 weeks of age, SFA and ω-6 mice had elevated leptin concentrations versus Control and ω-3 mice, while ω-3 mice exhibited higher adiponectin levels (Figure 3-4B,C). The ω-3 mice had lower levels of resistin (Figure 3-4D) but exhibited the highest concentrations of prostaglandin E2 (PGE2) relative to other mice (Figure 3-4E). While there were no differences in the active form of TGF-β1 among the mice, the ω-3 mice showed the highest latent TGF-β1 concentration (Appendix B, Figure B-4) but the lowest active-to-total TGF-β1 ratio (Figure 3-4F). To investigate the relationships between each cytokines and OA, we performed bivariate
models (Appendix B, Table B-7) and found leptin and resistin had a positive association with OA.

Figure 3-4 Serum cytokine analyses. Levels of serum (A) insulin, (B) leptin, (C) adiponectin, and (D) resistin at various time-points; (E) PGE2 and (F) the Active/Total TGF-β1 ratio were measured at 28 weeks of age. The ω-6 mice and SFA mice had higher levels of insulin and leptin in sera as compared to Control mice toward the end of study, while ω-3 mice had relatively lower levels of these two cytokines among the high-fat feeding mice, suggesting ω-6 and SFA mice might have insulin and leptin resistance. The ω-3 mice also had high levels of adiponectin and PGE2, but low levels of resistin and a lower Active/Total TGF-β1 ratio in comparison to the mice fed other diets. n = 11-14 mice/diet. # p < 0.05, versus all the other diets. Different letters are significantly different, p < 0.05, from each other at the same time-point. (A-D) Statistical significance was determined by two-way repeated measures ANOVA using age and diets as factors, while (E and F) statistical significance was determined by one-way ANOVA using diet as the factor. ANOVA was then followed by Fisher’s post-hoc test. All data are presented as mean ± SEM.
3.3.9 The relationships among mechanical factors, metabolic factors, and OA

A potential confounding variable in evaluating the links between metabolic factors and OA is the effect of body weight. To control for differences in body weight among the diet groups, we first examined weight-matched mice from each group. In weight-matched mice, SFA and \( \omega-6 \) mice still demonstrated significantly higher OA severity than the mice fed the other diets (Appendix B, Figure B-5). Multivariate models were then performed to further validate these associations (Table 3-1). Only diet and metabolic factors leptin, and resistin, but not body weight (AUC_{4-28} or AUC_{17-28}) were significantly associated with OA.

Table 3-1 Multivariate regression analyses for variables predicting OA severity

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Model 1</th>
<th>Model 2</th>
<th>Model 3</th>
<th>Model 4</th>
<th>Model 5</th>
<th>Model 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diet (^a)</td>
<td>0.60**</td>
<td>0.58**</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leptin</td>
<td></td>
<td>0.37**</td>
<td></td>
<td>0.31</td>
<td>((p = 0.08))</td>
<td></td>
</tr>
<tr>
<td>Resistin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.29*</td>
<td>0.29*</td>
</tr>
<tr>
<td>Biomechanical</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AUC(_{4-28})</td>
<td>-0.03</td>
<td></td>
<td>0.02</td>
<td>0.23</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AUC(_{17-28})</td>
<td></td>
<td>0.01</td>
<td>0.12</td>
<td>0.29*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whole model ((r^2))</td>
<td>0.34**</td>
<td>0.31**</td>
<td>0.15*</td>
<td>0.16*</td>
<td>0.14*</td>
<td>0.18*</td>
</tr>
</tbody>
</table>

\(^a\)Ordinal variable: Control and \( \omega-3 = 0 \), SFA and \( \omega-6 = 1 \)
\( \beta \) = standardized coefficient
\( p \) values less than 0.05 shown in bold. \(*p < 0.05, **p < 0.01\)

3.3.10 Behavioral activity

The data at 6 and 14 weeks of age were used to examine how diet affected changes in biomechanical and neurobehavioral functions of the mice. Results at 24
weeks of age were used to determine whether diets altered mouse activity levels after OA induction.

At 14 and 24 weeks of age, diet did not significantly influence voluntary activity or rotarod performance (Figure 3-5A,B). To determine the effect of high-fat feeding, we combined all high-fat diet groups together and compared to the low-fat diet group. Six to 14 week-old mice subjected to high-fat feeding had lower motor function but maintained similar spontaneous activities versus Control mice (Appendix B, Table B-8). With age, high-fat feeding decreased forelimb grip strength, but had no effect on hind-limb grip strength. (Figure 3-5C,D). Rotarod performance was positively associated with forelimb grip strength, indicating that musculoskeletal strength is related to motor function (Appendix B, Figure B-6) (Griffin et al., 2010).

The effect of diet on thermal hyperalgesia was evaluated using the hot plate and tail-flick tests, which investigate nociceptive reflexes that are associated with supraspinal and spinal pathways, respectively (Figure 3-5E,F) (Langerman et al., 1995). There was no significant difference in the hot plate latency among the mice at any time-points. However, after 10 weeks of feeding, ω-3 and SFA mice had decreased tail-flick latency versus Control mice. At 24 weeks, a significant difference in tail-flick latency was observed between ω-6 and SFA mice, but not between other diet groups. High-fat feeding decreased tail-flick latency in the period from 6 to 14 weeks of age.
Using bivariate models, we examined whether activity levels correlated with weight gain or OA (Table 3-2). Spontaneous locomotion did not correlate with either weight gain or OA, while rotarod latency was negatively associated with weight gain but not with OA. Grip strength was negatively associated with weight gain, but only forelimb grip strength was negatively associated with OA. Neither hot plate nor tail-flick latency correlated with either weight gain or OA.

Figure 3-5 Mouse behavioral measurements. Diets supplemented with different types of FAs did not significantly affect (A) spontaneous locomotion activity, (B) rotarod performance, (C) forelimb and (D) hind-limb grip strength prior to and post-surgery. For nociception, diet significantly influenced (E) the tail flick but not (F) the hot plate latency. However, if mice fed the 60% kcal high-fat diets were pooled together and were compared to the mice fed the 10% kcal Control low-fat diet, high-fat feeding significantly decreased rotarod performance and forelimb grip strength (main effect, p < 0.05). Nonetheless, no effects on spontaneous locomotion activity were observed. n = 11-14 mice/diet. * p < 0.05, versus ω-6 mice. # p < 0.05, high-fat feeding versus age-matched low-fat feeding. For 6 and 14 weeks of age, statistical significance was determined by two-way repeated measures ANOVA using age and diet as factors. For 24 weeks of age, statistical significance was determined by one-
way ANOVA using diet as factor. ANOVA was then followed by Fisher’s post-hoc test. All data are presented as mean ± SEM.

Table 3-2 Bivariate regression analysis for variables predicting biomechanical and neurobehavioral activity#

<table>
<thead>
<tr>
<th>Predictor variables</th>
<th>Weight Gain&lt;sup&gt;a&lt;/sup&gt;</th>
<th>OA Severity</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>r</td>
<td>r</td>
</tr>
<tr>
<td><strong>Biomechanical</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spontaneous locomotion (cm)</td>
<td>-0.25</td>
<td>-0.02</td>
</tr>
<tr>
<td>Rotarod latency to fall (s)</td>
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<td>0.16</td>
</tr>
<tr>
<td>Forelimb grip strength</td>
<td><strong>-0.79</strong></td>
<td><strong>-0.29</strong></td>
</tr>
<tr>
<td>Hindlimb grip strength</td>
<td><strong>-0.30</strong></td>
<td>0.18</td>
</tr>
<tr>
<td><strong>Neurobehavioral</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tail-flick withdrawal latency (s)</td>
<td>0.15</td>
<td>0.05</td>
</tr>
<tr>
<td>Hotplate withdrawal latency (s)</td>
<td>0.27</td>
<td>0.12</td>
</tr>
</tbody>
</table>

# Activity measurements at 24 weeks of age  
<sup>a</sup> Weight gain from 4 weeks to 24 weeks of age  
P values less than 0.05 shown in bold. *p < 0.05, **p < 0.001

3.3.11 ω-3 PUFAs accelerate wound repair in obese mice.

ω-3 mice had significantly improved regeneration (Figure 3-6A,B), characterized by increased epithelial thickness (Figure 3-6C,D). Three out of eleven ω-3 mice had complete epithelial fusion of the proximal and distal wound margins (Appendix B, Figure B-7). All the mice demonstrated some features of regeneration including re-epithelialization and formation of sebaceous glands (Figure 3-6E). We next evaluated
whether the cells in the wound margins were in a proliferative stage using Ki-67 marker; however, no obvious differences were observed (Figure 3-6F). Picosirius red staining was used to investigate the wound matrix composition. The ω-3 mice showed less collagen type I (COLI) deposition, while the other mice had densely packed COLI fibers (Figure 3-6G). Ear wound size showed a trend toward positive association with OA in the 1.5-mm model (Figure 3-6H).

Figure 3-6 Histology of ear wound. The ω-3 mice demonstrated enhanced ear wound healing capacity. (A) Representative images of ear hole (white arrows) at 12 weeks post-wounding. (B) The ω-3 mice had the smallest wound area as compared to the mice fed other diets. (C) H&E stained images showed that ω-3 mice had a thickened epithelium (yellow arrowheads). Black arrows indicated the wound edge of each sample. (D) ω-3 mice had significantly thicker epithelia as compared to other
mice. (E) Masson’s Trichrome indicated that all mice exhibited healing features such as regeneration of sebaceous glands (yellow arrowheads) and new cartilage islands (green arrowheads); however, only the ω-3 mice had several new cartilage condensations. (F) No difference in cell proliferation marker (Ki-67) among the diets was observed. (G) Picrosirius red staining indicated that the ω-3 mice had less deposition of collagen type I fibers in the wound area, suggesting less scar formation. (H) Wound healing capacity exhibited a trend towards a negative relationship with OA severity in the more severe ear wounding (1.5-mm punch) model, but not in the less severe (1.0-mm punch) model. n = 11-14 mice/diet. (A) The scale bar = 5 mm, and for others images the scale bar = 100 μm. (B) Statistical significance was determined by two-way repeated measures ANOVA. * p < 0.05, versus all the other groups. (D) Statistical significance was determined by one-way ANOVA. * p < 0.05 and # p < 0.01, versus all the other groups. All data are presented as mean ± SEM.

3.4 Discussion

The findings of this study showed that a small amount of ω-3 PUFA supplementation was sufficient to mitigate the effects of obesity on injury-induced OA and to accelerate would repair. Conversely, SFA and ω-6 PUFA independently acted as a detrimental factor in OA following joint injury, increasing osteophyte formation, heterotopic ossification, synovitis as well as increasing infiltration of macrophages into synovial tissue. By examining multivariate models and weight-matched mice from different diet groups, we found that injury-induced OA was only associated with dietary content and serum levels of pro-inflammatory adipokines, but not with body weight or activity levels. Our results indicate that dietary and metabolic factors may play a more significant role than body weight in the link between obesity and post-traumatic OA.
To investigate the specific effects of SFAs on OA, we maintained the same \( \omega-6 \) to \( \omega-3 \) PUFA ratio in the low-fat and SFA rich high-fat diet. We found that SFAs significantly exacerbated OA as compared to a low-fat diet with the same PUFA ratio. This result is consistent with several animal studies showing that a high-fat diet rich in SFA increases the severity of injury-induced arthritis (Louer et al., 2012; Mooney et al., 2011). Although the specific effect of SFAs on chondrocytes is less well characterized, SFAs can activate synovial macrophages to secrete IL-1 and TNF-\( \alpha \) that are involved in cartilage degradation (Shi et al., 2006). The Western diet is characterized by a high ratio of \( \omega-6 \) to \( \omega-3 \) PUFAs (Simopoulos, 2002). While maintaining the same PUFA content but altering \( \omega-6 \) and \( \omega-3 \) ratio in PUFA rich high-fat diets, we found that the \( \omega-6 \) mice developed severe OA and synovitis with elevated systemic inflammation. In contrast to \( \omega-6 \) PUFAs, the beneficial effect of \( \omega-3 \) PUFAs in OA and rheumatoid arthritis has been reported in animal models (Huang et al., 2013; Knott et al., 2011). However, most of these studies supplemented \( \omega-3 \) PUFAs in regular chow (i.e., low-fat diet) and thus the investigation of \( \omega-3 \) PUFAs in the context of a high-fat diet is a novel aspect of this study. Here we discovered that even a relatively small amount of supplementation (only 8% kcal of the energy provided), \( \omega-3 \) PUFAs provided protective effects on osteoarthritic changes of the joint after injury, while reducing leptin and resistin levels. In addition, \( \omega-3 \) mice had high levels of adiponectin (Neschen et al., 2006). The influence of adiponectin on chondrocytes is not fully understood, and some evidence suggests that adiponectin is
associated with cartilage matrix breakdown (Koskinen et al., 2011). Nevertheless, adiponectin may indirectly benefit cartilage by reducing inflammation through polarizing macrophages toward anti-inflammatory phenotypes (Ohashi et al., 2010).

Despite having relatively higher AUC values than Controls, obese mice supplemented with ω-3 PUFAs showed similar OA scores as Control mice, suggesting that factors other than body weight are responsible for OA severity in this model. In examining weight-matched mice from different diet groups, or using multivariate models, we showed that injury-induced OA was significantly associated with diet and pro-inflammatory adipokines, but it was not with body mass. These results emphasize the potential significance that systemic metabolic factors may play in exacerbating injury-induced OA.

Another significant finding was that ω-3 obese mice showed superior wound healing capacity. However, the cell proliferation marker did not differ among the diet groups, likely due to the fact that the skin wound of rodents enters the maturation phase 14 days post-injury (Sabol et al., 2012) with reduced Ki-67 expression (Seifert et al., 2012). The ω-3 mice also contained low levels of COLI fibers in the wounding area, suggesting less scar formation. In addition, adiponectin has been shown to accelerate wound repair by promoting keratinocyte proliferation (Salathia et al., 2013; Shibata et al., 2012a). Furthermore, low ratio of active/total TGF-β1 in ω-3 mice may further prevent them
from developing scar tissue because active TGF-β1 is involved in excessive matrix deposition (Penn et al., 2012).

Interestingly, we observed that the healing capacity of the ear wound tended to be negatively associated with OA. These findings are consistent with a recent study demonstrated that ear wound closure and cartilage regeneration may share a common heritable genetic basis that is associated with OA severity (Rai et al., 2012). Our findings suggest that the effects of diet may similarly reflect associations between wound healing and OA via epigenetic changes, potentially in the body’s stem cell populations (Wu et al., 2012).

The ω-3 mice exhibited a low incidence of mature osteophytes, potentially due to their low active/total TGF-β1 ratio systemically, as TGF-β1 is potent inducer of osteophyte (Bakker et al., 2001). Furthermore, ω-3 mice showed lower levels of heterotopic ossification, which is associated with surgical trauma during DMM. Tendon mineralization has been reported in patients with tendon rupture, and could be a cause of chronic pain (Camillieri et al., 2013). Studies have indicated that PGE₂, a lipid derivative from ω-6 PUFAs, enhances osteogenesis of tendon stem cells, providing a potential explanation for the greater heterotopic ossification in ω-6 mice. As anticipated, PGE₂ concentrations were relatively higher in the ω-6 mice than those in Control mice, although not statistically significant. To our surprise, ω-3 mice had the highest PGE₂ levels. The reason for this phenomenon remains unclear but may be related to their high
adiponectin levels, as a recent study reported that adiponectin stimulates PGE₂ production in cells in a dose-dependent manner (Kusunoki et al., 2010).

Although the whole body BMD did not differ among the obese mice, ω-3 mice had lower BMD and BV in the epiphyseal region of the both limbs. Increased subchondral bone density is a hallmark of OA, and an inverse relationship between osteoporosis and OA has been observed in humans (Zupan et al., 2013). Our observations of lower BMD, BV, and less heterotopic ossification in the joints of the ω-3 mice do not support the protective role of ω-3 PUFAs on bone measures in post-traumatic OA, (Reinwald et al., 2004) but are consistent with a recent study demonstrating that mice with up-regulated adiponectin expression had decreased osteocalcin levels and displayed low-bone-mass phenotypes (Kajimura et al., 2013). Nevertheless, further studies are required to determine the long-term effects of ω-3 PUFAs on bone metabolism in obese mice.

The fact that high-fat feeding did not alter spontaneous locomotor activity is consistent with our prior obese animal studies and those of others, (Griffin et al., 2010; Griffin et al., 2012; Yamada-Goto et al., 2012) suggesting that weight gain is not associated with lower voluntary activity or energy expenditure. In humans, a study conducted with lean and obese individuals indicates similar levels of spontaneous physical activity (Schutz et al., 1982). Furthermore, our result that spontaneous locomotion was independent of injury-induced OA is in agreement with recent findings...
using cruciate ligament transection OA model in mice (Ruan et al., 2013). It has also been reported that recreational activities do not contribute to OA in normal and overweight individuals (Ageberg et al., 2012; Felson et al., 2007).

Our results indicate that dietary FAs differentially influence the development of injury-induced OA, contributing a more critical role in osteoarthritic changes of the joint than does mechanical factor in obesity. The progress of OA and wound repair could be explained by regulation of obesity-associated inflammation (Figure 3-7). Our findings have significant implications on the mechanisms of injury-induced OA and wound healing, and provide a path toward clinical studies of dietary FA supplements to modify the course of OA.

Figure 3-7 The links among dietary FAs, OA and wound healing. Cytokine levels in the mice fed different diets and their potential effects on various disease conditions. The oval size corresponds to the concentrations of cytokines or the degree of disease/healing conditions. The solid line indicates a strong stimulus effect, while the dashed line represents weak induction. It is important to note that SFA and ω-6...
mice still had different cytokine levels and disease scores. However, since these differences were minor, the mice fed the SFA and ω6 high-fat diets were grouped together in this diagram. OA: osteoarthritis; HO: heterotopic ossification.

3.5 Summary

We show that dietary fatty acids (FAs) play a more significant role in obesity-induced osteoarthritis than weight gain. Saturated FAs and ω-6 FAs act as a detrimental factor in pathogenesis of osteoarthritis, increasing cartilage degradation, osteophytosis, heterotopic ossification, and synovitis, while ω-3 FAs protected obese mice from joint degeneration and enhanced their wound healing capacity. Our findings have significant implications on the mechanisms of osteoarthritis and wound healing, and provide a path toward clinical studies of dietary fatty acid supplements to modify the course of osteoarthritis.
4. The effect of conditional macrophage depletion on development of injury-induced knee OA in obese transgenic MAFIA mice

4.1 Introduction

Macrophages, one of the main phagocytes, are an integrative part of innate immune system. Currently, at least two categories of macrophages have been identified: M1 and M2 macrophages, although it is now believed that there is a spectrum of macrophage phenotypes. M1 macrophages, also called classically activated macrophages, express high levels of the M1 genes including inducible NO synthase (iNOS) and secrete pro-inflammatory cytokines such as TNF-α and IL-1 (Li et al., 2010), while M2 macrophages, i.e. alternatively activated macrophages, express CD206 and are responsible in releasing anti-inflammatory cytokines such as IL-10 (Aron-Wisnewsky et al., 2009).

In addition to defense the host against pathogens via their phagocytic ability, macrophages play a central role in metabolic homeostasis by regulating insulin sensitivity. Indeed, it has been proposed that during infection, pro-inflammatory cytokines secreted from M1 macrophages help tissue develop transient insulin resistance, conserving most glucose for immune cells in order to combat microbes (Wynn et al., 2013). However, as this mechanism is an important adaptive trait for mammals, it could be detrimental to surrounding host tissues if macrophages stay in the inflammatory phase for a prolonged time than necessary. For example, excess energy...
intake, such diet-induced obesity, often leads to increased cell stress, lipolysis, and apoptosis of adipocytes in fat tissue. FAs released from the adipocytes along with FA from the diet lead to phenotype switch of macrophage toward M1 pro-inflammatory pathway. However, TNF-α secreted by M1 macrophages further induces insulin resistance and lipolysis of adipocytes. Such interactions between adipocytes and M1 macrophages constitutes a vicious cycle, perpetuating chronic systemic inflammation and eventually leading to metabolic diseases in obese individuals(Suganami and Ogawa, 2010). A recent animal study has shown that conditional depletion of macrophages using clodronate improves metabolic parameters such as insulin resistance in obese mice, indicating the role of macrophage in metabolic syndrome(Feng et al., 2011).

In fact, macrophages are not only involved in developing obesity-associated inflammation but may also affect development of osteoarthritis (OA) following joint injury. Macrophages resident in joint synovium secrete TGF-β that enhances osteophytosis and proliferation of fibroblasts in experimental OA models(Blom et al., 2004). In our previous studies, we have demonstrated that mice with joint fracture had increased macrophage infiltration into synovium compared to non-fractured joint(Lewis et al., 2013). Furthermore, obese mice fed high-fat diet rich in pro-inflammatory FA such as SFA and ω-6 FAs showed significantly more macrophages in the joint after meniscal injury versus obese mice fed high-fat diet rich in anti-inflammatory FAs(Wu et al., 2014).
Recently, it has been showed that systemic removal of macrophages using clodronate also decreases joint swelling in a collagen-induced arthritis model (Li et al., 2012). Although these animal studies have greatly enhanced our understanding of the significance of macrophage in OA progress, most of them utilized lean animal models, i.e. animals that received standard low-fat diet (Blom et al., 2007). Therefore, little is known whether macrophage in the joint of obese individuals have a similar catabolic role in OA as they do in lean people. Furthermore, it is also unclear whether transient, systemic depletion of macrophage immediate after joint injury is sufficient to enhance metabolic profile and mitigate OA in obesity. Thus, in the present study, we aim to elucidate functionality of macrophages in regulating the effect of obesity on injury-induced OA. We hypothesize that short-term, systemically conditional ablation of macrophages would promote metabolic profiles and mitigate OA degeneration following injury in obese mice.

4.2 Materials and Method

4.2.1 Animals

All procedures were approved by the Duke University IACUC. Macrophage Fas-Induced Apoptosis (MAFIA) transgenic mice (strain name: C57BL/6-Tg (Csf1r-EGFP-NGFR/FKBP1A/TNFRSF6) 2Bck/J) were purchased from Jackson Lab and used in this study. Macrophages in MAFIA mice express enhanced green fluorescent protein (eGFP), and can be driven into an apoptotic pathway through the administration of a non-toxic.
reagent (AP20187, Clontech) according to previously established protocols (Burnett et al., 2004).

Beginning at 4 weeks of age, all mice were fed a custom-designed high-fat diet rich in SFA and ω-6 PUFAs (ω-6/ω-3 ratio = 20.3) (Research Diet, D11120107). The mice were weighed weekly. The study design is presented in Figure 4-1.

Figure 4-1 Experimental design. All the mice started a high-fat diet at 4 weeks of age and underwent DMM surgery at 13 weeks of age. The mice then received either macrophage depletion or vehicle control solution at 13 weeks and 14 weeks of age (one treatment per week). To evaluate the effect of macrophage depletion on acute joint inflammation, five mice were sacrificed at 14 weeks of age immediate after 2nd macrophage depletion (this time point refers to 0 week post-depletion). To investigate the effect of macrophage depletion on OA severity, twelve mice were sacrificed at 23 weeks of age (this time point refers to 9 weeks post-depletion).
4.2.2 Measurements of body fat composition and whole body bone mineral density

The body fat content and bone mineral density of the mice, excluding the head, were measured at 9 and 23 weeks of age using a dual-energy x-ray absorptiometry (DEXA) scanner (GE Lunar PIXImus, GE Healthcare, Madison, WI, USA).

4.2.3 Induction of OA and macrophage depletion

At 13 weeks of age, mice underwent surgery to destabilize the medial meniscus (DMM) to induce knee OA in the left hind limb as previously described (Glasson et al., 2007). Briefly, anesthetized mice were placed on a custom-designed device, which positioned their hind-limbs in 90-degree flexion. The medial side of the joint capsule was opened and the medial meniscotibial ligament, that attaches the medial meniscus to the tibial plateau, was transected with a #11 scapel blade. Bleeding due to the incision was controlled by absorption spears soaked with epinephrine 1:1000 (Neogen, Lexington, KY, USA). The joint capsule and subcutaneous layer of the skin were closed with a continuous 8-0 suture with taper point (Polysorb™, Covidien, Mansfield, MA, USA). Immediately after surgery, mice were treated via a retro-orbital route with either AP20187 injection solution (Clontech) consisting of 4% ethanol, 10% PEG-400, and 1.7% Tween-20 in water (dose: 10 mg per kg mouse body weight) to systemically deplete macrophages or vehicle solution as previously described (Burnett et al., 2004). At one week post-surgery, mice received a second depletion or vehicle as appropriate. To determine the effect of macrophage depletion on acute joint inflammation, mice (n =
5/group) were sacrificed the day after the second depletion (this time point refers to 0 week post-depletion). To evaluate the effects of macrophage depletion on OA development, mice (n = 12/group) were sacrificed at 23 weeks of age (this time point refers to 9 weeks post-depletion).

4.2.4 Quantification of macrophage percentage in various tissues via flow cytometry analysis

Upon sacrifice, spleen, visceral adipose tissue (epididymal fat), and both right and left joint capsules were harvested from the mice. Weight of the spleen and visceral fat were recorded. Tissues were then minced and digested at 37 °C with 0.2% collagenase type I (Worthington) for 1-1.5 hours. Tissue debris was removed by filtering digested solution through 70 µm and 40 µm filters sequentially. Cells were then collected, counted and suspended in flow cytometry sorting buffer (PBS + 1% P/B/S). Macrophage percentage within the tissues was determined by quantifying eGFP+ cells using flow cytometry.

4.2.5 Evaluation of OA severity

To investigate the effect of macrophage depletion on OA development, mice were euthanized at 23 weeks of age. Knee OA severity was determined as previously described (Louer et al., 2012). Briefly, both left and right hind-limbs were harvested and fixed in 10% buffered formalin. Limbs were then decalcified in Cal-Ex solution (Fisher Scientific, Pittsburgh, PA, USA), dehydrated and embedded in paraffin. The joint was sectioned in the coronal plane at a thickness of 8 µm. Sections were stained with
hematoxylin, fast green, and Safranin-O. Three independent, blinded graders then assessed sections for degenerative changes of joints using a modified Mankin scoring system. Scores were averaged between graders for individual joint quadrants as well as for the whole joint, resulting in scores between 0 and 30 for each quadrant (medial femoral condyle, medial tibial plateau, lateral femoral condyle, and lateral tibial plateau).

4.2.6 Evaluation of osteophyte formation

To access the severity of osteophyte pathology, a semiquantitative grading scale was created based on a review by van der Kraan and co-workers with slight modification (van der Kraan and van den Berg, 2007). Osteophytes in the joint were graded under high-power field (×200 magnification): 0 point = normal periosteal surface, 1 point = early stage (cells in the periosteum and synovial lining layer starting to proliferate), 2 points = middle stage (cells in the periosteum and synovial lining layer showing massive proliferation and hypertrophic chondrocytes can be observed), 3 points = mature stage (osteophyte integrated with the subchondral bone with the presence of bone marrow cavities.

4.2.7 Evaluation of knee synovitis

Joint sections were stained with hematoxylin and eosin (H&E) stain to reveal infiltrated cells and synovial structure. Three blinded, independent graders assessed sections for synovial lining thickness (0-3 points) and synovial stroma density (0-3
points) in each joint quadrant using a previously described scoring scale (Louver et al., 2012). Scores were averaged between graders for individual quadrants as well as for the whole joint, resulting in a maximum score of 24 per joint.

4.2.8 Evaluation of bone tomography

Bone tomography was analyzed as previously described (Louver et al., 2012). Briefly, both hind-limbs were scanned by a micro-computed tomography system (Skyscan 1176, Bruker). A hydroxyapatite calibration phantom was used to calibrate bone density values (mg/cm³). Morphometric bone parameters were investigated in the distal femoral condyles, the tibial epiphysis immediately distal to the subchondral bone, the tibial metaphysis, and heterotrophic bone fragments in the joints. Parameters reported for the femoral condyles were bone mineral density (BMD; mg/cm³) and cancellous bone fraction (bone volume/total volume, BV/TV, excluding the cortex), while parameters reported for the tibial epiphysis and metaphysis were BMD and BV (mm³).

Unless otherwise indicated, to determine how treatment, DMM surgery, and their interaction (surgery x treatment) affected bone remodeling, bone parameters from non-operated (right) and DMM-operated (left) joints were analyzed by two-factor repeated measures ANOVA.

4.2.9 Immunohistochemical staining against immune cells in the operated joints

DMM-operated joints from the each group were immunohistochemically stained (IHC) for murine macrophages (F4/80, AbD Serotec), M1 macrophages (iNOS, inducible
nitric oxide synthase, ab68672, abcam,) and M2 macrophages (CD206, ab64693, abcam),
neutrophils (neutrophil elastase, ab21595, abcam), T cells (CD3, a pan-T cell marker, 
ab1669, abcam), and B cells (CD22, a pan-B cell marker, ab65852, abcam). Joint sections
were pretreated by either a citric-based buffer (pH 10; Vector Labs, Burlingame, CA,
USA) at 95°C for 20 min or 0.01% Proteinase K (Sigma-Aldrich, St. Louis, MO, USA) at
37°C for antigen retrieval as appropriate. Endogenous peroxidase was then quenched
with 3% H$_2$O$_2$ for 1 hour. Next, either normal goat serum or normal horse serum (Vector
Labs) was applied to the sections to reduce nonspecific binding as appropriate. Sections
were then stained with either primary antibody or appropriate serum control, followed
by micro-polymer kit incubation (ImmPRESS Anto-Rat IgG Reagent; Vector Labs).
Chromogenic detection was performed with the DAB peroxidase substrate kit (Vector
Labs). Hematoxylin was used to identify cell nuclei. Immune cells stained joint
quadrants were evaluated by 2 independent, blinded graders using a semiquantitative
grading scheme based on counting per high-power field ($\times$200 magnification): Scores
were averaged between graders for the whole joint.

**4.2.10 Serum and synovial fluid cytokine analyses**

Mouse serum and synovial fluid were collected at time of sacrifice according to
previously established protocol (Seifer et al., 2008). Concentrations of both leptin and
insulin in serum samples collected over the course of the study were measured using the
Mouse Metabolic Assay (#K15124C; MSD, Gaithersburg, MD, USA) as a multiplexed
sandwich ELISA. Samples were run without dilution as suggested by the manufacturer. The lower limit of detection for each of the analytes was 43 pg/ml for leptin and 15 pg/ml for insulin. The intra-assay coefficients of variation (CVs) were 4.3% for leptin and 5.4% for insulin.

Two different sandwich ELISAs were employed for the determination of Free Active TGF-β1 (#437707; BioLegend, San Diego, CA, USA) and Total TGF-β1 (#436707; BioLegend) in serum. Samples were run undiluted for Free Active TGF-β1 and diluted 1000-fold for Total TGF-β1 as recommended by the manufacturer. The minimal detectable concentration of Free Active TGF-β1 was 2.3 pg/ml and 3.5 pg/ml for Total TGF-β1. The intra-assay CVs was 3.8%, for Free Active TGF-β1 and 2.3% for Total TGF-β1.

Concentrations of IL-27 in serum samples were measured using the Mouse IL-27 ELISA kit (#M2728; R&D). The minimal detectable concentration of Free Active TGF-β1 was 1.5 pg/ml. The intra-assay CVs was 2.4%, for IL-27.

For synovial fluid samples, Mouse Proinflammatory Panel 1 (V-plex K15048D; MSD, Gaithersburg, MD, USA), a multiplexed sandwich ELISA, was employed. Mouse Proinflammatory Panel 1 is capable of measuring IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL12p70, and TNF-α. The minimal detectable concentration of IFN-γ was 0.037 pg/ml, IL-1β was 0.125 pg/ml, IL-2 was 0.492 pg/ml, IL-4 was 0.139 pg/ml, IL-5 was 0.087 pg/ml, IL-8 was 0.271 pg/ml, IL-10 was 0.619 pg/ml, IL-12p70 was 14.6 pg/ml, and TNF-α
was 0.194 pg/ml. The intra-assay CVs was 4.7% for IFN-γ, 4.5% for IL-1β, 8.7% for IL-2, 6.0% for IL-4, 6.2% IL-5, 5.1% for IL-6, 4.2% for IL-8, 7.2% for IL-10, 3.9% for IL-12p70, and 2.7% for TNF-α. An IL-17 ELISA kit (MSD Cat#K152ATC-1) was used to evaluate the concentration of IL-17 in the synovial. The minimal detectable concentration of IL-17 was 1.66 pg/ml and the intra-assay CVs was 2.4%.

4.3 Results

4.3.1 Macrophage depletion significantly decreased body weight but increased spleen weight at the time of ablation

Upon macrophage depletion, obese MAFIA mice significantly lost their weight as compared to the control non-depleted mice (Figure 4-2A and B). However, the depleted mice gradually recovered to a similar weight as controls in 4 weeks after the depletion treatment was stopped. Furthermore, macrophage depletion also trended toward decreasing percentage body epididymal fat but significantly increased percent spleen weight at the time of depletion (Figure 4-2 C and D). There was no significant difference in percentage of body fat and body bone mineral density between depleted and non-depleted mice at 9 weeks and 23 weeks of age (Appendix C, Figure C-1).
Figure 4-2 Body weight, and percent body epididymal fat and spleen. (A) mouse body weight. Macrophage depletion significantly decreased body weight of the depleted mice at the time of depletion (black arrows); however, the depleted mice gradually recovered their weight once depletion treatment was stopped. Macrophage depletion also trended toward decreasing (C) percent body epididymal fat, but significantly increased (D) percent spleen weight in the depleted mice at 0 weeks post-depletion. n = 4-12 mice/group. * p < 0.05 compared to corresponding control group. (A and B) Statistical significance was determined by one-way repeated measures ANOVA. (C and D) Statistical significance was determined by student’s t-test within each time point. All data are presented as mean ± SEM.
4.3.2 Macrophage depletion significantly decreased macrophages in visceral fat and DMM-operated joint at the time of ablation

To evaluate whether macrophages were systemically depleted in our depletion scheme after AP20187 administration, various tissues including spleen, visceral adipose tissue, right non-operated and left DMM-operated knee joint capsules were harvested. Flow cytometry analysis for eGFP$^+$ cells (macrophages) demonstrated that our depletion strategy significantly decreased the amount macrophages in epididymal fat and DMM-operated joint capsules, but not in spleen, at the time of the depletion (Figure 4-3 and Figure 4-4A). At 9 weeks post-depletion, however, percentage of eGFP$^+$ cells in all tissues analyzed was comparable in the control and depleted mice. F4/80 IHC further confirmed that less macrophage existed in the DMM-operated (left) joints relative to the non-operated (right) joints in non-depleted mice (Figure 4-4B).
Figure 4-3 Flow cytometry analysis for percent eGFP+ cells (macrophages) in (A) spleen and (B) epididymal fat isolated from the control and macrophage-depleted mice. Our depletion scheme significantly ablated macrophages in the visceral fat but not spleen in the mice at time of depletion treatment. All mice had similar macrophage percentage in the spleen and visceral fat at 9 weeks post-depletion. n = 5-12 mice/group. Different letters are significantly different, p < 0.05, from each other. Statistical significance was determined by two-way ANOVA followed by Fisher’s post-hoc analysis. All data are presented as mean ± SEM.
Figure 4-4 Joint flow analyses and F4/80 IHC. (A) Flow cytometry analysis for percent eGFP+ cells (macrophages) in DMM-operated (left) and non-operated (right) hind limbs isolated from the control and macrophage-depleted mice. Depleted mice had significantly lower eGFP+ cells in the DMM joint as compared to the control mice immediately following depletion. At 9 weeks post-depletion, although the DMM joints had significantly more percentage of eGFP+ cells than non-operated joints from all mice, no significant difference in eGFP+ cells was observed in the DMM joints of the control and depleted mice. (B) F4/80 IHC against macrophages in DMM-operated joints and its quantification. Consistent to our flow analysis, DMM joints from the depleted mice had significantly lower macrophage numbers than those from the control mice. n = 5-12 mice/group. Different letters are significantly different, p < 0.05,
from each other. Statistical significance was determined by two-way ANOVA followed by Fisher’s post-hoc analysis. All data are presented as mean ± SEM.

4.3.3 Macrophage depletion altered bone parameters at the time of ablation

MicroCT imaging of the joints showed that macrophage depleted mice had less osteophytosis at the time of depletion treatment but showed similar degree of osteophyte score at 9 weeks post-depletion (Figure 4-5). Furthermore, macrophage-depleted mice had a lower BMD and bone fraction (bone volume/total volume, BV/TV) of the femoral condyle and tibia epiphysis as compared to the control mice. There was no significant difference in BMD and BV/TV between the control and depleted mice at 9 weeks post-depletion, although a trend toward lower BMD of femoral condyle was observed in the depleted mice.
Figure 4-5 Morphological bone changes and MicroCT analyses. (A) 3D reconstruction of MicroCT of left (DMM-operated) hind-limbs of the control and macrophage depleted mice at 0 week and 9 week post-depletion (white arrows indicate osteophytes). (B) Osteophyte score of the DMM-operated joints. Mice with
macrophage depletion had significantly lower osteophyte score than the control mice at 0 week of post-depletion. Macrophage depletion significantly decreased (C) bone mineral density and (D) cancellous bone fraction (bone volume / total volume, BV/TV) of femoral condyle and tibial epiphysis of the DMM-operated joints at the time of depletion. n = 5-12 mice/group. Different letters are significantly different, p < 0.05, from each other. Statistical significance was determined by two-way repeated measures ANOVA using right (non-operated) joints as the contralateral control. All data are presented as mean ± SEM.

4.3.4 Macrophage depletion did not prevent OA; instead, it increased synovitis in obese mice following DMM surgery

The DMM-operated joints of all mice had significantly higher OA score compared to their own contralateral non-operated joints at 9 weeks of depletion. However, macrophage depletion in obese mice did not mitigate OA severity at any time points studied (Figure 4-6A). At both 0 week and 9 week post-depletion, the DMM-operated joints of all mice had more severe synovial inflammation relative to their own contralateral non-operated joints (Figure 4-6B). Surprisingly, we observed that macrophage-depleted mice had significantly higher synovitis in the DMM-operated joints than the control mice at 9 weeks post-depletion. Given that the number of macrophages was lower in the synovium of the depleted DMM joint (see Figure 4-4), this result implies a potential protective role for macrophages, and that cells other than macrophages may infiltrate into DMM operated-joints, leading to more severe synovitis.
Figure 4-6 Evaluation of OA and synovitis. (A) Safranin-O (glycosaminoglycans) and fast green (bone and tendon) histology for the DMM-operated joint at 0 week and 9 weeks post-depletion. Severe cartilage loss was found in the control and macrophage-depleted mice at 9 weeks post-depletion. The DMM-operated joints of the macrophage-depleted mice were not significantly different from those of the control mice at 9 weeks of post-depletion. (B) H&E histology of the medial femoral condyle of the DMM-operated joints. Yellow arrowheads indicate massively infiltrated cells. The DMM-operated joints from macrophage-depleted mice had significantly higher synovitis score than those from the control mice at 9 weeks post-depletion. n = 5-12 mice/group. * p < 0.05 for the joints within each group. Different letters are significantly different, p < 0.05, from each other. Statistical significance was determined by two-way repeated measures ANOVA using right (non-operated) joints as the contralateral control. All data are presented as mean ± SEM.
4.3.5 CD3⁺ T cells and neutrophils are significantly increased after macrophage depletion

To identify the potential cells that were responsible for the increased synovitis in the DMM joint of the depleted mice, antibodies against various immune cells were applied to the joint sections. We observed that the control mice trended toward having more iNOS⁺ cells (commonly used as a marker for M1 macrophages) in the DMM joints as compared to the depleted mice at 0 week post-depletion. However, macrophage-depleted mice exhibited significantly less CD206⁺ cells (commonly used as a marker for M2 macrophages) in the DMM joints versus the control mice at 9 weeks post-depletion. Interestingly, we found that the mice with macrophage depletion showed significantly increased infiltration of CD3⁺ T cells (greater than 5-fold) at both time points studied. DMM joints of depleted mice also exhibited more neutrophils (approximate 8-fold increase) relative to those of the control mice immediately following macrophage depletion. Furthermore, no difference in CD22⁺ B cells in the DMM joints between the control and depleted mice was observed at any time points studied.
Figure 4-7 IHC of iNOS+ and CD206+ cells in the joints. (A) Representative IHC images of iNOS+ cells and CD206+ cells in the synovium at the femoral condyle of the DMM-operated joints. Red arrowheads indicate cells that were positive for staining. (B) Quantification of iNOS+ cells and CD206+ cells in the synovium of the entire DMM joints. Macrophage-depleted mice trended having less iNOS+ cells immediately after ablation. At 9 weeks post-depletion, depleted mice also had significantly less CD206+ cells in the DMM joint versus the control mice. n = 4-10 mice/group. Different letters are significantly different, p < 0.05, from each other. Statistical significance was determined by two-way ANOVA followed by Fisher’s post-hoc test. All data are presented as mean ± SEM.
Figure 4-8 IHC of CD22+ B cells, CD3+ T cells, and neutrophils in the joints. (A) Representative IHC images of CD22+ B cells, CD3+ T cells, neutrophils in the synovium at the femoral condyle of the DMM-operated joints. Red arrowheads indicate cells that were positive for staining. (B) Quantification of CD22+ B cells, CD3+ T cells, neutrophils in the synovium of the entire DMM joints. Macrophage-depleted mice exhibited significantly more CD3+ T cells in the DMM joints versus the control mice at any time points studied. Immediately following macrophage depletion, the DMM joints of the depleted mice also had significantly more neutrophils than those of the control mice. However, the control and depleted mice had comparable CD22+ B cells in their DMM-operated joints. n = 4-10 mice/group. Different letters are significantly different, p < 0.05, from each other. Statistical significance was determined by two-way ANOVA followed by Fisher’s post-hoc test. All data are presented as mean ± SEM.

4.3.6 Macrophage depletion significantly increased IL-27 levels in serum

The mice receiving macrophage depletion trended toward having lower serum insulin and leptin levels relative to the control mice immediately after depletion,
although the concentrations measured were not statistically significant (Figure 4-9).

However, at 9 weeks post-depletion, all mice showed significantly higher insulin and leptin levels compared the values at 0 week post-depletion. Furthermore, macrophage depletion significantly decreased serum TGF-β1 concentrations in the depleted mice immediately following depletion but all the mice express similar serum TGF-β1 concentrations at 9 weeks post-depletion. Interestingly, we observed that at both 0 and 9 weeks post-depletion, the mice receiving macrophage depletion demonstrated
significantly increased serum IL-27 levels as compared to the mice without depletion.

Figure 4-9 Serum cytokine analyses for the control and macrophage depleted mice at 0 and 9 weeks post-depletion. The depleted mice exhibited significantly lower serum TGF-β1 concentration and trended toward decreasing serum insulin and leptin levels relative to the control mice at 0 week post-depletion. Macrophage depletion also significantly increased serum IL-27 levels in the depleted mice at both time points studied. n = 5-12 mice/group. Different letters are significantly different, p < 0.05, from each other. Statistical significance was determined by two-way ANOVA followed by Fisher’s post-hoc test. All data are presented as mean ± SEM.
4.3.7 Macrophage depletion increased inflammatory cytokine levels in the synovial fluid of the DMM joint

The mice with macrophage depletion demonstrated significantly higher concentrations of IL-8, IL-10, and TNF-α in the synovial fluid of the DMM-operated joint compared to those cytokines from control mice at the time of depletion (Figure 4-10). Macrophage depleted mice also trended toward increasing IL-1β and IL-6 levels in the synovial fluid of operated joint relative to non-depleted mice at 0 weeks post-depletion. However, these up-regulated cytokines in the depleted mice had comparable levels versus those from control mice at 9 weeks post-depletion. Furthermore, only the macrophage depleted mice, not the control mice, exhibited detectable levels of IL-6, IL-8 and IL-10 in the synovial fluid of the non-operated right joints at the time of ablation. Interestingly, DMM surgery significantly decreased IL-17 concentrations in the operated joint as compared to that in the non-operated joint in both control and macrophage depleted mice at 0 week post-depletion. Finally, the concentrations of IFN-γ, IL-2, IL-4, IL-5, and IL12p70 in synovial fluid were below detectable levels in our study.
Figure 4-10 Synovial fluid cytokine analyses for the DMM-operated (left) joints and non-operated (right) joints of the control and macrophage-depleted mice at 0 and 9 weeks post-depletion. At 0 week post-depletion, macrophage-depleted mice showed significantly higher IL-8, IL-10 and TNF-α in the DMM joints as compared to the control mice. The depleted mice also trended toward increasing IL-1β and IL-6 in the DMM joint versus control mice immediately following depletion. DMM surgery significantly decreased IL-17 concentrations in the operated joint as compared to that in the non-operated joint in both control and macrophage depleted mice at 0 week post-depletion. Labels without bars indicate the cytokine measured was below detectable levels. n = 5-12 mice/group. * p < 0.05 for the joints within each group. Statistical significance was determined by two-way ANOVA followed by Fisher’s post-hoc test. All data are presented as mean ± SEM.
4.4 Discussion

Our findings indicate that transient, systemic depletion of macrophages in obese MAFIA mice did not prevent OA development following joint injury. Despite the fact that osteophyte formation, a clinical hallmark of OA, was less severe in the depleted mice at time of macrophage ablation, these obese mice still developed comparable osteophyte score to the control mice at 9 weeks post-depletion. Low osteophyte formation in the depleted mice at the time of treatment may be related to their low serum TGF-β1 levels, as it has been shown that macrophages are one of the main sources of growth factor production and their removal in joint decreased osteophytosis (Blom et al., 2007). According to the studies conducted by Burnett et al., macrophages repopulate in about 5 days after AP2018 administration in the MAFIA transgenic mouse model (Burnett et al., 2006). Therefore, it is possible that repopulated macrophages in the joints still contributed the formation of osteophytes at 9 weeks post-depletion.

To our surprise, macrophage depletion not only did not decrease OA; it rather exacerbated synovitis and intensified inflammatory cytokine production in the depleted mice. Given that the number of macrophages was lower in the synovium of the depleted joint, which was confirmed by flow cytometry for eGFP+ cells and F4/80 staining in the joint, this result implies that cells other than macrophages invaded into the DMM operated-joints of the depleted mice. IHC against various immune cells shows that CD3+ T cells and neutrophils, but not CD22+ B cells, massively infiltrated into the operated
joint of the depleted mice, while non-depleted mice trended toward having more iNOS+ M1 macrophages in their operated joints. This finding suggests that despite their recognized pro-inflammatory role, macrophages are vital in regulating the homeostasis of immune cells in the joint following injury. Indeed, two recent studies using distinct transgenic mice models capable of macrophage depletion support our observation. For example, Gordy et al. reported that lean mice lack of macrophages in marginal zone of spleen and bone marrow exhibited significantly increased numbers of neutrophils in circulating blood (Gordy et al., 2011). Similarly, in Lee’s study, they observed up-regulated Ly-6G expression, a specific marker for neutrophils, in the white adipose tissue, liver and skeletal muscles upon removal of macrophages (Lee et al., 2014). Both studies described occurrence of severe neutrophilia and systemic inflammatory responses following macrophage depletion in lean mice. Therefore, it is likely that accumulated neutrophils in the operated-joints of the depleted mice are the main source of inflammatory cytokines in synovial fluid as neutrophils are capable of secreting IL-1, IL-6, TNF-α, and neutrophil elastase, leading synovial inflammation and cartilage degradation. In addition, neutrophils can also produce IL-8, a potent chemokine for themselves, recruiting more granulocytes to inflammatory sites (Scapini and Cassatella, 2014; Simard et al., 2014; Wright et al., 2014). Despite the fact that IL-10, an anti-inflammatory molecule, was also found up-regulated in the synovial fluid, its presence may not be sufficient to counteract the catabolic effects of inflammatory cytokines on the
joint due to its relative low quantities compared IL-1β (about 10-fold less) in this model. Interestingly, IL-17 in the synovial fluid of the DMM joints from both mice decreased to below detectable levels when the mice underwent DMM surgery, suggesting this reduction may be associated with injury. However, whether this down-regulation was the result of modulation of IL-17 producing cells, mainly T helper-17 (Th17) cells by injury-related cytokines, or its concentration was simply diluted by cytokines massively produced during injury requires further investigation. Overall, macrophage depletion in obese MAFIA mice appears to alter OA progress from macrophage-associated to neutrophil-dependent, providing a distinct mechanism on OA induction in macrophage deficient obese mice.

Another novel finding in this project is that there was a sustained systemic elevation of IL-27 production in the macrophage-depleted mice. It has been reported that IL-27 is among the diverse secretion repertories of neutrophils(Rinchai et al., 2012). Thus, over production of IL-27 in our model may potentially result from systemic neutrophilia in macrophage-depleted mice. IL-27 possesses both anti- and pro-inflammatory properties(Hunter and Kastelein, 2012). For instance, treatment of IL-27 ameliorates several Th17-mediated immune diseases including Th17-dependent rheumatoid arthritis(Hirahara et al., 2012; Moon et al., 2013). On the contrary, IL-27 has been shown to promote naive T cells differentiate into Th1 cells and have a prominent role in development of Th1-dependent experimental arthritis(Cao et al., 2008). Recent
studies further demonstrated that IL-27 facilitates a subset of T regulatory cells to produce IL-10 (Hall et al., 2012a; Hall et al., 2012b). The pleiotropic functions of IL-27 may provide a potential explanation for increased T cell numbers and IL-10 production in the synovial fluid of operated in the macrophage-depleted mice.

In the current model, we observed that along with temporarily reduced insulin and leptin levels, obese MAFIA mice lost approximately 30% of their body weight upon macrophage depletion, which is 13% more than that was reported when lean MAFIA mice were used (Burnett et al., 2004). A recent study reported that adipose tissue increased lipolysis in the absence of adipose tissue macrophages (ATMs), suggesting a central role of macrophages in lipid tracking (Kosteli et al., 2010). In obese state, significantly enlarged adipocytes due to excess lipid storage are surrounded by ATMs (Lumeng et al., 2008). Perhaps, upon macrophage depletion, adipocytes lost the regulatory signaling from ATMs and rapidly released large amount of lipid content, leading to a dramatic weight loss in our obese MAFIA mice. On the contrary, in lean MAFIA mice, since there was only little lipid stored in adipocytes, the effect of macrophage depletion on body mass was therefore less profound. In addition, another possible explanation for the substantial weight loss may be the result of hypothalamus information following macrophage depletion as it has been reported that mice ablated with macrophages showed elevated inflammatory cytokines in the hypothalamus,
causing reduced food intake (Lee et al., 2014). Our results provide further evidence that macrophages are essential in maintaining energy metabolism.

Paradoxically, there are studies showing that macrophage ablation in mice did not elicit inflammatory responses and significant weight loss. For instance, by constructing diphtheria toxin receptor (DTR) under the promoter of CD11c gene, a marker commonly expressed in M1 macrophages, Patsouris and co-workers depleted CD11c+ cells by administrating DT in CD11c-DTR transgenic mice without inducing severe neutrophilia (Patsouris et al., 2008). The discrepancy between their and our findings may potentially result from whether neutrophils were also depleted along with macrophages. In our MAFIA mice, colony stimulating factor 1 receptor (csf1r), the promoter specific for myeloid cells, was used to express Fas-FKBP protein that can drive macrophage into apoptotic pathway upon AP20187 delivery. It has been suggested that granulocytes such as neutrophils may have lower expression levels of Fas-FKBP levels compared to macrophages (Hume, 2011) and thus were not significantly depleted in csf1r transgenic system. On the contrary, a study recently came out discovered that neutrophils may give rise of a hybrid, neutrophil-dendritic cell population that co-express CD11c and Ly6G (Matsushima et al., 2013). Furthermore, it has been reported that neutrophils express CD11 in human (Pillay et al., 2012). Therefore, a subset of neutrophils that are potentially inflammatory may likely be removed simultaneously with macrophages in CD11c-DTR system. Indeed, the results of transgenic mouse study
needs to be explained with caution. We believe that our findings provide insightful knowledge in understanding the links between macrophages and other immune cells in the joint following injury.

One limitation of the current study is that it is unknown whether repopulated macrophages are able to reverse the catabolic effects of severe neutrophilia on OA in obesity. Furthermore, whether sustained expression of serum IL-27 at later time point is the results of combined secretion of granulocytes and regenerated macrophages, or it is still solely contributed by excess neutrophils alone. Detailed longitudinal studies following macrophage depletion may provide answers to these questions.

4.5 Summary

In contrary to our hypothesis, we found that short-term, systemic macrophage depletion did not mitigate cartilage degeneration following injury in obese mice; instead, it enhanced joint synovitis by increasing infiltration of CD3+ T cells and neutrophils into the operated joint. Our study is significant for elucidating the immunomodulatory activity of macrophage in inflammation and joint injury in obesity.
5. Summary and Conclusions

This research developed novel techniques to harvest a variety of pure stem cell populations from mice. We further utilized animal and surgically induced-osteoarthritis (OA) models to elucidate the links among obesity, macrophage and OA. Our first research goal, to investigate the effect of obesity on stem cell function, was accomplished by isolating and expanding various stem cell populations from lean and obese mice. The comparison of proliferation, immunophenotype, and multipotency in lean and obese stem cells was performed. Our second research goal, to study the effect of obesity and dietary fatty acids (FAs) on development of injury-induced OA, was pursued by utilizing an obese mouse model with surgical techniques to induce knee OA in the mice fed high-fat diets rich in different types of dietary FAs. Finally, our third research goal, to examine the functionality of macrophages in obesity-associated OA, was addressed by transient, systemically depleting macrophages in an obese transgenic mouse model.

In Chapter 2, we successfully developed novel techniques using a combination of specific surface markers by flow cytometry cell sorting to harvest pure, various stem cell population including bone marrow-derived mesenchymal stem cells (MSCs), subcutaneous adipose-derived stem cells (sqASCs), and infrapatellar-derived stem cells (IFP cells). The results of the study supported our hypothesis by showing that obesity significantly altered function and phenotypes of stem cells. MSCs from obese mice demonstrated decreased adipogenic, osteogenic and chondrogenic potential compared
to lean MSCs. Decreased expression of CD105 and increased PDGFR α in obese MSCs provided a plausible explanation for decreased chondrogenic capacity of these cells. Compared to lean stem cells, obese sqASCs and IFP cells showed increased adipogenic and osteogenic differentiation, but decreased chondrogenic ability. We also observed that FA treatment of lean stem cells significantly altered their multipotency but did not completely recapitulate the properties of obese stem cells. This result suggests that the exposure to FAs alone cannot explain the alterations of stem cell functions in the obese environment, and it also implies that other obesity-associated cytokines might act synergically with FAs on stem cells. Our findings contribute to the understanding of mesenchymal tissue remodeling with obesity, as well as the development of autologous stem cell therapies for obese patients.

In Chapter 3, by utilizing surgical techniques to induce knee OA and a obese animal model in which mice fed high-fat diets rich in various dietary FAs such as saturated FAs (SFAs), ω-6 polyunsaturated fatty acids (PUFAs) and ω-3 PUFAs, we identified that the dietary FA content plays a more significant role in injury-induced OA than mechanical factor such as weight gain. We also discovered that small amounts of ω-3 PUFAs (8% kcal) in a high-fat diet (60% kcal) were sufficient to ameliorate OA in obese mice, decreasing serum leptin and resistin but increasing adiponectin levels. On the contrary, mice fed high-fat diet rich in SFAs or ω-6 PUFAs independently increased OA severity and heterotopic ossification. Furthermore, we also found that ω-3 PUFAs
significantly enhanced wound repair with less type I collagen deposition, while SFAs or ω-6 PUFAs promoted scar tissue formation. This study provides supports the need for future investigations of dietary FA supplements as a potential therapeutic approach for OA.

In Chapter 3, Using MAFIA mice, a transgenic mouse model that are capable of conditional depletion of macrophages, we identified the significance of macrophages in immune modulation in obesity. Obese MAFIA mice receiving transient, systemic macrophage depletion exhibited temporarily decreased percentage of body visceral fat, less osteophyte formation, and declined serum insulin, TGF-β1 and leptin concentrations. However, macrophage depletion did not mitigate cartilage degradation after the depletion treatment was stopped. To our surprise, macrophage depletion unexpectedly enhanced joint synovitis in the surgery-operated joint with significantly elevated inflammatory cytokine levels such as IL-1β, IL-6, and TNF-α in the synovial fluid. Immunohistochemical staining against various immune cells demonstrated that macrophage depleted mice, although having less macrophages, exhibited significantly increased infiltration of T cells and neutrophils cells into the operated joints as compared to the control mice. Our findings extend the knowledge in the immunoregulatory role of macrophages in obesity and OA.

Given that prevalence of obesity is rapidly increasing worldwide, our work in comparison of lean and obese murine stem cells are significant for developing
autologous stem cell therapy for obese patients. Furthermore, we also established novel techniques that can isolate and expand purified murine stem cells from various tissues. These techniques can be used as a tool to further explore stem cell biology, function and therapeutic applications. Our demonstration of dietary FA content is the primary regulator in obesity-associated OA and that ω-3 PUFAs improves wound healing and prevents OA development urge the need of clinical studies on dietary FA supplementation for OA and/or post-operative obese patients. The finding that macrophages are essential in immune balance in joint injury and obesity has a significant implication on the clinical applications that seek to target or ablate macrophages in patients. Future studies may wish to explore a strategy that is able to specifically deplete inflammatory macrophages without inducing systemic neutrophilia in obesity.

Collectively, the studies presented in this dissertation extend the understanding of effects of obesity on stem cell properties and further elucidate the relationships among obesity, OA and macrophages.
Appendix A

A version of this appendix was published as supplemental material to the following:

Figure A-1 Proliferation and multipotency of visASCs.

Cells were isolated from the epididymal fat pads (visASCs) of lean and obese mice using the same surface markers used for sqASCs (i.e. Sca-1+ CD34+ CD31- CD45- TER119-). (A) visASCs were expanded through passage 5 at hypoxia conditions and the cumulative fold increase was quantified. Obese visASCs showed a trend toward decreased proliferation compared to other types of ASC populations. Results were averaged from 2 independent isolations for obese visASCs and 3 independent isolations for other types of ASCs with mean ± SEM displayed (n ≥ 3 mice per isolation). (B, C) lean visASC showed significantly higher absorbance than non-induced control group when differentiated into adipogenic and osteogenic lineages. Interestingly, lean visASCs had decreased adipogenesis but increased osteogenesis as compared lean sqASCs. Results from ≥ 3 samples per group with mean ± SEM displayed. # p < 0.05 vs. all groups by two-way ANOVA with Tukey’s post-hoc test. (D) Representative Alcian Blue staining of lean visASC pellets. While visASCs were able to differentiate into adipocytes and osteoblasts, they failed to differentiate into chondrocytes, even when cultured under a variety of different conditions. All the chondrogenic pellets were supplemented with 10 ng/ml TGF-β3 and 500 ng/ml BMP-6. Red indicates nucleus and blue indicates GAGs. Scale bar is 100 μm.
MSCs cultured with several concentrations of FFA for 7 days did not show any measurable cell death. Cells also maintained their morphology and grew at similar rates to confluence. Scale bar is 100 µm.
of (A) MSCs, (B) sqASCs and (C) IFP cells from lean and obese mice. Obese IFP pellets had significantly lower GAG content compared to lean IFP pellets, while obese MSCs and sqASCs exhibited a trend toward decreased GAG content compared to their corresponding lean cell types. (F) SA/MUFA treated IFP pellets had significantly lower GAG content compared to vehicle control. Results from ≥ 4 pellets per group of the cells pooled from two independent isolations (n = 6 mice per isolation) with mean ± SEM displayed. # p < 0.05 vs. corresponding group by t-test.
Subcutaneous fat (inguinal fat pad), infrapatellar fat pad and visceral fat (epididymal fat pad) were harvested from lean and obese mice and fixed in 10% formalin. Serial section (5 µm) was performed and sections were then immunohistochemically labeled for murine macrophage marker F4/80 (Biolegend, 1:50 dilution) and counter-stained with hematoxylin QS (Vector Lab) to reveal infiltrated macrophages. Color was developed by horseradish peroxidase with DAB substrate (all from Vector Lab). No macrophage was found in lean and obese joint fat pad (C, D), while only a few were detected in obese subcutaneous fat pad (B; arrow). In contrast, macrophages massively infiltrated into visceral adipose tissue in response to obesity and formed characteristic crown-like structures around dead adipocytes (F; * indicates representative crown-like structure). Scale bar is 100 µm.
Table A-1 Summary of cell sorting yield per mouse

<table>
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<tr>
<th>Body fatness</th>
<th>Cell type</th>
<th>Lean</th>
<th>Obese</th>
</tr>
</thead>
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<tr>
<td>Cell type</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MSCs</td>
<td></td>
<td>0.8-0.9k</td>
<td>1.3-2.8k</td>
</tr>
<tr>
<td>sqASCs</td>
<td></td>
<td>25-40k</td>
<td>113-123k</td>
</tr>
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</table>

Table A-2 Summary of multilineage of differentiation

(A) Multilineage differentiation capacity of obese stem cells as compared to lean stem cells

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<th>Obese cells</th>
<th>MSCs</th>
<th>sqASCs and IFP cells</th>
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</thead>
<tbody>
<tr>
<td>Adipo</td>
<td>↓</td>
<td>↑</td>
<td></td>
</tr>
<tr>
<td>Osteo</td>
<td>↓</td>
<td>↑</td>
<td></td>
</tr>
<tr>
<td>Chondro</td>
<td>≈</td>
<td>↓</td>
<td></td>
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</table>

(B) Multilineage differentiation capacity of FFA-treated lean stem cells as compared to vehicle control

<table>
<thead>
<tr>
<th>lineage</th>
<th>FFA-treated cells</th>
<th>MSCs</th>
<th>sqASCs and IFP cells</th>
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<td>↑</td>
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<tr>
<td>Osteo</td>
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<td>≈</td>
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<tr>
<td>Chondro</td>
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<td></td>
</tr>
</tbody>
</table>
Appendix B

A version of this appendix was published as supplemental material to the following:


Figure B-1 Study design.

Animals received the prescribed diets at 4 weeks of age until the end of the study at 28 weeks of age. DMM surgery and ear punch were performed at 16 weeks of age (green). Behavioral measurements were assessed at 6, 14 and 24 weeks of age (brown). Sera were collected at 8, 12, 17, 23, and 28 weeks of age (red).
Figure B-2 Representative images for osteophyte grading.

0 point = normal periosteal surface, 1 point = early stage (cells in the periosteum and synovial lining layer start to proliferate), 2 points = middle stage (cells in the periosteum and synovial lining layer show massive proliferation and hypertrophic chondrocytes can be observed), 3 points = mature stage (osteophyte integrated with the subchondral bone with the presence of bone marrow cavities). Scale bar = 100 µm
Figure B-3 Representative image of severe subchondral erosion.

Three out of 14 ω-6 mice had severe subchondral erosion through the medial tibial plateau on the DMM-operated joint. Ectopic bone formation (green arrowheads, and chondrogenesis and massive deposition of proteoglycans (yellow arrowheads) in the ligaments were also observed in these joints. (F) femoral condyle. (T) tibial plateau. (M) meniscus. (OM) original location of menicus (black dashed line). (AB) abnormal fusion of cartilage, meniscus and subchondral bone. (GP) growth plate. (SB) remaining subchondral bone. (OS) osteophyte. (ACL/PCL) anterior/posterior cruciate ligament.
Figure B-4 serum TGF-β1

(A) Active and (B) latent form of serum TGF-β1 measured at 28 weeks of age. The ω-3 mice had the highest levels of the latent TGF-β1 as compared to the mice fed other diets. n = 11-14 mice/diet. # p < 0.01, versus other diets. Mean ± SEM.
Figure B-5 The relationship between diet and OA using weight-matched strategy

(A) at 28 weeks of age. The mice whose weights were in the range of 31 to 45 grams were used for OA analysis. The line the box indicates median and the length of the box represents interquartile range. (B) Left (DMM-operated) to right (non-operated) joint OA score ratio of the weight-matched mice. The ω-6 mice had a significantly higher OA ratio score compared to the ω-3 and Control mice. Different letters are significantly different (p < 0.05) from each other. Mean ± SEM. n = 5-9 mice/diet.
Figure B-6 Regression between rotarod performance and grip strength.

Rotarod performance showed a positive association with forelimb grip strength, suggesting musculoskeletal strength is associated with motor function.
Figure B-7 Representative images of complete ear wound fusion of ω-3 PUFA fed mice. (A) H&E staining reveals regenerated matrix between two developing cartilage ends (dashed line). Regenerative features including chondrogenesis (green arrowhead), adipogenesis (blue arrowhead), sebaceous gland formation (yellow arrowheads), and folliculogenesis (orange arrowhead) were observed in the restored tissue region (magnified in B and C). Scale bar = 100 μm
Table B-1 Diet composition

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<th>Control D11120103</th>
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<th>ω-6 PUFA D11120105</th>
<th>ω-3 PUFA D11120106</th>
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<tr>
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</tr>
<tr>
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<td>26.2 g</td>
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<tr>
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<td>14.6</td>
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<td>MUFA (% of kcal)</td>
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<tr>
<td>ω6:ω3 ratio</td>
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Table B-2 PCR primers

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<td>Mm00802529_m1</td>
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<tr>
<td>GAPDH</td>
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| IL-6   | F: GAGGATAACCCTCCCAACAGACC  
        | R: AAGTGCATCATCGTTGTCATACA |         |
| TNF-α  | F: CATTTCTCAAAATTCGAGTGACAA  
        | R: TGGGAGTAGACAAGGTACAAACCC |         |
| MCP-1  | F: GGCTCAGCCAGATGCAGTTAA  
        | R: CCTACTCATGGGATCATCTTGCT |         |

Table B-3 Incidence, bone volume and bone mineral density of heterotopic bone

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<th>Heterotopic ossification</th>
<th>Low-fat</th>
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<tr>
<td></td>
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<td>ω-3</td>
<td>ω-6</td>
<td>SFA</td>
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<td>p value</td>
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<tr>
<td>Incidence§</td>
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<td>75%</td>
<td>100%</td>
<td>100%</td>
<td></td>
<td>0.00</td>
</tr>
<tr>
<td>Bone volume (mm³) *</td>
<td>0.0016a (0.008)</td>
<td>0.0251a, b (0.068)</td>
<td>0.1122b (0.204)</td>
<td>0.059b (0.058)</td>
<td></td>
<td>0.00†</td>
</tr>
<tr>
<td>Bone mineral density (mg/cm³) *</td>
<td>377.07a (867.60)</td>
<td>812.09a, b (657.63)</td>
<td>887.09b (66.61)</td>
<td>886.20b (46.97)</td>
<td></td>
<td>0.00†</td>
</tr>
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</table>

§ The data were analyzed by chi-square tests.
* Values represent median (25%-75% interquartile Range)
† The data were log transformed but were still not normally distributed due to several Control mice and ω-3 mice that had no heterotopic ossifications in DMM-operated joints. Thus, the data were analyzed using a Kruskal-Wallis H test for the main effect of the diet and then followed by Mann-Whitney U tests with Holm–Bonferroni corrections for multiple comparisons among diet groups. Different letters are significantly different from each other.
Table B-4 Osteophyte disease stage of joints

<table>
<thead>
<tr>
<th></th>
<th>Left (DMM-operated) joint</th>
<th>Right joint</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Early</td>
<td>Middle</td>
<td>Mature</td>
<td>Total</td>
<td>Early</td>
<td>Middle</td>
<td>Mature</td>
<td>Total</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>3</td>
<td>12</td>
<td>20 (100)</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>4 (100)</td>
</tr>
<tr>
<td>ω-3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>4</td>
<td>11</td>
<td>17 (100)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0 (−)</td>
</tr>
<tr>
<td>ω-6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>3</td>
<td>19</td>
<td>25 (100)</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1 (100)</td>
</tr>
<tr>
<td>SFA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1</td>
<td>23</td>
<td>25 (100)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0 (−)</td>
</tr>
</tbody>
</table>

Data are expressed as number of incidences of each disease stage and the % of incidences within diet is displayed in parenthesis.
Table B-5 Incidence of osteophyte formation for each diet group

<table>
<thead>
<tr>
<th>Incidence of osteophyte formation</th>
<th>Low-fat</th>
<th>High-fat</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>ω-3</td>
</tr>
<tr>
<td>Number of mice per diet</td>
<td>11</td>
<td>12</td>
</tr>
<tr>
<td>Number of mice developing osteophytes</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Percentage</td>
<td>90.91%</td>
<td>83.33%</td>
</tr>
<tr>
<td>Number of mice developing osteophyte at left joints</td>
<td>9</td>
<td>10</td>
</tr>
<tr>
<td>Percentage</td>
<td>90%</td>
<td>100%</td>
</tr>
<tr>
<td>Number of mice developing osteophyte at right joints</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Percentage</td>
<td>10%</td>
<td>0%</td>
</tr>
</tbody>
</table>

Table B-6 Frequency of macrophages at the specific site of DMM-operated (left) joints

<table>
<thead>
<tr>
<th>Site</th>
<th>Low-fat</th>
<th>High-fat</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>ω-3</td>
</tr>
<tr>
<td>Periosteum</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>(23)</td>
<td>(19)</td>
</tr>
<tr>
<td>Synovial lining layer</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>(62)</td>
<td>(50)</td>
</tr>
<tr>
<td>Synovial stroma</td>
<td>2\textsuperscript{a}</td>
<td>5\textsuperscript{a}</td>
</tr>
<tr>
<td></td>
<td>(15)</td>
<td>(31)</td>
</tr>
<tr>
<td>Total</td>
<td>13</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>(100)</td>
<td>(100)</td>
</tr>
</tbody>
</table>

Data expressed as frequency count and % of the total count within each diet in parenthesis (n = 4). * Diet has a significant effect on macrophage frequency in synovial stroma when analyzed by one-way ANOVA. Superscripts with different letters are
significantly different with each other when analyzed by Tukey’s HSD post-hoc analysis (p < 0.05).

Table B-7 Bivariate regression analyses for variables predicting OA severity

<table>
<thead>
<tr>
<th>Predictor variables</th>
<th>OA severity</th>
<th>r</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin</td>
<td></td>
<td>0.15</td>
</tr>
<tr>
<td>Leptin</td>
<td></td>
<td><strong>0.38</strong></td>
</tr>
<tr>
<td>Adiponectin</td>
<td></td>
<td>0.11</td>
</tr>
<tr>
<td>Resistin</td>
<td></td>
<td><strong>0.31</strong></td>
</tr>
<tr>
<td>PGE₂</td>
<td></td>
<td>0.27</td>
</tr>
<tr>
<td>Active/Total TGFβ1 ratio</td>
<td></td>
<td>0.18</td>
</tr>
</tbody>
</table>

Table B-8 The effect of low- and high-fat feeding on biomechanical and neurobehavioral functions

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Low-fat feeding (10% kcal)</th>
<th>High-fat feeding (60% kcal)*</th>
<th>Repeated measures ANOVA (p value)^b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6 week 14 week</td>
<td>6 week 14 week</td>
<td>Age  Diet Age × Diet</td>
</tr>
<tr>
<td>Biomechanical</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spontaneous locomotion (cm)</td>
<td>1770.73 (183.20)</td>
<td>1289.64 (79.88)</td>
<td>1745.45 (126.76) 1139.58 (91.41)</td>
</tr>
<tr>
<td>Rotarod latency to fall (s)</td>
<td>261.57 (9.84)</td>
<td>249.61 (6.54)</td>
<td>234.61 (7.73) 214.49 (7.86)</td>
</tr>
<tr>
<td>Forelimb grip strength^c</td>
<td>3.78 (0.34)</td>
<td>1.83 (0.07)</td>
<td>3.24 (0.08) 1.70 (0.04)</td>
</tr>
<tr>
<td>Hindlimb grip strength^c</td>
<td>3.29 (0.34)</td>
<td>2.79 (0.23)</td>
<td>3.18 (0.13) 2.47 (0.11)</td>
</tr>
<tr>
<td>Neurobehavioral</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hotplate withdrawal latency (s)</td>
<td>14.76 (0.94)</td>
<td>10.88 (0.72)</td>
<td>14.83 (0.50) 12.83 (0.54)</td>
</tr>
<tr>
<td>Tail-flick withdrawal latency (s)</td>
<td>4.49 (0.65)</td>
<td>3.52 (0.15)</td>
<td>3.90 (0.23) 3.46 (0.10)</td>
</tr>
</tbody>
</table>

a All the high-fat diets (i.e., ω-3 PUFA, ω-6 PUFA and SFA) were analyzed by one-factor ANOVA for the effect of different types of fatty acids on the behavioral assays conducted at specific time-points. If they were not significantly different from each
other, all data from the high-fat diets were pooled together and considered as for a single 60% kcal high-fat feeding group. If the significance was observed on a certain FA type, the diet with that FA was then excluded from the single high-fat feeding group.

b The data were presented as mean with SEM in parenthesis and were analyzed by two-factor repeated measures ANOVA for the effect of diet (two levels: low- and high-fat feeding), the effect of age (two levels: 6 week and 14 weeks) and their interaction. Bonferroni corrections were applied as the post-hoc test.

c Grip strength was normalized to body weight (force in gram per gram body mass).
Appendix C

Figure C-1 DEXA measurements.

All mice exhibited comparable percent body fat and whole body bone mineral density at 9 weeks and 23 weeks of age.
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and fish oils lifelong supplementation decreases inflammation and improves bone health in a murine model of senile osteoporosis. Bone 50, 553-561.


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Biography

Chia-Lung Wu was born in Taipei, Taiwan on September 22nd, 1979. He earned a Bachelor of Science in Materials and Mineral Resources Engineering summa cum laude from National Taipei University of Technology in 2001. In 2002, Chia-Lung served in the military of Republic of China for two years. Chia-Lung received his Master’s degree in Material Science and Engineering (MSE) at National Taiwan University (NTU) in 2005, and worked as teaching assistant in the MSE department at NTU for 2 years. In 2008, Chia-Lung earned his Master’s degree in Biomedical Engineering at Duke University in 2010. Chia-Lung received Duke BME fellowship and Taiwan Government Student Studying Aboard Scholarship. Chia-Lung received The New Investigator Recognition Awards from Orthopaedic Research Society in 2014.

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