OBESITY ENHANCES THE TUMORIGINICITY AND METASTASIS OF BREAST CANCER CELLS BY ALTERING ADIPOSE STROMAL CELL FUNCTION

AN ABSTRACT

SUBMITTED ON THE THIRTY FIRST DAY OF OCTOBER 2013

TO THE GRADUATE PROGRAM IN BIOMEDICAL SCIENCES

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

OF THE GRADUATE SCHOOL

OF TULANE UNIVERSITY

FOR THE DEGREE

OF

DOCTOR OF PHILOSOPHY

BY

AMY LIN STRONG

APPROVED:

Bruce A. Bunnell, Ph.D.

Aline M. Betancourt, Ph.D.

Barbara S. Beckman, Ph.D.

Matthew E. Burow, Ph.D.

Jeffrey M. Gimble, M.D., Ph.D.
ABSTRACT

Obesity, defined by the excessive accumulation of adipose tissue, has been shown to increase the incidence and aggressiveness of postmenopausal breast cancer. The increased morbidity and mortality of obesity-associated breast cancer has been attributed to increased adipose tissue volume, as increased adiposity results in the increased generation of adipose stromal cells (ASCs) and the production of hormones, cytokines, and adipokines. Previous studies suggest that factors secreted by cancer cells alter ASC biology, resulting in the increased recruitment of ASCs to the tumor site (through the SDF-1/CXCR4 axis) and increased secretion of growth factors (IL-6, TNF-α, leptin, and PAI-1), which results in the enhanced proliferation and migration of breast cancer cells (BCCs). While studies have shown that ASCs alter key regulatory genes associated with these processes, it remains to be determined the precise mechanism by which obesity and ASCs within the adipose tissue alters this interaction. Additional studies are necessary to fully elucidate the role of obesity and breast cancer.

Herein, ASCs isolated from obese women (obese ASCs) and lean women (lean ASCs) were characterized based on: (a) the mobilization of ASCs following exposure to BCCs and (b) the effect of these ASCs on BCC growth, migration, and metastasis. The gene expression profiles of ASCs and of the BCCs following co-culture with ASCs were examined to determine obesity-induced alterations in ASC biology and obesity-induced
ASC alterations in BCCs, respectively. Obese ASCs were found to invade significantly more compared to lean-ASCs through the upregulation of MMP-15 and calpain-4 and the downregulation of CAST. Assessment of the alterations at the cellular level determined that obese ASCs, when compared to lean ASCs, enhanced the proliferation of BCCs and the tumor volume in vivo. Gene expression profile analysis demonstrated a significant upregulation of leptin in the obese ASCs, which in turn led to the upregulation of CDKN2A, SFRP1, IL-6, MMP-2, and PAI-1 in BCCs (MCF7, ZR75, and T47D) when co-cultured together. Furthermore, obese-ASC conditioned BCCs demonstrated enhanced migratory and invasive potential that translated into enhanced metastasis in an immunodeficient mouse model. These studies provide a mechanistic link by which obesity, in particular obese ASCs, may enhance breast cancer cell growth and metastasis.
OBESITY ENHANCES THE TUMORIGENICITY AND METASTASIS OF BREAST CANCER CELLS BY ALTERING ADIPOSE STROMAL CELL FUNCTION

A DISSERTATION
SUBMITTED ON THE THIRTY FIRST DAY OF OCTOBER 2013
TO THE GRADUATE PROGRAM IN BIOMEDICAL SCIENCES
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
OF THE GRADUATE SCHOOL
OF TULANE UNIVERSITY
FOR THE DEGREE
OF
DOCTOR OF PHILOSOPHY

BY

[Signature]

AMY LIN STRONG

APPROVED:

[Signature]
Bruce A. Bunnell, Ph.D.

[Signature]
Aline M. Betancourt, Ph.D.

[Signature]
Barbara S. Beckman, Ph.D.

[Signature]
Matthew E. Burow, Ph.D.

[Signature]
Jeffrey M. Gimble, M.D., Ph.D.
ACKNOWLEDGEMENTS

Due to the seemingly impossible task of prioritizing my many acknowledgements, I will do my best to thank all those who have contributed during this journey. Foremost, I would like to express my sincerest gratitude to my advisor, Dr. Bruce Bunnell for the continuous support, encouragement, and mentoring necessary for me to complete my dissertation. His guidance, patience, motivation, enthusiasm, and immense knowledge truly make him a great mentor.

I’d like to thank my committee members, Drs. Jeffrey Gimble, Matthew Burow, Barbara Beckman, and Aline Betancourt for their support and helpful suggestions. Each has contributed in his or her own way to aspects of my training and has helped me grow and develop into the scientist I am today. I owe them my heartfelt appreciation.

In addition, I’d like to thank our collaborators at Xavier University, Dr. Harris McFerrin, who provided me with a model to use to complete my first manuscript, and Tatyana Santoke for helping me with those experiments.

My sincerest thanks goes to past and present lab members and colleagues for the stimulating discussions, tireless effort, and fun the past few years: Julie Semon, Jason Ohlstein, Thomas Strong, Annie Bowles, Maria Dutreil, Zhenzhen Shi, Lyndsay Rhodes, Xiujuan Zhang, Shijia Zhang, Brandi Biagas, Ben Jones, Betsy Bateman, Dorothy Pei, Rachel Wise, Stephen Lee, and Connor MacCrimmon. I would also like to take this time
to acknowledge the core directors and key personnel in the Center for Stem Cell and Regenerative Medicine for their invaluable help: Alan Tucker, Dina Gaupp, Claire Llamas, and Marjorie McCants.

Finally, I would like to thank my parents and brother for their unconditional love, support, and encouragement throughout this process. While they may not fully understand why I needed to train for so long, they still stood by my decision. I also want to thank my in-laws for their unconditional support and encouragement.

Last but not least, I would like to thank my husband, Michael Strong, for the love, patience, understanding, and support he has given me and for being my “partner in crime” throughout this journey.
TABLE OF CONTENTS

ACKNOWLEDGEMENTS ........................................................................................................... ii

TABLE OF CONTENTS .......................................................................................................... iv

LIST OF TABLES .................................................................................................................... viii

LIST OF FIGURES .................................................................................................................. ix

ABBREVIATIONS .................................................................................................................. xii

CHAPTER 1. GENERAL INTRODUCTION ............................................................................. 1

1.1 Mesenchymal Stem Cells (MSCs) ................................................................. 1

1.2 MSCs in Cell-Based Therapies ................................................................. 3

1.2.1 Autoimmune Diseases or Severe Inflammation-Related Diseases .............. 3

1.2.2 Regenerative Medicine ................................................................. 8

1.2.3 Suicide Cancer Gene Therapy .................................................. 10

1.3 MSCs in the Tumor Microenvironment and Cancer Metastasis .................... 14

1.4 Obesity, MSCs, and Cancer ............................................................... 17

1.4.1 Obesity and Cancer ............................................................... 17

1.4.2 Obesity, Inflammation, and Cancer ........................................ 17

1.4.3 Obesity, Adipokines and Breast Cancer .................................... 22

1.4.4 Obesity-associated dysregulation of ASCs .................................... 29

CHAPTER 2. THE ROLE OF OBESITY ON ASC INVASION ........................................ 32

2.1 Abstract ................................................................................................. 32

2.2 Introduction ......................................................................................... 33

2.3 Material and Methods ........................................................................ 34

2.3.1 Materials ................................................................................. 34

2.3.2 Human Subjects .......................................................................... 35

2.3.3 Cell Culture of ASCs .................................................................. 36

2.3.4 Cell Culture of MDA-MB-231 ................................................... 36

2.3.5 Conditioned Media .................................................................... 38
2.3.6 Adipogenic Differentiation Protocol ...............................................38
2.3.7 Osteogenic Differentiation Protocol ...............................................38
2.3.8 Colony Forming Units Assay ..............................................................39
2.3.9 Flow Cytometry ..................................................................................39
2.3.10 Transwell Invasion Assay ..................................................................39
2.3.11 RNA Isolation and RT-PCR ...............................................................40
2.3.12 Protein Isolation and Western Blot ....................................................41
2.3.13 Zymography .......................................................................................42
2.3.14 Stable Transfection of shRNA ..........................................................42
2.3.15 Chick Chorioallantoic Membrane (CAM) Assay ..............................43
2.3.16 Statistical Analysis ............................................................................44

2.4 Results ....................................................................................................44
2.4.1 Characterization of ASCs .................................................................44
2.4.2 Ob\(^{-}\)Ab\(^{+}\) ASCs demonstrate increased invasion towards
  breast cancer cell conditioned media .......................................................45
2.4.3 GM6001 and acetyl-calpastatin inhibit
  Ob\(^{-}\)Ab\(^{+}\) ASC invasion ........................................................................45
2.4.4 mRNA expression of MMPs and calpains vary
  among the four groups ...........................................................................54
2.4.5 Expression and activity of MMPs and calpains vary
  among the four groups ...........................................................................55
2.4.6 Assessing variability of MMP-15, calpain-4, and calpastatin expression
  .................................................................................................................56
2.4.7 Reduced calpastatin and enhanced calpain-4
  expression increase invasion ....................................................................56
2.4.8 Inhibition of MMP-15 shRNA reduced invasion
  of ASCs ......................................................................................................57
2.4.9 Invasion of ASCs \textit{in vivo} .................................................................62

2.5 Discussion ...............................................................................................63

CHAPTER 3. OBESE ASCS INFLUENCE
BREAST CANCER TUMORIGENESIS .........................................................69

3.1 Abstract .....................................................................................................69
3.2 Introduction ...............................................................................................70
3.3 Material and Methods ..............................................................................72
  3.3.1 Human subjects ..................................................................................72
  3.3.2 Cell culture ........................................................................................73
  3.3.3 Synthesis of GFP breast cancer cells .................................................75
  3.3.4 Synthesis of DsRed ASCs .................................................................76
  3.3.5 Breast cancer cell and ASC co-culture ..............................................76
  3.3.6 ASC conditioned media .....................................................................78
  3.3.7 RT\(^{2}\) Profiler\textsuperscript{TM} PCR Arrays ..........................................78
  3.3.8 Western blot ......................................................................................79
  3.3.9 \textit{In vivo} tumorigenicity assay ..........................................................80
  3.3.10 Immunohistochemistry ....................................................................81
3.3.11 Quantitative RT-PCR ........................................... 82
3.3.12 Oncomine analysis .............................................. 83
3.3.13 KM plot analysis ................................................ 83
3.3.14 Statistical analysis .............................................. 83
3.4 Results .................................................................. 84
3.4.1 Characterization of ASCs ...................................... 84
3.4.2 ASCs isolated from obese subjects enhance the proliferation of MCF7 cells in vitro ...................... 84
3.4.3 Donor’s obesity status and depot site of ASCs influence their effect on the gene expression profile of MCF7 cells ........................................ 86
3.4.4 Increased expression of CDKN2A, GSTP1, SFRP1, ESR1 and PGR in MCF7 cells after co-culture with ASCs .......... 90
3.4.5 Estrogen enhances the ASC-induced MCF7 cell proliferation in vitro ........................................... 91
3.4.6 Tumor volume of MCF7 and ASC xenografts is related to the obesity status and depot source of the ASCs .......... 91
3.4.7 Enhanced progesterone receptor expression in xenografts formed with Ob\(^\)Ab\(^+\) ASCs ......................... 97
3.4.8 Gene expression profile differ between ASCs based on obesity status and depot source ......................... 98
3.4.9 Estrogen stimulates leptin expression in ASCs which then enhances MCF7 proliferation ......................... 98
3.4.10 Enhanced ASC aromatase expression and activity increases MCF7 cells ......................................... 99
3.4.11 High leptin levels correlate with poor relapse-free survival in ER\(^+\)/PR\(^+\) breast cancer ......................... 105
3.5 Discussion ............................................................. 106
3.6 Conclusion ............................................................ 110

CHAPTER 4. OBESE ASCS ENHANCE ER BREAST CANCER EMT AND METASTASIS ........................................... 111

4.1 Abstract .................................................................. 111
4.2 Introduction ............................................................ 112
4.3 Material and Methods .............................................. 114
4.3.1 Human subjects .................................................... 115
4.3.2 Cell culture .......................................................... 115
4.3.3 Synthesis of GFP\(^+\) BCCs .......................................... 116
4.3.4 Conditioned Media ............................................... 117
4.3.5 Stable transfection of shRNA ................................... 117
4.3.6 Alamar Blue Cell Proliferation Assay ....................... 118
4.3.7 RNA Isolation Followed by Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR) ...................... 118
4.3.8 Characterization of ASCs ....................................... 119
4.3.9 BCC and ASC Co-culture ....................................... 121
4.3.10 Transwell Migration and Invasion Assays ..................................121
4.3.11 RNA Isolation Followed by Custom 
RT² Profiler™ PCR Arrays ........................................122
4.3.12 In Vivo Tumorigenicity Assay ......................................123
4.3.13 Flow Cytometry ................................................124
4.3.14 Immunohistochemistry .........................................124
4.3.15 Protein Isolation and Western Blot ..........................125
4.3.16 Metastasis Assessment ........................................126
4.3.17 Statistical Analysis .............................................126

4.4 Results ........................................................................127
4.4.1 ER+ BCCs selective respond to obese ASCs .................127
4.4.2 obASCs expressing leptin shRNA have diminished capacity to enhance BCC proliferation ..........128
4.4.3 obASCs enhance BCC migration and invasion ............130
4.4.4 Leptin shRNA obASCs reduce expression of proliferation and EMT genes .........................138
4.4.5 Leptin shRNA obASCs result in diminished tumorigenicity and expression of EMT genes ......139
4.4.6 Leptin inhibition reduces obASCs-induced metastasis of BCCs ........................................146

4.5 Discussion ....................................................................147

CHAPTER 5. GENERAL DISCUSSION ........................................156

5.1 Obesity alters ASC Biology .............................................156
5.2 Obese ASCs increase metastasis and reoccurrence of breast cancer ..................161
5.3 ASC as a therapeutic target for breast cancer ....................162
5.4 Implications on stem cell therapy .................................162

APPENDICES

A. Calpastatin Expression Varies Among Different Types of Breast Cancer ........................................164
B. Calpastatin Expression in Several Cancers .............................165

REFERENCES ....................................................................166

BIOGRAPHY .....................................................................192
LIST OF TABLES

1.1 Characteristics of mesenchymal stem cells .................................................................2
1.2 BMSC therapy in experimental animal models of autoimmune diseases and severe inflammation-related diseases ...............6
1.3 ASC therapy in experimental animal models of autoimmune diseases and severe inflammation-related diseases ...........................................7
2.1 Donor demographics in ASCs ..................................................................................37
2.2 Primer set used in amplification of ASC mRNA regions .............................................49
3.1 mRNA expression of MCF7 cells after exposure to ASCs ...........................................88
3.2 Fold change in mRNA expression of ASCs based on obesity status and depot site of origin .................................................................101
4.1 mRNA expression of MCF7 cells after exposure to leptin knockdown ASCs ................142
4.2 mRNA expression of ZR75 cells after exposure to leptin knockdown ASCs ................143
4.3 mRNA expression of T47D cells after exposure to leptin knockdown ASCs ................144
4.4 mRNA expression of xenografts with MCF7 cells and leptin knockdown ASCs ...........149
## LIST OF FIGURES

1.1 MSC multilineage differentiation potential ......................................................... 5
1.2 BMI and postmenopausal breast cancer ................................................................. 18
1.3 Relative risk of developing obesity-associated cancer
categorized by cancer site in men.............................................................................. 19
1.4 Relative risk of developing obesity-associated cancer
categorized by cancer site in women ........................................................................ 20
1.5 Oxygen diffusion from the vasculature to distant tissue ........................................ 23
1.6 HIF-1α regulation during normoxia and hypoxia .................................................... 24
1.7 Phenotypic modulation of adipose tissue by obesity ............................................... 27
1.8 Leptin receptor activation and inactivation ................................................................ 28
2.1 Characterization of ASCs isolated from donors based
on obesity status and depot site .................................................................................. 46
2.2 Invasion of ASCs towards conditioned media ......................................................... 47
2.3 Protease inhibitors limit ASC invasion ...................................................................... 48
2.4 RT-PCR analyses of MMP, TIMP, calpain, and calpastatin in ASCs .................... 50
2.5 Quantification of RT-PCR analyses of MMPs and TIMPs, Part 1 ....................... 51
2.6 Quantification of RT-PCR analyses of MMPs and TIMPs, Part 2 ....................... 52
2.7 Quantification of RT-PCR analyses of calpains and calpastatin .......................... 53
2.8 Zymographic analyses of MMP-2 and MMP-9 ..........................................................58
2.9 Western blot analyses of MMPs, TIMPs, calpains, and calpastatin in different ASC population ..........................................................................................................................59
2.10 Western blot analyses of ASCs isolated from 24 donors for MMP-15, calpain-4, and calpastatin expression ..........................................................60
2.11 shRNA constructs effectively target genes of interest .................................................61
2.12 ASC invasion influenced by shRNA knockdown ..........................................................64
2.13 The role of MMP-15, calpain-4, and calpastatin in ASC invasion in the CAM model ..........................................................65
3.1 Sorting of GFP+ MCF7 cells from DsRed+ ASCs .........................................................77
3.2 Characterization of ASCs isolated from donors based on obesity status and deposit site .............................................................................................................................86
3.3 Direct co-culture of breast cancer cells with ASCs result in increased proliferation in vitro .................................................................................................87
3.4 Cluster diagram of relative gene expression of MCF7 cells co-culturing with ASCs characterized by obese status and depot site of origin .........................89
3.5 Changes in the expression of cell cycle regulators and steroid receptors of MCF7 cells .........................................................................................................................93
3.6 Estrogen enhances the effects of ASCs on MCF7 proliferation in vitro .................94
3.7 Tumorigenesis of MCF7 cells when co-mixed with the 4 categorical ASC group in the absence of estrogen .................................................................95
3.8 Donor’s obesity status and depot site of ASCs differentiates its influence on tumorigenicity in vivo .................................................................................................96
3.9 Cluster diagram of relative gene expression of ASCs characterized by obesity status and depot site of origin .........................................................100
3.10 Estrogen exposure influences leptin expression in ASCs ..........................102
3.11 Leptin neutralizing antibody and letrozole negate enhanced proliferation in obese ASCs .................................................................103
3.12 Levels of leptin expression correlate with decrease survival in ER+/PR+ breast cancers ........................................................................104
4.1 obASCs enhance BCC proliferation ...............................................................131
4.2 Letrozole was unable to inhibit the effects of obASCs .................................132
4.3 Leptin in condition media is essential for the obASC-drive BCC proliferation ......................................................................................................133
4.4 InASCs and obASCs were stably transfected with ctrl-shRNA and lep-shRNA .............................................................................................134
4.5 Leptin is essential for the obASC-drive BCC proliferation ............................136
4.6 obASC-driven leptin increases invasion of BCC cells .....................................137
4.7 Leptin inhibition reduces the expression of key regulatory genes involved in invasion and metastasis .........................................................141
4.8 Leptin inhibition in obASCs reduces tumor volume ......................................145
4.9 obASC-derived leptin induces SERPINE1 and MMP2 expression in MCF7 xenografts ......................................................................................150
4.10 Leptin is essential for the obASC-driven metastasis MCF7 cells in vivo .................................................................151
4.11 obASCs enhance metastasis of BCCs ............................................................152
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-FC</td>
<td>5-fluorocytosine</td>
</tr>
<tr>
<td>5-FU</td>
<td>5-fluorouracil</td>
</tr>
<tr>
<td>Ab⁻</td>
<td>non-abdominal source of adipose tissue</td>
</tr>
<tr>
<td>Ab⁺</td>
<td>abdominal source of adipose tissue</td>
</tr>
<tr>
<td>ABCG2</td>
<td>ATP-binding cassette, sub-family G, member 2</td>
</tr>
<tr>
<td>ACTB</td>
<td>actin</td>
</tr>
<tr>
<td>ADAM23</td>
<td>ADAM metallopeptidase domain 23</td>
</tr>
<tr>
<td>AKT</td>
<td>protein kinase B</td>
</tr>
<tr>
<td>α-MEM</td>
<td>alpha-Minimum Essential Medium</td>
</tr>
<tr>
<td>α-SMA</td>
<td>alpha-smooth muscle actin</td>
</tr>
<tr>
<td>AMP</td>
<td>adenosine monophosphate-activated protein</td>
</tr>
<tr>
<td>AMPK</td>
<td>AMP kinase</td>
</tr>
<tr>
<td>ASCs</td>
<td>Adipose stromal cells, adipose-derived stem cells</td>
</tr>
<tr>
<td>BAD</td>
<td>BCL2-associated agonist of cell death</td>
</tr>
<tr>
<td>BBB</td>
<td>blood brain barrier</td>
</tr>
<tr>
<td>BCC</td>
<td>breast cancer cell</td>
</tr>
<tr>
<td>BIRC5</td>
<td>baculoviral IAP repeat containing 5</td>
</tr>
<tr>
<td>BMI</td>
<td>body mass index</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
</tr>
<tr>
<td>---------</td>
<td>-----------</td>
</tr>
<tr>
<td>BMSC</td>
<td>bone marrow-derived mesenchymal stem cells</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CAM</td>
<td>chick chorioallantoic membrane</td>
</tr>
<tr>
<td>CAST</td>
<td>calpastatin</td>
</tr>
<tr>
<td>CCL5</td>
<td>chemokine (C-C motif) ligand 5</td>
</tr>
<tr>
<td>CCM</td>
<td>complete culture media</td>
</tr>
<tr>
<td>CCND2</td>
<td>cyclin D2</td>
</tr>
<tr>
<td>CD</td>
<td>cytosine deaminase</td>
</tr>
<tr>
<td>CD29</td>
<td>integrin beta-1</td>
</tr>
<tr>
<td>CD73</td>
<td>5'-nucleotidase, ecto-5'-nucleotidase</td>
</tr>
<tr>
<td>CD90</td>
<td>thy-1</td>
</tr>
<tr>
<td>CD105</td>
<td>endoglin</td>
</tr>
<tr>
<td>CD106</td>
<td>vascular cell adhesion protein-1; VCAM-1</td>
</tr>
<tr>
<td>CD166</td>
<td>ALCAM</td>
</tr>
<tr>
<td>CD11b</td>
<td>integrin alpha M</td>
</tr>
<tr>
<td>CD45</td>
<td>protein kinase phosphatase, receptor C</td>
</tr>
<tr>
<td>CDKN2A</td>
<td>cyclin dependent kinase inhibitor 2A</td>
</tr>
<tr>
<td>CDS-FBS</td>
<td>charcoal dextran stripped-fetal bovine serum</td>
</tr>
<tr>
<td>CDH13</td>
<td>cadherin 13</td>
</tr>
<tr>
<td>CFU</td>
<td>colony forming units</td>
</tr>
<tr>
<td>CM</td>
<td>conditioned media</td>
</tr>
<tr>
<td>CSF1</td>
<td>colony stimulating factor 1</td>
</tr>
<tr>
<td>CXCL1</td>
<td>chemokine (C-X-C motif) ligand 1</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Name</td>
</tr>
<tr>
<td>-----------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>CXCL2</td>
<td>chemokine (C-X-C motif) ligand 2</td>
</tr>
<tr>
<td>CXCR2</td>
<td>C-X-C chemokine receptor type 2</td>
</tr>
<tr>
<td>CXCR4</td>
<td>C-X-C chemokine receptor type 4</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
</tr>
<tr>
<td>DP-MSC</td>
<td>dental pulp-mesenchymal stem cells</td>
</tr>
<tr>
<td>EAE</td>
<td>experimental autoimmune encephalomyelitis</td>
</tr>
<tr>
<td>E2</td>
<td>estradiol</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>EMT</td>
<td>epithelial-to-mesenchymal transition</td>
</tr>
<tr>
<td>ER</td>
<td>estrogen receptor</td>
</tr>
<tr>
<td>ER^+</td>
<td>estrogen receptor positive</td>
</tr>
<tr>
<td>ERK1/2</td>
<td>extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>ESR1</td>
<td>estrogen receptor alpha</td>
</tr>
<tr>
<td>FAK</td>
<td>focal adhesion kinase</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FFPE</td>
<td>formalin-fixed, paraffin-embedded</td>
</tr>
<tr>
<td>G-MSC</td>
<td>gingival-mesenchymal stem cells</td>
</tr>
<tr>
<td>GCV</td>
<td>ganciclovir</td>
</tr>
<tr>
<td>GSK3</td>
<td>glycogen synthase kinase</td>
</tr>
<tr>
<td>GSTP1</td>
<td>glutathione S-transferase P</td>
</tr>
<tr>
<td>GVHD</td>
<td>graft-versus-host disease</td>
</tr>
<tr>
<td>HIF-1α</td>
<td>hypoxia induced factor-1alpha</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
</tr>
<tr>
<td>---------------</td>
<td>-------------------------------------------------------------------</td>
</tr>
<tr>
<td>HSC</td>
<td>hematopoietic stem cells</td>
</tr>
<tr>
<td>HSV-tk</td>
<td>herpes simplex virus-thymidine kinase</td>
</tr>
<tr>
<td>ICI182,780</td>
<td>Fulvestrant</td>
</tr>
<tr>
<td>IDC</td>
<td>invasive ductal carcinoma</td>
</tr>
<tr>
<td>IDO</td>
<td>indoleamine 2,3-dioxygenase</td>
</tr>
<tr>
<td>IFNβ</td>
<td>interferon beta</td>
</tr>
<tr>
<td>IGFBP3</td>
<td>insulin-like growth factor binding protein 3</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>IL-6</td>
<td>interleukin-6</td>
</tr>
<tr>
<td>IL-10</td>
<td>interleukin-10</td>
</tr>
<tr>
<td>IL-12</td>
<td>interleukin-12</td>
</tr>
<tr>
<td>IL-13</td>
<td>interleukin-13</td>
</tr>
<tr>
<td>JAK2</td>
<td>Janus kinase 2</td>
</tr>
<tr>
<td>LEP</td>
<td>leptin</td>
</tr>
<tr>
<td>Lep⁻</td>
<td>leptin shRNA knockdown</td>
</tr>
<tr>
<td>LEPR</td>
<td>leptin receptor</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MCHR1</td>
<td>melanin-concentrating hormone 1</td>
</tr>
<tr>
<td>MCP-1</td>
<td>monocyte chemotactic protein-1</td>
</tr>
<tr>
<td>MI</td>
<td>myocardial infarction</td>
</tr>
<tr>
<td>MMP</td>
<td>matrix metallopeptidase</td>
</tr>
<tr>
<td>MMP-2</td>
<td>matrix metallopeptidase 2</td>
</tr>
<tr>
<td>MMP-9</td>
<td>matrix metallopeptidase 9</td>
</tr>
</tbody>
</table>
MMP-14 matrix metallopeptidase 14
MMP-15 matrix metallopeptidase 15
MSC mesenchymal stem cell
NC negative control for shRNA construct targeting a non-human gene
neg ctrl no template controls
NF-κB nuclear factor kappa-light-chain-enhancer of activated B cells
NK cell natural killer cell
NME1 NME/NM23 nucleoside diphosphate kinase 1
Ob−, ln lean donor
Ob+, ob obese donor
P/S penicillin/streptomycin
PAI-1 plasminogen activator inhibitor type 1
PBS phosphate buffered saline
PBST phosphate-buffered solution with 0.01% TWEEN-20
PGE2 prostaglandin E2
PGR progesterone receptor
PI3K phosphatidylinositol 3-kinase
PLAU plasminogen activator
PPAR-gamma peroxisome proliferator-activated receptor gamma
PTEN phosphatase and tensin homolog
PTGS2 prostaglandin-endoperoxide synthase 2
ROS reactive oxygen species
RT-PCR reverse transcriptase polymerase chain reaction
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>SBTI</td>
<td>soybean trypsin inhibitor</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>SERPINE1</td>
<td>plasminogen activator inhibitor type 1</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SF</td>
<td>serum free</td>
</tr>
<tr>
<td>SDF-1</td>
<td>stromal-derived factor-1</td>
</tr>
<tr>
<td>SFRP1</td>
<td>secreted frizzled-related protein 1</td>
</tr>
<tr>
<td>shRNA</td>
<td>short hairpin RNA</td>
</tr>
<tr>
<td>shRNA neg ctrl</td>
<td>shRNA construct targeting a non-human gene</td>
</tr>
<tr>
<td>SLIT2</td>
<td>slit homolog 2</td>
</tr>
<tr>
<td>SNAI2</td>
<td>snail homolog 2</td>
</tr>
<tr>
<td>SORT1</td>
<td>sortilin1</td>
</tr>
<tr>
<td>STAT3</td>
<td>signal transducer and activator of transcription 3</td>
</tr>
<tr>
<td>STC-1</td>
<td>stanniocalcin-1</td>
</tr>
<tr>
<td>TCGA</td>
<td>The Cancer Genome Atlas</td>
</tr>
<tr>
<td>TGFβ-1</td>
<td>transforming growth factor β-1</td>
</tr>
<tr>
<td>TH17</td>
<td>T-helper 17 cell</td>
</tr>
<tr>
<td>THBS1</td>
<td>thrombospondin 1</td>
</tr>
<tr>
<td>THRB</td>
<td>thyroid hormone receptor-beta</td>
</tr>
<tr>
<td>TIMP</td>
<td>tissue inhibitor of metalloproteinase</td>
</tr>
<tr>
<td>TNF- α</td>
<td>tumor necrosis factor- alpha</td>
</tr>
<tr>
<td>TRH</td>
<td>thyrotropin-releasing hormone</td>
</tr>
<tr>
<td>TRAIL</td>
<td>tumor necrosis factor related apoptosis-induced ligand</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>Treg</td>
<td>regulatory T cell</td>
</tr>
<tr>
<td>TWIST1</td>
<td>twist basic helix-loop-helix transcription factor 1</td>
</tr>
<tr>
<td>UCB-MSC</td>
<td>umbilical cord blood-mesenchymal stem cells</td>
</tr>
<tr>
<td>uPA</td>
<td>urokinase plasminogen activator</td>
</tr>
<tr>
<td>uPAR</td>
<td>urokinase plasminogen activator receptor</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
<tr>
<td>WAT</td>
<td>white adipose tissue</td>
</tr>
<tr>
<td>WHR</td>
<td>waist-to-hip ratio</td>
</tr>
</tbody>
</table>
CHAPTER 1: INTRODUCTION

1.1 Mesenchymal Stem Cells (MSCs)

As early as the 1970s, mesenchymal stem cells (MSCs) were characterized as fibroblast-like stromal cells residing in the trabecular bone anastomoses of long bones and secreting various cytokines and growth factors that support the proliferation and the differentiation of hematopoietic stem cells (HSCs) [1]. While MSCs were initially used as feeder cells to maintain the HSCs, it was later discovered that MSCs have the potential to differentiate into a variety of mesenchymal lineage cells, such as adipocytes, osteocytes, and chondrocytes [2]. As such, these cells have warranted further investigations for their potential use in clinical practice.

According to the International Society for Cellular Therapy, for stem cells to be considered mesenchymal in origin, they must possess additional characteristics in addition to the capacity to differentiate into osteoblasts, adipocytes, or chondroblasts. MSCs must maintain adherence to plastic when cultured in standard conditions, be fibroblast-like in appearance, be able to self-renew as demonstrated through the ability to form colony forming units (CFUs), and express specific cell surface markers (Table 1).

The cell surface marker profile of MSCs includes: CD29+, CD44+, CD73+, CD90+, CD105+, CD106+, CD166+, CD11b−, CD14−, CD34−, and CD45−.
Table 1. Characteristics of Mesenchymal Stem Cells.

<table>
<thead>
<tr>
<th>Characteristics of Mesenchymal Stem Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibroblast-like in appearance</td>
</tr>
<tr>
<td>Plastic-adherent</td>
</tr>
<tr>
<td>Differentiate into osteoblasts, adipocytes, and chondroblasts</td>
</tr>
<tr>
<td>Express specific cell surface markers</td>
</tr>
<tr>
<td>Self-renewal capacity</td>
</tr>
</tbody>
</table>
While MSCs were originally isolated from human bone marrow (BMSCs), MSCs have since been found in many other adult tissues, such as adipose tissue (ASCs) [3], umbilical cord blood (UCB-MSC) [4], skeletal muscle [5], dental pulp (DP-MSC) [6], and gingiva (G-MSC) [7]. Recent studies have utilized MSCs for clinical application in cell-based therapies and tissue engineered products to regenerate adipose tissue, bone, and cartilage (Figure 1). Furthermore, additional studies have also demonstrated that under appropriate inductive conditions, these cells can acquire the phenotype of unrelated germ line lineages, such as neurons [8], epithelial cells [9], hepatocytes [10], endothelial cells [11], pancreatic cells [12], suggesting possible transdifferentiation of cells and the increasing interest for additional clinical applications (Figure 1). Due to their differentiation and transdifferentiation potential, widespread clinical use of \textit{ex vivo} cultured allogenic or autologous MSCs are now in clinical trials for refractory acute graft versus host disease [13], acute myocardial infarction [14, 15], chronic obstructive pulmonary disease [16], and wound healing [17].

With the easy accessibility through a minimally invasive procedure, the safety and effective transplantation to host, and the abundance in supply, the interest in investigating and utilizing ASCs for clinical application has grown rapidly. It has been demonstrated that the isolation of stromal cells from adipose tissue has been shown to be 100 to 500 fold higher than of BMSCs, averaging an upwards of $5 \times 10^3$ ASCs per gram of adipose tissue [18, 19].

1.2 MSCs in Cell-Based Therapies
1.2.1 Autoimmune Diseases and Severe Inflammation-Related Diseases

MSCs modulate the inflammatory response by downregulating pro-inflammatory cytokines and upregulating anti-inflammatory factors [20]. While many factors are secreted by MSCs as a result of the local and systemic environment, several key factors, including interleukin-6 (IL-6), interleukin-10 (IL-10), and indoleamine 2,3-dioxygenase (IDO), are worth highlighting. Previous studies have shown that IL-6 is a MSC-induced immune molecule believed to modulate the immune system through the inhibition of monocyte differentiation into dendritic cells exerted by MSCs [26]. IL-10 is another anti-inflammatory cytokine that has been shown to be involved in many immunosuppressive processes and is therefore considered to play a critical role in preventing inflammatory and autoimmune pathologies [21, 22]. Previous studies have shown that IL-10 inhibits the proliferation of peripheral blood mononuclear cells (PBMCs), and neutralization of IL-10 in a co-culture of MSCs with activated PBMCs resulted in the abrogation of the inhibitory effect exerted by MSCs [23]. Another soluble factor suggested to be involved in MSC-mediated immunosuppression is IDO. IDO is a rate-limiting enzyme that catabolizes the essential amino acid tryptophan and its upregulation depletes natural killer (NK) cells of tryptophan, rendering this innate immune cell inactive [24, 25]. Additional factors important in the immunomodulation of MSCs include TGF-β and PGE2, which will be discussed in detail below.

MSCs suppress T cell and NK cell function, increase the number of regulatory T cells (Tregs), and modulate dendritic cell activity [27-34]. These characteristics allow MSCs to be ideal candidates for immunotherapeutic agents in a variety of autoimmune and severe inflammation-related diseases, such as experimental autoimmune
Figure 1. MSC multilineage differentiation potential. Mesenchymal stem cells are able to undergo extensive self-renewal prior to differentiation into many cell types. These cell types include bone, cartilage, muscle, stroma, tendon, and adipose tissue. MSCs can also differentiate into non-mesenchymal tissue, including liver, heart, skin, and neurons. (Adopted from Caplan and Bruder, 2001).
Table 2. BMSC therapy in experimental animal models of autoimmune diseases and severe inflammation-related diseases.

<table>
<thead>
<tr>
<th>Administration Route</th>
<th>Cell Source</th>
<th>Results</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Experimental autoimmune encephalomyelitis</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intravenous</td>
<td>Syngeneic (C57Bl/6)</td>
<td>Clinical amelioration</td>
<td>[35, 36]</td>
</tr>
<tr>
<td>Intravenous</td>
<td>Xenogeneic (human)</td>
<td>Clinical amelioration</td>
<td>[37]</td>
</tr>
<tr>
<td>Intravenous and intraventricular</td>
<td>Syngeneic (C57Bl/6)</td>
<td>Decreased inflammatory infiltrates</td>
<td>[38]</td>
</tr>
<tr>
<td>Intrapertoneal</td>
<td>Syngeneic (C57Bl/6)</td>
<td>Clinical amelioration</td>
<td>[39, 40]</td>
</tr>
<tr>
<td>Intrapertoneal</td>
<td>Allogeneic (BALC/c to C57Bl/6)</td>
<td>Clinical amelioration</td>
<td>[41]</td>
</tr>
<tr>
<td>Intrapertoneal</td>
<td>Xenogeneic (human)</td>
<td>Decreased inflammatory infiltrates and demyelination</td>
<td>[42]</td>
</tr>
<tr>
<td><strong>Myasthenia gravis</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intravenous</td>
<td>Xenogeneic (human)</td>
<td>Improved clinical score, reduced anti-AChR antibodies in serum</td>
<td>[43]</td>
</tr>
<tr>
<td><strong>Experimental diabetes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intravenous</td>
<td>Syngeneic (NOD1) Allogeneic (BALB/c to NOD)</td>
<td>Enhance insulin secretion and sustained normoglycemia T cell shift toward IL-10/IL-13 production and higher frequency of Tregs</td>
<td>[44]</td>
</tr>
<tr>
<td>Intravenous</td>
<td>Xenogeneic (human) Allogeneic (C57Bl/6 to DBA/1)</td>
<td>Reversed hyperglycemia Decreased Th1 cell expansion Induction of de novo generation of Treg cells</td>
<td>[45]</td>
</tr>
<tr>
<td><strong>Rheumatoid arthritis</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intrapertoneal</td>
<td>Allogeneic (C57B/6l to DBA/1)</td>
<td>Prevention of severe tissue damage, induced hypo-responsiveness of T cells and altered serum cytokine profile</td>
<td>[46]</td>
</tr>
<tr>
<td><strong>Systemic Lupus Erythematosus</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intravenous</td>
<td>Xenogeneic (human)</td>
<td>Amelioration of disease activity Improvement in serologic markers Improvement in renal function Suppression of Th17 cells Increase in Treg cells</td>
<td>[47]</td>
</tr>
<tr>
<td>Intravenous</td>
<td>Xenogeneic</td>
<td>Reduced proliferation of T cells</td>
<td>[48]</td>
</tr>
</tbody>
</table>

Abbreviations: IL, interleukin; Th, T helper; Treg, regulatory T cells
Table 3. ASC therapy in experimental animal models of autoimmune diseases and severe inflammation-related diseases.

<table>
<thead>
<tr>
<th>Administration Route</th>
<th>Cell Source</th>
<th>Results</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Experimental autoimmune encephalomyelitis</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intravenous</td>
<td>Syngeneic (C57Bl/6)</td>
<td>Improve clinical score</td>
<td>[49]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reduce demyelination and axon loss</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Immune modulation and decrease spinal cord inflammation</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Xenogeneic (human)</td>
<td>Improved clinical scores</td>
<td>[50]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Limited podocyte injury by high glucose</td>
<td></td>
</tr>
<tr>
<td>Intravenous</td>
<td>Syngeneic (HIGA)</td>
<td>Diminished proteinuria</td>
<td>[51]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Glomerulosclerosis was decreased</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Levels of fibrotic and inflammatory molecules were markedly decreased</td>
<td></td>
</tr>
<tr>
<td><strong>Rheumatoid arthritis</strong></td>
<td></td>
<td>Prevention of severe tissue damage</td>
<td>[46]</td>
</tr>
<tr>
<td>Intraperitoneal</td>
<td>Syngeneic (DBA/1)</td>
<td>Reduced Th1 and Th17 cells response</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Induced Treg cell generation</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Altered serum cytokine profile</td>
<td></td>
</tr>
<tr>
<td><strong>Graft-versus-host disease (GVHD)</strong></td>
<td></td>
<td>Rescued lethal GVHD</td>
<td>[52, 53]</td>
</tr>
<tr>
<td>Intravenous</td>
<td>Xenogeneic (human)</td>
<td>Limited inflammation by suppressing the expression of inflammatory cytokines (TNFα, IFNγ, IL-12)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Allogeneic (C57Bl/6 to B6D2F1 [H2b/d])</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: Th, T helper; Treg, regulatory T cells; TNFα, tumor necrosis factor alpha; IFNγ, interferon gamma; IL, interleukin
encephalomyelitis (EAE), myasthenia gravis, diabetes, nephropathy, rheumatoid arthritis, systemic lupus erythematosus, and graft versus host disease (GVHD; Table 2; Table 3). Recent studies suggest that MSCs modulate a variety of T cell responses by producing cytokines from direct cellular contact as well as through secreted factors. After mitogen stimulation, MSCs produce numerous cytokines, such as IL-6 and TGF-β, that regulate the activity of pro-inflammatory T helper 17 (Th17) cells and enhance anti-inflammatory Treg cells [54]. Furthermore, MSCs prevent T cell responses to cellular and nonspecific mitogenic stimuli, targeting both naïve and memory CD4+ and CD8+ T cells to modulate the immune system [54]. Therefore, MSCs regulate differentiation and development of different T cell subsets, all of which mediate immune-regulatory function.

Other cell types that MSCs modulate are NK cells and macrophages. NK cells secrete cytotoxic cytokines in response to external environmental cues. Studies have shown that MSCs inhibit the IL-2-induced proliferation of resting NK cells, reduce the expression of the activating receptors of NK cells, and mitigate secretion of interferon-γ of resting NK cells by approximately 80% [33, 55, 56]. Therefore, MSCs are expected to regulate innate immunity by limiting the activity of NK cells. Similarly, MSCs mediate macrophage activity by several mechanisms, including the enhanced secretion of anti-inflammatory cytokine IL-10, prostaglandin E2 (PGE2), transforming growth factor-β (TGF-β) to quell inflammation, promote tissue remodeling, and eliminate tissue debris [57-59].

1.2.2 Regenerative medicine
MSCs also function as reparative cells that regenerate not only cells of mesenchymal origin (e.g. osteoblasts, myocytes, chondroblasts) but also cells from other lineages (e.g. lung epithelial cells, keratinocytes, hepatocytes) [60-63]. Studies have shown that the rate of new bone formation following MSC treatment of calvarial defect wounds were accelerated [64, 65]. MSCs implanted onto an osteogenic scaffold have enhanced osteogenic capacity compared to osteogenic scaffolds without MSCs. These findings were associated with an upregulation of several factors, including bone morphogenetic protein 2 and hypoxia induced factor (HIF)-1α [64, 66]. Additional studies have found that conditioned media isolated from MSCs were able to equally induce closure of calvarial defects by accelerating bone regeneration [67].

Additionally, recent studies have shown that MSCs are capable of aiding in the healing of the heart following myocardial infarction (MI). Delivered MSCs are believed to limit the damage acquired during an MI by limiting the inflammation around the local ischemic region [60, 61]. MSCs take on a reparative role and differentiate into many different cell types, such as cardiomyocytes, endothelial cells, and vascular smooth muscle cells to replenish the damaged cells during MI [68-70].

In another model, MSCs have been shown to aid in the treatment of neurological disorders. Recently, it has been suggested that the trophic effects of MSCs, including the secretion of glial cell-derived neutrophic factor, brain derived neurotrophic factor, nerve growth factor, and vascular endothelial growth factor (VEGF) enhance neural survival and differentiation [71-73]. These factors secreted by MSCs are also believed to simulate angiogenesis and migration of endogenous neural stem cells to the injury site. MSCs utilize both mechanisms to improve neural function.
MSCs have also been examined for their potential to aid in wound healing. Recently, it was shown that MSCs injected into a 10-mm full thickness punch biopsy wound in female C57Bl/6 mice were able to transdifferentiate into several cell types. More specifically, MSCs injected into wounds were able to differentiate into epithelial cells, pericytes, and endothelial cells \textit{in vivo} [74-76]. Studies have also shown that MSCs enhance angiogenesis or differentiate into pericytes and aid in skin regeneration. Additionally, MSCs have been shown to limit the amount of inflammation caused by a wound, recruit of keratinocytes, dermal fibroblast, and host stem cells to regenerate the wound, secrete matrix metalloproteinases (MMPs) and tissue inhibitor of metalloproteinases (TIMPs) to regulate remodeling, and regulate collagen deposition to limit fibrosis and increase strength [77]. Chen et al. demonstrated that MSCs were able to locally migrate throughout the wound and engraft into the wound [78]. Furthermore, it has been shown that topical administration of MSCs onto skin ulcers accelerate the healing process of the skin wounds by increasing granulation thickness and capillary density, suggesting that the factors secreted by MSCs are capable of inducing regeneration [79]. Nevertheless, understanding the precise mechanism by which MSCs interact with their milieu will aid in our ability to be use in clinical practice.

\textit{1.2.3 Suicide cancer gene therapy}

Stem cell-driven cancer gene therapy is based on the tumor-trophic property of MSCs. Recent studies have found that MSCs circulate and traffic to injured or inflamed tissue, such as a tumor, and incorporates into the tumor microenvironment. It is thought
that increased inflammatory mediators found at the tumor site are responsible for the recruitment and engraftment of MSCs [80]. Recent studies suggest that cancer cells produce monocyte chemotactic protein-1 (MCP-1) to simulate the migration of MSCs to the tumor site [81]. Additional studies have shown that the upregulation of chemokine (C-X-C motif) ligand 2 (CXCL2), C-X-C chemokine receptor 4 (CXCR4), and stromal cell derived factor 1 (SDF-1) on MSCs is responsible for their enhanced mobility following cancer cell conditioned media [82-85]. Inhibiting SDF-1 expression reduced migration of MSCs, thus verifying the importance of its expression by MSCs in the migration process [82].

The homing ability of MSCs to a tumor has been investigated using *in vivo* models of colon cancer, glioblastomas, prostate cancer, and breast cancer [86]. Studies conducted in mice implanted with human colon cancer HT-29 found that ASCs mobilize to the cancer site [87]. In a glioblastoma model, tumor-bearing mice were delivered MSCs on the contralateral side, and it was shown that MSCs migrate through the corpus callosum towards malignant glioma cells in the opposite hemisphere [88, 89]. Both *in vitro* and *in vivo* studies on prostate cancer, hepatocellular carcinoma, and melanoma also confirm the migratory capabilities of MSCs to the tumor site [90-92].

MSC-targeted cancer gene therapy utilizes gene-directed enzyme prodrug therapy or suicide gene therapy to target the cancer. It does so by converting minimally toxic drugs into highly toxic compounds to eliminate both the vehicle (MSCs) and cancer cells around the MSCs, known as the bystander effect [93, 94]. A non-human enzyme is first transduced into MSCs. These transduced MSCs expressing the enzyme is delivered systemically and allowed to home to the tumor [95]. Next, the enzymatic activity of the
gene product is able to convert a far less toxic prodrug to its cytotoxic metabolite at the tumor site [95]. Consequently, the active drug produced by the enzymatic process within transduce MSCs allows for the effective killing of neighboring tumor cells along with the transduced MSC through the bystander effect. Thus, the genetically modified MSCs are eliminated from the organisms as well. In addition, dying tumor and therapeutic stem cells can induce host immune responses mediated by NK cells and T cells to further eliminate any remaining cancer cells. This therapeutically beneficial effect is coined the distant bystander effect [96-98].

The two most commonly investigated prodrug systems include: cytosine deaminase/5-fluorocytosine prodrug system and herpes simplex virus thymidine kinase/gancyclovir prodrug system. The cytosine deaminase(CD)/5-fluorocytosine (5-FC) prodrug system utilizes either bacterial or yeast CD to convert 5-FC to 5-fluorouracil (5-FU). Since this gene is only found in either bacteria or yeast, human or mammalian cells are unable to convert the prodrug into 5-FU [99]. Utilizing a human colon cancer model established by implanting HT-29 cells into mice, ASCs transduced with genetically engineered yeast CD were delivered into the mice and shown to home to the tumor and decrease tumor size [87]. Furthermore, it has been shown that these transduced ASCs were able to traffic to secondary lesions in the bone and eliminate the metastatic lesions. Another advantage of ASCs is their ability to cross the blood brain barrier (BBB) and deliver the active drug to the tumor site. While 5-FU is effective against brain tumors and metastasis, 5-FU is unable to cross the BBB [100, 101]. However, the substrate 5-FC is able to cross the BBB and its conversion into 5-FU with this prodrug system has the potential to reduce tumor burden. Paralleling in concept, the herpes simplex virus-
thymidine kinase (HSV-tk)/ganciclovir (GCV) prodrug system. In this system, HSV-tk is transduced into MSCs and allowed to engraft into the tumor microenvironment. Once there, GCV is delivered, which will be converted into GCV-triphosphate by the HSV-tk enzyme in MSCs. GCV-triphosphate inhibits DNA synthesis and results in cell death via apoptosis [102-104]. This system has been proven effective again killing glioblastoma, gastric adenocarcinomas, metastatic breast cancer, pancreatic cancer, and prostate cancer cells in animal models [105-110]. Currently, human clinical trials are being conducted to determine the safety and efficacy of this suicide gene therapy in cancer.

Alternative gene therapies delivered through MSCs have targeted the immune system or induced apoptosis and have been shown to be successful at reducing the tumor burden of metastatic breast cancer, medulloblastomas, gliomas, and melanoma models [111]. In particular, MSCs engineered to overexpress interferon β (IFNβ) was used as a potent anti-proliferative and pro-apoptotic agent. Utilizing tumor-bearing mice, MSCs overexpressing IFNβ were shown to migrate towards established tumors and exerted anti-tumor effects through associated increases in local IL-12 [112]. Additional, tumor-bearing mice treated directly with MSCs overexpressing IL-12 also exerted anti-tumor effects and were resistant to repeated establishment of gliomas, translating to lower reoccurrence rate [112, 113]. MSCs transduced with tumor necrosis factor related apoptosis-induced ligand (TRAIL) also significantly reduced tumor burden [114, 115]. TRAIL-MSCs migrated into the tumor and activated the apoptotic cascade reducing tumor volume and increasing overall patient survival [116, 117].

Although it has been established that systemically administered MSCs specifically home to neoplastic lesions and genetically engineered MSCs are able to
reduce tumor burden, there is a possibility that some remaining MSC might contribute to
tumorigenesis. This topic will be further discussed in Section 1.3. As such, suicide gene
therapy may be an option for advance-staged cancers, where treatment with MSCs will
alleviate symptoms without the possibility of further advancing the disease.

### 1.3 MSCs in the Tumor Microenvironment and Cancer Metastasis

Cancerous epithelial cells coexist in carcinomas with a biologically complex
stroma composed of MSCs (both BMSCs and ASCs), extracellular matrix, and immune
cells, which together create the complexity of the tumor microenvironment. The
significant contributions of the stroma has been examined and highlighted through the
increased incidence of tumor formation in tissues exhibiting a chronically inflamed
stroma. MSCs are believed to acquire an activated phenotype through the interactions of
the tumor microenvironment. Normal human mammary stromal cells mixed with human
breast carcinoma cells and injected subcutaneously into immunodeficient mice were
found to transform into myofibroblast as determined by α-smooth muscle actin (α-SMA)
expression and provide support to the establishment of a malignant tumor [118]. Cancer
conditioned stromal cells have been shown to closely resembled the stromal cells isolated
from primary tumors. Collectively, these findings suggest that pre-existing normal
stromal cells acquire an activated phenotype due to conditioning by the tumor cells
during the course of tumor progression.

Within the tumor, MSCs have been shown to enhance tumor growth as well as
metastasis in cancers arising from a wide range of tissues, such as the breast, colon, lung,
and prostate [119-124]. MSCs influence tumorigenesis by secreting a plethora of molecules, such as chemokines, cytokines, and growth factors that act through direct paracrine influences on the cancer cells to promote tumor proliferation, invasion and metastasis [125, 126]. In particular, MSC-derived chemokines, chemokine (C-X-C motif) ligand 1 (CXCL1), chemokine (C-X-C motif) ligand 2 (CXCL2), and SDF-1 increased cancer cell proliferation by activating receptors on the cancer cells [127, 128]. Similarly, cytokines secreted by MSCs, which include IL-6 and IL-8, have been demonstrated to enhance cancer cell tumorigenesis and metastasis [129, 130]. MSCs also respond to cues secreted by the tumor that transform them into an activated state, resulting in the secretion of additional factors that influence tumor pathogenesis. Of particular note is the secretion of chemokine (C-C motif) ligand 5 (CCL5) and stanniocalcin-1 (STC-1) by MSCs, which has been shown to enhance breast cancer metastasis and lung cancer metastasis, respectively [125, 131]. It should be noted that not all cancer cells express receptors for CCL5 or STC-1 and that secretion of these factors by the MSC is cancer dependent, suggesting that their secretion is dependent on the cancer signals within the tumor milieu.

MSCs also exert indirect pro-malignant actions by promoting tumor angiogenesis through recruitment of endothelial progenitor cells and by facilitating the formation and maturation of tumor vasculature [132]. MSCs play multifaceted roles in fostering tissue revascularization after injury and appear to serve similar pro-angiogenic functions in the setting of tumor development. MSCs have been shown to promote tumor neoangiogenesis through the secretion of proangiogenic factors, such as VEGF, angiopoietins, epidermal growth factor, keratinocyte growth factors, insulin-like growth
factor-1, and galectin-1 [124, 133, 134]. These factors promote the recruitment of endothelial cells and maturation of newly formed blood vessels. Furthermore, MSCs have been shown to differentiate into endothelial-like cells and share cell surface antigens similar to pericytes, which results in stabilization of newly formed blood vessels.

Furthermore, MSCs have also been shown to exert immunomodulatory properties that protect the tumor cells from detection and destruction by the adaptive immune system [135, 136]. MSCs inhibit the proliferation and maturation of B-cells and NK cells, while exhibiting protective activities towards other cells, such as neutrophils [137-139]. MSCs have also been shown to inhibit both CD4$^+$ and CD8$^+$ T cell proliferation, suggesting that MSCs influence the behavior of almost all key immune activities involved in tumor development and immune surveillance [140]. The mechanism by which MSCs exert their immunomodulatory effects on the tumor microenvironment has been partially characterized. MSCs have been shown to suppress T cell proliferation through the production of IL-6, IL-8, and C-X-C motif chemokine 10 [141]. MSCs have also been shown to indirectly suppress T cell proliferation by influencing dendritic cell proliferation and maturation into a subtype incapable of stimulating T cell expansion [142]. Lastly, it is believed that MSCs can direct T cells to differentiate into Treg cells, which confers growth and metastatic advantages [143]. These advantages are manifested through Treg-mediated suppression of normal innate and adaptive immune responses.

It is worth noting that the inhibition or the activation of any one single factor does not appear to be sufficient to completely disrupt MSC activity within the tumor, suggesting that multiple concerted mechanisms cooperate to enhance tumorigenesis.
1.4  Obesity, MSCs and Cancer

1.4.1  Obesity and Cancer

Obesity is the excessive accumulation of adipose tissue, resulting in physical and psychological health impairments. The prevalence of obesity in the United States has tripled over the past decade, with more than one third of adults meeting the criteria for obesity [144, 145]. In 2007, the World Cancer Research Fund used a standardized approach to review the affects of obesity on cancer incidence and mortality [146]. It was determined that increased adiposity was associated with increased risk of esophageal adenocarcinoma, pancreatic, colorectal, postmenopausal breast, endometrial, and renal carcinoma (Figure 2) [147]. Additionally, meta-analysis studies confirmed the role of obesity in many cancers in both men and women, including thyroid, colon, renal, hepatocellular, rectal carcinoma, esophageal adenocarcinoma, malignant melanoma, hematological malignancies, and large B-cell lymphomas (Figure 3, Figure 4) [147-153]. In men, obesity has been associated with increased prostate cancer incidence and mortality [152, 153] (Figure 3). In women, strong associations have also been made between obesity and ovarian, endometrial, postmenopausal breast, pancreatic, and gallbladder carcinoma [154-156] (Figure 4). Nevertheless, it remains to be determined the precise mechanism by which obesity enhances tumorigenesis.

1.4.2  Obesity, Inflammation, and Cancer

Obesity is strongly associated with changes in the physiological function of adipose tissue, resulting in chronic inflammation, altered secretion of adipokines, and
Figure 2. BMI and postmenopausal breast cancer. Related risk ± confidence intervals in epidemiological studies considered in the meta-analysis. (Adopted from Bianchini et al., 2002).
Figure 3. Relative risk of developing obesity-associated cancer categorized by cancer site in men. (Adopted from Renehan et al., 2008).
Figure 4. Relative risk of developing obesity-associated cancer categorized by cancer site in women. (Adopted from Renehan et al., 2008.)

<table>
<thead>
<tr>
<th>Cancer site and type</th>
<th>Number of studies</th>
<th>RR (95% CI)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endometrium</td>
<td>19</td>
<td>1.59 (1.50-1.68)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Gallbladder</td>
<td>2</td>
<td>1.59 (1.02-2.47)</td>
<td>0.04</td>
</tr>
<tr>
<td>Oesophageal adenocarcinoma</td>
<td>3</td>
<td>1.51 (1.21-1.74)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Renal</td>
<td>12</td>
<td>1.34 (1.25-1.43)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Leukaemia</td>
<td>7</td>
<td>1.17 (1.04-1.32)</td>
<td>0.01</td>
</tr>
<tr>
<td>Thyroid</td>
<td>3</td>
<td>1.14 (1.06-1.23)</td>
<td>0.001</td>
</tr>
<tr>
<td>Postmenopausal breast</td>
<td>31</td>
<td>1.12 (1.08-1.16)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Pancreas</td>
<td>11</td>
<td>1.12 (1.02-1.27)</td>
<td>0.01</td>
</tr>
<tr>
<td>Multiple myeloma</td>
<td>6</td>
<td>1.11 (1.07-1.15)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Colon</td>
<td>19</td>
<td>1.09 (1.05-1.13)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Non-Hodgkin lymphoma</td>
<td>7</td>
<td>1.07 (1.00-1.14)</td>
<td>0.05</td>
</tr>
<tr>
<td>Liver</td>
<td>1</td>
<td>1.07 (0.85-1.30)</td>
<td></td>
</tr>
<tr>
<td>Gastric</td>
<td>5</td>
<td>1.04 (0.90-1.20)</td>
<td>0.56</td>
</tr>
<tr>
<td>Ovarian</td>
<td>13</td>
<td>1.03 (0.99-1.08)</td>
<td>0.30</td>
</tr>
<tr>
<td>Rectum</td>
<td>14</td>
<td>1.02 (1.00-1.05)</td>
<td>0.76</td>
</tr>
<tr>
<td>Malignant melanoma</td>
<td>5</td>
<td>0.96 (0.92-1.01)</td>
<td>0.05</td>
</tr>
<tr>
<td>Premenopausal breast</td>
<td>20</td>
<td>0.92 (0.80-0.97)</td>
<td>0.001</td>
</tr>
<tr>
<td>Lung</td>
<td>6</td>
<td>0.80 (0.66-0.97)</td>
<td>0.03</td>
</tr>
<tr>
<td>Oesophageal squamous</td>
<td>2</td>
<td>0.57 (0.47-0.69)</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>
altered stem cell biology. While altered secretion of adipokines and altered stem cell biology will be discussed in subsequent sections, the effect of obesity on adipose tissue will be discussed here.

Adipose tissue expansion in obesity results in increased distance between the enlarging adipocytes and the vasculature, leading to local pockets of hypoxia. Large adipocytes can grow up to 100 to 200 µm in diameter and subsequently exceed the normal diffusion distances of oxygen into tissue (Figure 5) [157, 158]. Studies have also demonstrated that oxygen content is close to zero at 100 µm distance from the vasculature [158]. As a result, increased adipocyte size and adipocyte number can result in significant hypoxia. Furthermore, studies have shown that despite the substantial expansion of adipose tissue in obesity, neither cardiac output or total blood flow to the adipose tissue is increased [159, 160]. In obese mice, reduced blood perfusion and hypoxia appeared to be specific to white adipose tissue (WAT) [161]. The lack of oxygen to the adipose tissue results in hypoxia, which in turn results in the inflammatory response to increase blood flow and stimulate angiogenesis. This hypoxic response is presumed to act through HIF-1α (Figure 6) [162, 163]. However, the activation of HIF-1α and its downstream targets are insufficient to compensate for the growing adipocytes, consequently resulting in chronic low-grade inflammation. It is believed that the chronic low-grade inflammation has been hypothesized to increase the secretion of pro-inflammatory cytokines (tumor necrosis factor-α [TNF-α] and IL-6), chemokines (MCP-1), and proteases (MMP2 and PAI-1) that lead to adipose tissue dysfunction and tumor promotion (Figure 7) [164, 165].
One of the factors suspected to contribute to the low grade inflammation is TNF-α. TNF-α plays an important role in the adaptive response of the immune system and other organ systems. Recent studies have suggested that TNF-α is involved in carcinogenesis because of its ability to activate the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway, leading to the expression of a variety of inflammation-related genes [166, 167]. TNF-α appears to contribute to the development of the tissue architecture necessary for tumor growth and metastasis [168]. It also has the potential to induce other cytokines, angiogenic factors and MMPs, which may drive the growth and survival of tumor cells [169]. Similarly, IL-6 is also an important regulator of immune cell growth and differentiation. Recent studies demonstrate that IL-6 regulates chronic inflammation, which can create a cellular microenvironment conducive to cancer growth [170]. High concentrations of circulating IL-6 in obese patients correlated with increased risk of cancer precursor lesions. The activation of IL-6 complex activates both Janus kinase (JAK) and the signal transducer and activator of transcription 3 (STAT3) pathways, which regulate cell proliferation and apoptosis. Likewise, MCP-1 has been shown to play a significant role in the recruitment of immune cells. Specifically, MCP-1 has been shown to recruit macrophages in both obesity and cancer [171][172]. Obesity induced inflammation could contribute to the development of cancer and in part explain the enhanced incidence of breast cancer in obese patients.

1.4.3. Obesity, Adipokines, and Breast Cancer

In recent years, adipose tissue has been considered a complex, essential, and highly active metabolic, endocrine organ. As such, adipose tissue secretes many factors
Figure 5. Oxygen diffusion from the vasculature to distant tissue. Oxygen diffusion through tissue is between 100 and 200 um, depending on the tissue. (Adopted from Folkman et al., 2000).
Figure 6. HIF-1α regulation during normoxia and hypoxia. (A) In normoxia, HIF-1α is hydroxylated by proline hydroxylase (PHD1, 2, 3) in the presence of oxygen. Hydroxylated HIF-1α is recognized by pVHL, and forms a multi-subunit ubiquitin ligase complex, and tags HIF-1α with polyubiquitin for proteasome degradation. (B) In response to hypoxia, proline hydroxylated is inhibited. VHL is unable to target HIF-1α for proteasomal degradation, which leads to an accumulation of HIF-1α accumulation and translocation into the nucleus. Once in the nucleus, HIF-1α dimerizes with HIF-1β, and binds to hypoxia response elements (HREs) within the promoters of the target genes and recruits transcriptional co-activators for full transcriptional activity. This results in angiogenesis and other processes to increase proliferation. (Adopted from Carroll and Ashcroft, 2005).
that have been termed adipokines. Approximately one third of WAT is adipocyte related, leaving the remainder to be associated with fibroblast, macrophages, stromal cells, and monocytes [173]. Previous work by Fain et al. discovered that adipokines are derived from several cell types, and in fact, most of the adipokines come from cells other than the mature adipocytes [174]. Although many adipokines have been identified, WAT was initially recognized as an organ with endocrine properties following the discovery of leptin, which was followed by the discovery of adiponectin and PAI-1 [175]. As such, these compounds have been investigated extensively with regards to normal cellular function as well as aberrant cell function, such as in obesity. In this section, the processes mediated by leptin, adiponectin, and PAI-1 will be discussed in more detail as it relates to obesity, inflammation, and breast cancer.

*Leptin.* Leptin is a product of the* ob* gene, and is expressed and produced by differentiated adipocytes in WAT. Subcutaneous adipose tissue is the main source of leptin production. Leptin acts through the central nervous system, whereby it binds to Y-aminobutyric acid (GABA)-ergic neurons to reduce appetite and increase energy expenditure [176-178]. In obesity, leptin resistance results in hyperphagia and increased adipose tissue [179-181]. The leptin resistance also results in hyperleptinemia such that leptin plasma concentration and mRNA expression in adipose tissue directly correlate to obesity severity [182, 183]. This hyperleptinemia has been shown to be partially responsible for the chronic low-grade inflammation associated with obesity.

Leptin has also been shown to be involved in inflammation by acting on both the adaptive and innate immune systems [184-186]. Leptin receptor is found to be expressed on a variety of immune cells, resulting in enhanced T cell and macrophage activation.
Leptin has also been linked to TNF-α secretion and reactive oxygen-species (ROS) production, MCP-1 expression, and endothelial cell proliferation and migration. These observations suggest that obesity-related hyperleptinemia has the potential to induce a pro-inflammatory environment that enhances tumorigenesis.

With respect to cancer, leptin has also been found to be a significant growth factor in several cancers. Recently, leptin has been shown to not only enhance cancer cell growth but also have the potential to increase migration, invasion, and angiogenesis [187, 188]. Furthermore, through the JAK2-STAT3, PI3K-Akt-GSK3, ERK1/2, and AP-1 pathways, leptin has been shown to increase the expression of proteolytic enzymes that are essential in tumor growth, metastasis, and neoangiogenesis (Figure 8) [189-191]. In estrogen receptor-positive human breast cancer cell lines, leptin has been shown to exert its effect through the activation of the MAPK pathway [190]. Leptin itself can also enhance aromatase activity in MCF7 cells and increase the production of estradiol [190, 192]. Thus, high levels of leptin caused by obesity may result in increased breast cancer incidence, and additional studies may provide clues to the therapeutic potential of anti-leptin antibodies.

Adiponectin. In contrast to leptin, adiponectin has anti-inflammatory effects and circulating levels of adiponectin has been negatively associated with obesity, BMI, and visceral fat accumulation [193, 194]. Several studies have observed that serum adiponectin levels were significantly decreased in breast cancer patients [195, 196]. In
Figure 7. Phenotypic modulation of adipose tissue by obesity. In lean individuals without metabolic dysfunction, adipose tissue secretes anti-inflammatory adipokines and maintains adequate vascular function. With obesity and mild metabolic dysfunction, cells continue to secrete anti-inflammatory adipokines but begin to secrete pro-inflammatory adipokines as well. Mild inflammation is observed at this stage. With prolonged obesity and overt metabolic dysfunction, adipose tissue is a source of pro-inflammatory adipokines (TNFα, IL-6, and CCL2) and inflammation, metabolic dysfunction, and decreased vascular function ensues. (Adopted from Ouchi et al., 2011).
Figure 8. Leptin receptor activation and inactivation. (a) Resting state, (b) Activated state, whereby leptin binds to its receptor and the subunit undergoes a conformational change, resulting in the transphosphorylation and transactivation of JAK2, SHIP2, STAT5, and finally STAT3. STAT3 activates several downstream pathways and cellular processes. (c) LEPRB is inactivated at proximal sites by protein tyrosine phosphatase 1B (PTP1B), a tyrosine phosphatase that directly dephosphorylates JAK2. (Adopted from Coppari et al., 2012).
fact, additional studies have demonstrated that adiponectin treatment suppressed cell proliferation, caused cell growth arrest and apoptosis, and reduced angiogenic activities in breast cancer cells [197]. While the role of adiponectin in cancer etiology is not fully understood, it has been postulated that adiponectin provides indirect protection against carcinogenesis through the activation of AMP-activated protein kinase (AMPK). AMPK plays an important role in the regulation of growth arrest and apoptosis by stimulating p53 and p21 and by decreasing the production of MAPK, thereby inhibiting cell proliferation [198]. Moreover, the anti-carcinogenic effects of adiponectin have been attributed to its anti-inflammatory and immunomodulatory properties. Adiponectin has been shown to inhibit endothelial NF-κB signaling and to markedly reduce TNF-α production in cultured macrophages [199]. Its immunomodulatory properties have been correlated with increased production of anti-inflammatory cytokines IL-10 and IL-1 receptor antagonist in human leukocytes [199].

**PAI-1.** PAI-1 is a serine protease inhibitor produced by several cell types, including endothelial cells, stromal cells, and adipocytes. PAI-1 has been shown to affect adipocyte differentiation, and increased PAI-1 levels have been associated with obesity, [199]. The role of PAI-1 in cancer is associated with increased tumor cell invasion and metastasis. Furthermore, studies have shown that PAI-1 is a poor prognostic indicator for a number of cancers including breast cancer [201].

### 1.4.4 Obesity-associated dysregulation of MSCs and ASCs

Rapid accumulation of excessive adipose tissue associated with obesity leads to hypoxia and increased pro-inflammatory cytokine production (IL-6 and TNF-α) and pro-
inflammatory adipokine secretion (leptin and PAI-1) in the microenvironment where the stromal cells are maintained. As such, the ASCs within this inflamed adipose tissue are likely to be altered due to the microenvironment in which they are found.

Recent studies have reported that hypoxia induced by obesity is sufficient to increase the circulation of ASCs. Zhang et al. demonstrated that while a greater number of ASCs could be isolated from the WAT of obese mice compared to lean mice, possibly due to increased WAT from obese mice [202]. Furthermore, Bellows et al. demonstrated that that obesity increased the mobilization of ASCs into circulation [203]. Taken together, obesity increases the number of ASCs, and obesity results in the enhance trafficking of these cells through the blood [202].

To further characterize the role of obesity on ASC function, several studies have investigated the differentiation capacity and proliferation of ASCs isolated from obese individuals (obese ASCs). It has been shown obese ASCs display less potential to differentiate into adipocytes and osteocytes, suggesting a loss of stem cell function in ASCs conditioned by the obesity-induced inflamed microenvironment [203-206].

In order to assess the importance of the tumor chemoattractants to stimulate mobilization of ASCs, tumor-bearing obese mice were compared to tumor-bearing lean mice and shown to have a 6-fold increase in circulating ASCs. Furthermore, it was shown that once localized to the tumor microenvironment, the mobilized ASCs enhanced the tumor vasculature by transdifferentiation into perivascular cells and incorporating into the tumor microenvironment in an obesity dependent manner [202]. The transdifferentiation into perivascular cells increases oxygen and nutrients to the tumor, enhancing survival and limiting apoptosis of cancer cells.
While these studies suggest that the secretion of these pro-inflammatory cytokines and adipokines within the adipose tissue microenvironment could potentially alter ASC capacity to mobilize to damaged tissue, maintain multilineage differentiation, and impact breast cancer tumorigenesis through enhancing alterations in the vasculature, additional studies are necessary to full characterize the alterations caused by obesity and investigate the impact of these alterations on breast cancer tumorigenesis. Studies delineating the migratory or invasive factors involved in the enhanced circulation of ASCs isolated from obese ASCs will help elucidate the pathways involved. Additional analysis of the role of obese ASCs on breast cancer tumorigenesis is necessary to clarify the role of obesity on ASC biology and the effect of that interaction on ASC and breast cancer.
CHAPTER 2: THE ROLE OF OBESITY ON ASC INVASION

Obesity-associated dysregulation of calpastatin and MMP-15 in adipose-derived stromal cells results in their enhanced invasion

Amy L. Strong, Julie A. Semon, Thomas A. Strong, Tatyana T. Santoke, Shijia Zhang, Harris E. McFerrin, Jeffrey M. Gimble, and Bruce A. Bunnell

Published in Stem Cells 2013; 30(12): 2774-2783.

2.1 Abstract

Adipose tissue maintains a subpopulation of cells, referred to as adipose-derived stromal/stem cells (ASCs), which have been associated with increased breast cancer tumorigenesis and metastasis. For ASCs to affect breast cancer cells, it is necessary to delineate how they mobilize and home to cancer cells, which requires mobilization and invasion through extracellular matrix barriers. In this study, ASCs were separated into four different categories based on the donor’s obesity status and depot site of origin. ASCs isolated from the subcutaneous abdominal adipose tissue of obese patients (Ob\(^{+}\)Ab\(^{+}\)) demonstrated increased invasion through Matrigel as well as a chick chorioallantoic membrane, a type I collagen-rich extracellular matrix barrier. Detailed mRNA and protein analyses revealed that calpain-4, calpastatin, and MMP-15 were associated with increased invasion, and the silencing of each protease or protease inhibitor confirmed their role in ASC invasion. Thus, the data indicate that both the
donor’s obesity status and depot site of origin distinguishes the properties of subcutaneous-derived ASCs with respect to enhanced invasion and this is associated with the dysregulation of calpain-4, calpastatin, and MMP-15.

2.2 Introduction

Worldwide, breast cancer mortality rates have increased continuously and substantially and this is correlated with increasing body mass index (BMI) and waist-to-hip ratio (WHR) [207-210]. Abdominally obese women have a higher risk for developing breast cancer compared to non-obese women or women with normal waist-to-hip ratios. Although both BMI and WHR are predictors of cancer risk, BMI quantifies the volume of adipose tissue, whereas WHR reflects the anatomical distribution of adipose tissue. While studies have shown that increased visceral adipose tissue is associated with increased incidence of cancer, subcutaneous adipose tissue has recently been recognized for its role in angiogenesis and support of tumor growth [211]. In addition, both subcutaneous and visceral adipose tissues are composed of a heterogeneous population of cells, which includes adipose-derived stromal stem cells (ASCs). These ASCs have been shown to enhance tumorigenesis and metastasis of both breast cancer cell lines and primary breast cancer samples [212, 213]. However, to contribute to breast cancer tumorigenesis and metastasis, it is necessary for endogenous ASCs to egress from adipose tissue and invade through the extracellular matrix (ECM) into the target tissues [214].

In order for cells to invade into neighboring tissues, they must express a tissue-invasive phenotype that allows them to traverse the basement membrane and infiltrate the
ECM. In humans, four major groups of protease are credited for cellular invasion through the ECM, aspartate protease, serine protease, cysteine protease, and matrix metalloproteinase [215, 216], but the identity of the particular protease that confers tissue-invasive activity to ASCs remains undefined.

Although much remains to be discovered about the molecular machinery that allows both cancer and trafficking cells to disassemble and transmigrate through the ECM, it can be assumed that ASCs utilize similar, if not identical mechanism(s), since they have been demonstrated to traffic to damaged tissues and sites of inflammation [217]. The current work hypothesizes that the invasive phenotype of ASCs may be influenced by both obesity status and adipose depot site of origin. Furthermore, ASCs under the influence of obesity-derived factors may possess properties that distinguish them from ASCs isolated from a lean individual. The ASCs may be conditioned by the local microenvironment within their original site and exhibit unique invasive characteristics. Thus, the aim of this study was to determine the invasive potential of ASCs isolated from subjects based on obesity status and depot site and to explore the underlying mechanism for invasion.

### 2.3 Materials and Methods

#### 2.3.1 Materials

Anti-CD45-PeCy7, anti-CD11b-PeCy5, anti-CD166-PE, anti-CD105-PE, anti-CD90-PeCy5, anti-CD34-PE, isotype-control FITC human IgG1 and isotype-control PE human IgG2a were purchased from Beckman Coulter (Indianapolis, IN). Anti-CD44-APC was purchased from BD Biosciences (San Jose, CA). Anti-MMP-12, anti-MMP-15,
anti-MMP-21, anti-MMP-23, anti-MMP-28, anti-TIMP-4, anti-calpain-1, anti-calpain-2, anti-calpain-3, anti-calpain-4, anti-calpain-5, anti-calpain-6, anti-calpain-7, and anti-calpain-10 were purchased from Abcam (Cambridge, MA). Anti-calpastatin and anti-actin were purchased from Sigma (St. Louis, MO). Rabbit anti-mouse and goat anti-rabbit HRP-conjugated secondary antibodies were purchased from Abcam. Aspartate proteinase inhibitor, pepstatin A (100 mM), serine proteinase inhibitor, soybean trypsin inhibitor, (SBTI; 100 µg/mL), and cysteine proteinase inhibitor, E64d (1 µM), were purchased from Sigma. Synthetic matrix metalloproteinase (MMP) inhibitor, GM6001 (25 µM), was purchased from Millipore (Bedford, MA), and cathepsin inhibitor, 1,3-Bis(CBZ-Leu-NH)-2-propanone (10 µM), was purchased from EMD Chemicals (Gibbstown, NJ). The calpain inhibitor, acetyl-calpastatin (0.2 µM), was purchased from AnaSpec (Fremont, CA), and the caspase inhibitor, Z-VAD-FMK (20 µM), was purchased from Promega (Madison, WI).

2.3.2 Human Subjects

Primary human adipose stromal cells (ASCs) were obtained from 24 Caucasian females undergoing elective liposuction procedures, as previously described [218]. ASCs were isolated from processed lipoaspirates from the subcutaneous abdominal adipose tissue of obese (Ob⁺Ab⁺) or non-obese (Ob⁻Ab⁺) subjects and from non-abdominal subcutaneous adipose depots of obese (Ob⁺Ab⁻) and non-obese (Ob⁻Ab⁻) subjects. Additional demographic information can be found in Table 1. No statistical significance in age was observed between the groups. Six donors per group were individually characterized and expanded. All protocols were reviewed and approved by the
Pennington Biomedical Research Center Institutional Review Board and all human participants provided written informed consent.

2.3.3 *Cell Culture of ASCs*

ASCs were isolated and expanded as previously reported [219]. In brief, frozen vials of approximately $10^6$ ASCs were thawed, plated onto 150 cm$^2$ culture dishes (Nunc; Rochester, NY) in 25 ml complete culture media (CCM) that consisted of $\alpha$-minimal essential medium (GIBCO; Grand Island, NY), 20% fetal bovine serum (Atlanta Biologicals; Lawrenceville, GA), 100 units per ml penicillin (GIBCO), 100 µg/mL streptomycin (GIBCO), and 2 mM L-glutamine (GIBCO) and incubated at 37.5°C with 5% humidified CO$_2$. After 24 hours, the media was removed and adherent, viable cells were washed twice with phosphate buffered saline (PBS), harvested with 0.25% trypsin / 1mM EDTA (Gibco), and replated at 100 cells/cm$^2$ in CCM. Media was changed every 3-4 day. For all experiments, sub-confluent cells ($\leq$70% confluent) between passages 2-6 were used.

2.3.4 *Cell Culture of MDA-MB-231 cells*

MDA-MB-231 cells were obtained from American Type Culture Collection and were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM; GIBCO), supplemented with 10% FBS, 100 units per mL penicillin, and 100 µg/mL streptomycin. Cells were grown at 37°C with 5% humidified CO$_2$, fed every 2-3 days, and split 1:4 to 1:6 when they reached 90% confluent.
Table 1. Donor demographics of ASCs.

<table>
<thead>
<tr>
<th>Obesity Status</th>
<th>Depot Site</th>
<th>Number of Subjects</th>
<th>Race</th>
<th>Gender</th>
<th>Age (mean ± SD)</th>
<th>BMI (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-obese</td>
<td>Non-abdominal</td>
<td>n = 6</td>
<td></td>
<td>Female</td>
<td>52.4 ± 18.0</td>
<td>22.5 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>Abdominal</td>
<td>n = 6</td>
<td>Caucasian</td>
<td></td>
<td>38.8 ± 7.0</td>
<td>22.7 ± 1.9</td>
</tr>
<tr>
<td>Obese</td>
<td>Non-abdominal</td>
<td>n = 6</td>
<td></td>
<td></td>
<td>44.0 ± 12.4</td>
<td>31.1 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>Abdominal</td>
<td>n = 6</td>
<td></td>
<td></td>
<td>42.5 ± 8.9</td>
<td>32.7 ± 3.7</td>
</tr>
</tbody>
</table>

*D = donor
2.3.5 *Conditioned Media*

MDA-MB-231 cells were cultured in DMEM, supplemented with 10% FBS, 100 units per mL penicillin, and 100 µg/mL streptomycin for 24 hours. After 24 hours, the cells were washed twice with serum free DMEM and further cultured for 48 hours in serum free DMEM. The conditioned media was filtered (0.22 µM) to remove any cellular debris.

2.3.6 *Adipogenic Differentiation Protocol*

ASCs were cultured in 6-well plates in CCM until 70% confluent. Media was replaced with fresh media containing adipogenic supplements, consisting of 0.5 µM dexamethasone (Sigma), 0.5 mM isobuylmethylxanthine (Sigma), and 50 µM indomethacin (Sigma). After three weeks, cells were fixed in 10% formalin for 1 hour at 4°C, stained for 10–15 minutes at room temperature with Oil Red O (Sigma) to detect neutral lipid vacuoles, and images were acquired at 10x magnification on Nikon Eclipse TE200 (Melville, NY) with Nikon Digital Camera DXM1200F using the Nikon ACT-1 software version 2.7.

2.3.7 *Osteogenic Differentiation Protocol*

ASCs were cultured in 6-well plates in CCM until 70% confluent. Media was replaced with fresh media containing osteogenic supplements, consisting of 50 µM ascorbate 2-phosphate (Sigma), 10 mM β-glycerol phosphate (Sigma), and 10 nM dexamethasone. After three weeks cells were fixed in 10% formalin for 1 hour at 4°C and stained for 10 minutes with 40 mM Alizarin Red (pH 4.1) to visualize calcium
deposition in the extracellular matrix. Images were acquired at 4x magnification on Nikon Eclipse TE200 with Nikon Digital Camera DXM1200F using Nikon ACT-1 software version 2.7.

2.3.8 Colony Forming Unit Assay

ASCs were plated at a density of 100 cells on a 10 cm² plate in CCM and incubated for 14 days. Plates were rinsed three times with PBS, and 10 mL of 3% crystal violet (Sigma) was added for 30 minutes at room temperature. Plates were washed three times with PBS and once with tap water. Colonies that were larger than 2 mm in diameter were counted. Each experiment was performed in triplicate.

2.3.9 Flow Cytometry

ASCs were harvested with 0.25% trypsin / 1mM EDTA for 3-4 minutes at 37°C. A total of 3 x 10⁵ cells were concentrated by centrifugation at 500 x g for 5 min, suspended in 50 µl PBS and labeled with the primary antibodies. The samples were incubated for 30 minutes at room temperature and washed three times with PBS. The samples were then analyzed by FACScan (FACScalibur; BD Biosciences) with CellQuest software (BD Biosciences). To assay cells by forward and side scatter of light, FACScan was standardized with microbeads (Dynosphere uniform microspheres; Bangs Laboratories Inc.; Thermo Scientific; Waltham, MA). At least 10,000 events were analyzed and compared with isotype controls.

2.3.10 Transwell Invasion Assay
Invasion assays were performed in transwell inserts with 8-µm pore membrane filters pre-coated with a growth factor-reduced Matrigel layer to mimic basement membranes (BD Biosciences). ASCs were grown to 70% confluent prior to harvesting by trypsinization and pooled based on obesity status and depot site. ASCs were loaded (5 x 10^4 cells/well in 500 µL) onto the upper chamber, while 750 µL MDA-MB-231 conditioned media was loaded onto the bottom chamber. Where indicated, ASCs were pretreated with protease inhibitors for 4 hours prior to loading onto the upper chamber, and fresh protease inhibitors were added to the upper chamber for the duration of the invasion assay. After overnight incubation, the lower side of the transwell insert was carefully washed with cold PBS and non-invading cells remaining on the top chamber were removed with a cotton tip applicator. Invading cells were stained with Calcein-AM (2 µg/ml; Invitrogen; Grand Island, NY) and measured on a fluorescent plate reader (FLUOstar optima; BMG Labtech Inc.; Durham, NC). Data was normalized by dividing the values obtained with or without MDA-MB-231 conditioned media as a chemoattractant by the values obtained without conditioned media. Where ASCs were treated with protease inhibitors, the data was normalized by dividing the values obtained with each treatment group by the values obtained without protease inhibitor treatment to determine the percentage of inhibition.

2.3.11 RNA Isolation and Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Subconfluent cultures of ASCs were grown in conditioned media for 24 hours. The three donors in each category were pooled based on obesity status and depot site prior to total RNA extraction from ASCs using TRIzol reagent (Invitrogen) and
purification with DNase I digestion (Invitrogen). A total of 200 ng of cellular RNA was used for cDNA synthesis and PCR with the SuperScript III One-Step RT-PCR System with Platinum Taq (Invitrogen). No-template controls (negative control, neg ctrl) were run to rule out cross contamination of reagents and surfaces. All PCR products were analyzed by gel electrophoresis on 2.0% agarose gels with ethidium bromide stain. Gels were imaged with ImageQuant LAS 4000 (GE Healthcare Life Science; Piscataway, NJ) and representative image of one of three independent experiments are shown. Quantitative analysis of RT-PCR was performed by densitometry. MMP primer sets were designed by Kohrmann et al. [220]. The primer sets used in the RT-PCR are listed in Table 2.

2.3.12 Protein Isolation and Western Blot

Subconfluent cultures of ASCs were grown in conditioned media for 24 hours. The three donors in each category were pooled based on obesity status and depot site prior to cell lysis with RIPA buffer (Pierce; Thermo Scientific). The cell lysate was clarified by centrifugation at 15,000 x g for 15 min. Protein concentration was determined by the BCA Protein Assay (Pierce). Lysate (20 µg) was resolved on 4-12% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Invitrogen). Blots were blocked with Blok Noise Canceling Reagents (Millipore). Blots were then incubated with the primary antibodies overnight at 4°C and washed with PBS with Tween 20 three times before incubated with species-specific IgG conjugated to horseradish peroxidase for 1 hour at room temperature. Antigen-antibody complexes were visualized after incubation in chemiluminescence reagent (Invitrogen). Blots were imaged on an
ImageQuant LAS 4000 and representative blots of one of three independent experiments are shown. Quantitative analysis of Western blots was performed by densitometry.

### 2.3.13 Zymography

Subconfluent cultures of ASCs were grown in conditioned media for 24 hours. The three donors in each category were pooled based on obesity status and depot site prior to suspension media collection and concentrated with protein concentrators (Pierce). A total of 20 µg of protein was loaded under non-denaturing conditions into Novex Precast Polyacrylamide Zymography Gels supplemented with 0.1% gelatin (Invitrogen). Electrophoresis was performed at a constant voltage of 120 V for 90 min. Gels were washed with Novex Zymogram Renaturing Buffer (Invitrogen) and incubated overnight in Novex Zymogram Developing Buffer (Invitrogen) according to Manufacturer’s instructions. Gels were stained with Simply Blue SafeStain (Invitrogen) for 1 hour and then washed with water for 1 hour. Gels were incubated with Gel-Dry Drying Solution (Invitrogen) for 5 minutes before being placed between cellophane for drying overnight. After gels dried overnight, zymograms were recorded with ImageQuant LAS 4000 and representative zymographs of one of three independent experiments are shown. Quantitative analyses of zymographs were analyzed by densitometry.

### 2.3.14 Stable Transfection of shRNA

Short hairpin RNA constructs targeting MMP-14, MMP-15, calpain-4, and calpastatin and an shRNA construct targeting a non-human gene serving as a control were purchased from SABiosciences (Frederick, MD). The following shRNA constructs
were used: MMP-14 (5’-gcccaatggaagacctactt-3’), MMP-15 (5’-aaccgcgtcctggacaactat-3’), calpain-4 (5’-cgacgctactcagtaaagt-3’), calpastatin (5’-aaaccacaagacatgattct-3’), and non-coding gene (5’-ggaatctcattcgatgcatac-3’). ASCs were transfected with shRNA using the Neon Transfection System (Invitrogen), using 1400 V for the pulse voltage, 10 ms for the pulse width, and 3 pulses. Cells were allowed to recover for 48 hours before selection with Geneticin (Invitrogen). For selection, 600 µg/mL of Geneticin diluted in fresh CCM was replaced every 2 days, and the selection was performed for 14 days.

2.3.15 Chick Chorioallantoic Membrane (CAM) Assays

Fertilized white leghorn chick embryos were obtained from Charles River Laboratories (Wilmington, MA). Following 2 days of incubation in a 37°C humidified incubator, eggs were swabbed with 70% ethanol, and embryos were carefully removed from the shell using a Dremel tool and placed in a sterile, covered weigh boat and returned to the incubator. On day 10 of incubation, 10⁶ ASCs were labeled with 10 µM CellTracker Green CMFDA (Invitrogen), resuspended in 100 µl PBS, and added onto the CAM on a nylon mesh for localization of the cells as previously described [221-224]. After 3 days of incubation, the areas of interest were dissected out, fixed in 4% paraformaldehyde, frozen in OCT, and cut into 8 µm sections. The percentage of invading cells was quantified in three or more randomly selected fields. Depth of invasion from the CAM surface was defined as the leading front of three or more invading cells in randomly selected fields. Images were acquired on Olympus BXS1W1 spinning disk confocal microscope (Center Valley, PA) with Hamamatsu EM-CCD.
C9100 camera (Hamamatsu City, Japan) using Slidebook version 5.0 software (Olympus).

2.3.16 Statistical Analysis

All values are presented as means ± SD. The statistical differences among two or more groups were determined by ANOVA, followed by post-hoc Dunnet multiple comparison tests versus the respective control group. The statistical differences between two groups were performed by Student’s t-test. Statistical significant was set at $P < .05$. Analysis was performed using Prism (Graphpad Software, San Diego, CA).

2.4 Results

2.4.1 Characterization of ASCs isolated from donors based on obesity status and depot site of origin

ASCs were isolated from processed lipoaspirates of obese (Ob$^+$) and non-obese (Ob$^-$) subjects undergoing elective plastic surgery and obtained from the subcutaneous abdominal adipose tissue (Ab$^+$) or non-abdominal subcutaneous depots (Ab$^-$). The ASC from all groups (Ob$^+$Ab$^+$, Ob$^-$Ab$^+$, Ob$^+$Ab$^-$, Ob$^-$Ab$^-$) were found to be positive for CD44, CD90, CD105, and CD166 and negative for CD34, CD45, and CD11b via flow cytometry (Figure 1A). Each group of ASCs was able to differentiate into adipocytes and osteoblasts (Figure 1B) as well as generate colony-forming units (Figure 1C). No differences were observed among the four groups for ASC differentiation or self-renewal capacity as defined by colony forming units (Figure 1).
2.4.2 *Ob*⁺*Ab*⁺ ASCs demonstrate increased invasion towards breast cancer cell conditioned media

To investigate the invasive potential of ASCs in response to breast cancer cells, conditioned media from MDA-MB-231 cells was placed in the lower chamber of a growth factor reduced Matrigel coated transwell insert, while ASCs were plated onto the top chamber. All four ASC groups invaded towards conditioned media with statistically significant increases compared to control after 24 hour (*P*<0.05; Figure 2). Invasion of Ob⁺Ab⁺ ASCs was increased by 7.5 fold relative to control (unconditioned media), whereas the invasion of Ob⁺Ab⁻, Ob⁻Ab⁺, and Ob⁻Ab⁻ ASCs was increased by 2.8-, 3.6-, and 3.8-fold, respectively. Invasion of Ob⁺Ab⁺ ASCs invaded was significantly more compared to Ob⁺Ab⁻, Ob⁻Ab⁺, and Ob⁻Ab⁻ ASCs (*P*<0.001, Figure 2).

2.4.3 GM6001 and acetyl-calpastatin inhibit Ob⁺Ab⁺ ASC invasion

To determine the protease responsible for the robust invasion of Ob⁺Ab⁺ ASCs towards breast cancer conditioned media, all four groups of ASCs were pretreated with blanket inhibitors for 4 hours to the four protease classes found in humans: cysteine, serine, aspartate, and matrix metalloproteinases (MMP). Inhibition of Ob⁺Ab⁺ ASCs with E64d and GM6001 significantly impaired the invasion of these ASCs by 34% and 62%, respectively, when compared with untreated control cells (Figure 3A). To further reveal the protease(s) involved, Ob⁺Ab⁺ ASCs were pretreated with blanket inhibitors to the three classes of cysteine proteases: cathepsins, calpain, and caspases. Pretreatment of the ASCs with calpain inhibitor acetyl-calpastatin reduced the invasion of Ob⁺Ab⁺ ASC by 40% relative to cells that were not treated (Figure 3B). ASCs pretreated with cathepsin
Figure 1. Characterization of ASCs isolated from donors based on obesity status and deposit site. (A) ASCs were stained with antibodies against the indicated antigens and analyzed by flow cytometry. Histograms are shown as colored lines and the respective isotype controls are shaded in gray. (B) ASCs were grown until 70% confluent in CCM and then switched to differentiation media. After 21 days, cells were fixed and stained with Alizarin Red for osteogenesis and Oil Red O for adipogenesis. Representative images for each group are shown. Original magnification for osteogenesis is 4x and for adipogenesis is 10x for all panels. (C) CFUs were seeded at low density and incubated in CCM for 14 days. Cells were fixed and stained with crystal violet. Representative image for each group are shown.
Figure 2. Invasion of ASCs towards conditioned media. ASCs were placed onto transwell filters coated with growth factor reduced Matrigel and incubated for 24 hours in serum free or MDA-MB-231 conditioned media (CM) or serum free media (SF). Data was normalized by dividing the values obtained with or without MDA-MB-231 conditioned media as a chemoattractant by the values obtained without conditioned media (set as 1). Values are means of three independent experiments, each performed in triplicate. Bars, ± SD. *P < 0.05, **P < 0.01, ***P < .00001.
**Figure 3. Protease inhibitors limit ASC invasion.** Ob\(^+\)Ab\(^+\) ASCs were pretreated with protease inhibitors for 4 hours and placed onto transwell inserts with 8 µm pore membrane filters pre-coated with growth factor-reduced Matrigel in the presence of protease inhibitors. The bottom chamber was loaded with MDA-MB-231 conditioned media and incubated for 24 hours. (A) ASCs were pretreated with broad-spectrum protease inhibitor Pepstatin A (100 mM), SBTI (100 µg/mL), E64d (1 µM), or GM6001 (25 µM) for 4 hours prior to being placed onto transwell inserts. (B) ASCs were pretreated with cysteine protease inhibitor 1,3-Bis(CBZ-Leu-NH)-2-propanone (10 µM), acetyl-calpastatin (0.2 µM), or Z-VAD-FMK (20 µM) for 4 hours prior to being placed onto transwell inserts. Data was normalized by dividing the values obtained with each treatment group by the values obtained without protease inhibitor treatment and represented as a percentage. Values are means of three independent experiments, each performed in triplicates. Bars, ± SD. *P < 0.05, **P < 0.005.
Table 2. Primer sets used in amplification of ASC mRNA regions.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-1</td>
<td>5'-AAGGGCCAGTATGCACAGCCT-3'</td>
<td>5'-TGCTTGGACCTCCATGAGACT-3'</td>
</tr>
<tr>
<td>MMP-2</td>
<td>5'-TTTCCATTTCCGTTTTCCAGGGCAA-3'</td>
<td>5'-TGGACACACCCATCTTCCGGTACT-3'</td>
</tr>
<tr>
<td>MMP-3</td>
<td>5'-GGGTTTTCCAGAAGATTACG-3'</td>
<td>5'-TGGCCCATATTGTTGAGTCT-3'</td>
</tr>
<tr>
<td>MMP-7</td>
<td>5'-TCAACATGCTTGGAGGAAGT-3'</td>
<td>5'-GGAATGAGTGAAGCAGTTGC-3'</td>
</tr>
<tr>
<td>MMP-8</td>
<td>5'-TCTTGCAAGTTATCCCAAGG-3'</td>
<td>5'-ACCTGCTCAGTTGAAATTTG-3'</td>
</tr>
<tr>
<td>MMP-9</td>
<td>5'-TCTCGAGTTCCATTCCCTC-3'</td>
<td>5'-GCCATTCACAGTCTGTATCC-3'</td>
</tr>
<tr>
<td>MMP-10</td>
<td>5'-CCAGTTGTGCTGCTGCTATCC-3'</td>
<td>5'-CATCTCAGATCAGCAAGAAA-3'</td>
</tr>
<tr>
<td>MMP-11</td>
<td>5'-ACAGGTGTTGAGGCCATTA-3'</td>
<td>5'-AGTGGTGAAGGCGAAAGT-3'</td>
</tr>
<tr>
<td>MMP-12</td>
<td>5'-ACAGATGAGTGGACCTGTT-3'</td>
<td>5'-AGAGTCAAGCAAGATGGCACA-3'</td>
</tr>
<tr>
<td>MMP-13</td>
<td>5'-AACATCCAAAAACCCAGAC-3'</td>
<td>5'-GGGATTTGCCAAATATG-3'</td>
</tr>
<tr>
<td>MMP-14</td>
<td>5'-GGCAGCTCAGGGCGAGTATAG-3'</td>
<td>5'-AGCAGATGACCCTTGGAC-3'</td>
</tr>
<tr>
<td>MMP-15</td>
<td>5'-AGGAGAACAGCGAGCTGAG-3'</td>
<td>5'-TTCAGATTTTGGTCAG-3'</td>
</tr>
<tr>
<td>MMP-16</td>
<td>5'-AGCAGACATGCTTGGATGAG-3'</td>
<td>5'-ACCTTTTGGAGGAGTGAATC-3'</td>
</tr>
<tr>
<td>MMP-17</td>
<td>5'-GGACAGCTGTCACAGTTCCAC-3'</td>
<td>5'-ATCTTGGAGGAGATGTA-3'</td>
</tr>
<tr>
<td>MMP-19</td>
<td>5'-AGCAGACATGCTTGGATGAG-3'</td>
<td>5'-ACCTTTTGGAGGAGTGAATC-3'</td>
</tr>
<tr>
<td>MMP-20</td>
<td>5'-AGCAGACATGCTTGGATGAG-3'</td>
<td>5'-ACCTTTTGGAGGAGTGAATC-3'</td>
</tr>
<tr>
<td>MMP-22</td>
<td>5'-AGCAGACATGCTTGGATGAG-3'</td>
<td>5'-ACCTTTTGGAGGAGTGAATC-3'</td>
</tr>
<tr>
<td>MMP-23</td>
<td>5'-GGGACACACTTCAAACCT-3'</td>
<td>5'-CTGTGTTGTGACCTGAG-3'</td>
</tr>
<tr>
<td>MMP-24</td>
<td>5'-GACACCTTGGGCGAACAT-3'</td>
<td>5'-TGACAAAGCAGAAACTGAG-3'</td>
</tr>
<tr>
<td>MMP-25</td>
<td>5'-CCATTATGGGAGCAGCCTAG-3'</td>
<td>5'-TGGAGAGTGGTTGATC-3'</td>
</tr>
<tr>
<td>MMP-26</td>
<td>5'-GATTAGAAGCCATCCGGGAT-3'</td>
<td>5'-GCTGAAGGTGTTGACTG-3'</td>
</tr>
<tr>
<td>MMP-27</td>
<td>5'-TTTCTTCTTGAGTGCAGTC-3'</td>
<td>5'-GCTAGACCAAGGAGAACC-3'</td>
</tr>
<tr>
<td>MMP-28</td>
<td>5'-CCATCGTACCTGCAGCTTACG-3'</td>
<td>5'-AAAAGCTTGTGACCAGCC-3'</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>5'-AAGGACTCTGAAAGGGCTTCT-3'</td>
<td>5'-GAAAGGTGGATGAGTGGGAA-3'</td>
</tr>
<tr>
<td>TIMP-2</td>
<td>5'-TGATCCACACAGCTTGGTGC-3'</td>
<td>5'-TTGGATTTGTTGACATAGG-3'</td>
</tr>
<tr>
<td>TIMP-3</td>
<td>5'-TAGCCCAAGTATTGTTGAGGG-3'</td>
<td>5'-CATGATTTTCTTCAAACAGAAGCA-3'</td>
</tr>
<tr>
<td>TIMP-4</td>
<td>5'-CCTTGACTTGGTACTGGAAGTG-3'</td>
<td>5'-TGCTCAAACACCTCTTCTGAT-3'</td>
</tr>
<tr>
<td>Calpain-1</td>
<td>5'-CAGAGGCTGTTGGCTCAAAGGA-3'</td>
<td>5'-TTGGGCAATAGCCCTCTGCA-3'</td>
</tr>
<tr>
<td>Calpain-2</td>
<td>5'-GCCCTACTCAGACAAAAAC-3'</td>
<td>5'-GCTGAAATGCACAAGAGCAG-3'</td>
</tr>
<tr>
<td>Calpain-3</td>
<td>5'-AAAGAAGAGCTTAGGGGAGTGC-3'</td>
<td>5'-CTCTGACGGTTGCTTCCACCT-3'</td>
</tr>
<tr>
<td>Calpain-4</td>
<td>5'-GATCCAGGAGGACCTTGGGACG-3'</td>
<td>5'-GATTTTAAGGCGAGACCAGAT-3'</td>
</tr>
<tr>
<td>Calpain-5</td>
<td>5'-GACTGGAAGGAGGAGAAGT-3'</td>
<td>5'-CCATCTTCAAATGCGCTC-3'</td>
</tr>
<tr>
<td>Calpain-6</td>
<td>5'-GACCTGCTTCTGAGAAGGTG-3'</td>
<td>5'-TATGTTGGCGAACAGATG-3'</td>
</tr>
<tr>
<td>Calpain-7</td>
<td>5'-AGCGTGCACCTTTCTCTTGTT-3'</td>
<td>5'-TGGACATGAGACCTGACTGAG-3'</td>
</tr>
<tr>
<td>Calpain-8</td>
<td>5'-GTCCTCAAGCTTTTGGGACTAC-3'</td>
<td>5'-GCGAATGAGTGGGTCTTCCACAG-3'</td>
</tr>
<tr>
<td>Calpain-9</td>
<td>5'-CCAGAAGCTTGGAGCAATGGA-3'</td>
<td>5'-GSTGCAGACAGTGGAAT-3'</td>
</tr>
<tr>
<td>Calpain-10</td>
<td>5'-CAGATGAGCAGCCAGAGCGG-3'</td>
<td>5'-TGGGATTTGACCTCTTCCAC-3'</td>
</tr>
<tr>
<td>Calpain-11</td>
<td>5'-CTTCCAGGAGCCCCTATTCC-3'</td>
<td>5'-TTCACCACAGTCTCACAAGCC-3'</td>
</tr>
<tr>
<td>Calpain-12</td>
<td>5'-AAAGGCGTGGAAATGAGTGAAG-3'</td>
<td>5'-GAAATCAGCAGTCTCCTC-3'</td>
</tr>
<tr>
<td>Calpain-13</td>
<td>5'-CTTCCATAGCCAGGAAAGGTG-3'</td>
<td>5'-AACGGGAAATGCGACAT-3'</td>
</tr>
<tr>
<td>Calpain-14</td>
<td>5'-GCTGCTCAGCTCTTGAGAAG-3'</td>
<td>5'-TCTGATCCAGGGCAACAC-3'</td>
</tr>
<tr>
<td>Calpain-15</td>
<td>5'-CTGCTCCTCCTTCCTCACAAGG-3'</td>
<td>5'-AGGAAACCAAGCTCTTCC-3'</td>
</tr>
<tr>
<td>Calpastatin</td>
<td>5'-CTGCTCAGCTCTCTGCAGG-3'</td>
<td>5'-CCAGAGCTTATCAGCATCTC-3'</td>
</tr>
<tr>
<td>β-actin</td>
<td>5'-CACCTTCTCAAATGAGTCGC-3'</td>
<td>5'-AGGCACTGCTGTAGTCTTGG-3'</td>
</tr>
</tbody>
</table>
Figure 4. RT-PCR analyses of MMP, TIMP, calpain, and calpastatin in ASCs. ASCs were cultured in CCM and grown in breast cancer CM. Cells were harvested and RNA was isolated. A total of 200 ng of total RNA was used for cDNA synthesis and PCR analysis. No-template controls (neg ctrl) were run to rule out contamination. PCR products were analyzed by gel electrophoresis on 2.0% agarose gels with ethidium bromide stain. β-actin is shown as a standard reference. Bars, ± SD. *P < 0.05
Figure 5. Quantification of RT-PCR analyses of MMPs and TIMPs, Part 1.
Densitometry was performed on RT-PCR gel electrophoresis and represented as a ratio relative to β-actin. Bars, ± SD. *P < 0.05.
Figure 6. Quantification of RT-PCR analyses of MMPs and TIMPs, Part 2. Densitometry was performed on RT-PCR gel electrophoresis and represented as a ratio relative to β-actin. Bars, ± SD. *$P < 0.05$. 
Figure 7. Quantification of RT-PCR analyses of calpains and calpastatin.
Densitometry was performed on RT-PCR gel electrophoresis and represented as a ratio relative to β-actin. Bars, ± SD. *P < 0.05.
and caspase inhibitors had no effect on invasion. No changes in cell viability or proliferation were observed after treatment with protease inhibitors (data not shown). These experiments indicate that both MMPs and calpains are involved in the invasion of Ob⁺Ab⁺ ASCs.

2.4.4 mRNA expression of MMPs and calpains vary among the four groups

To further identify the protease(s) involved in the invasion, ASCs were grown in the absence and presence of MDA-MB-231 conditioned media prior to the isolation of RNA and detection of mRNA expression by RT-PCR. Primer sets were constructed for the detection of specific MMPs and calpains along with the endogenous inhibitors of these proteases, which included the TIMPs and calpastatin, respectively (Table 2).

Of the MMPs and TIMPs, the mRNAs for 18 MMPs and 4 TIMPs were detected in all groups of ASCs (Figure 4). However, the levels of mRNA expression of MMP-2, -9, -12, -15, -21, -23, and -28 as well as TIMP-4 varied among the four groups (Figure 4, Figure 5, Figure 6). More specifically, levels of MMP-2, MMP-9, and MMP-28 were increased by 1.3-fold, 1.8-fold, and 1.8-fold, respectively, in Ob⁺ ASCs. In contrast, the mRNA level of MMP-12, MMP-21, MMP-23 and TIMP-4 were increased by 1.3-fold, 2.2-fold, 2.4-fold, and 1.4-fold, respectively, in Ob⁻ ASCs. Of particular interest was the increase in MMP-15 expression in the Ob⁺Ab⁺ ASCs compared to the other 3 groups of ASCs, indicating the potential involvement of MMP-15 in the enhanced invasion of Ob⁺Ab⁺ ASC.

Of the 15 calpains and calpastatin, the mRNAs for 9 calpains and calpastatin were present at detectable levels in all groups of ASCs (Figure 4; Figure 7); however, levels of
calpain-4, -6, -7, and -10 varied among the four groups (Figure 4; Figure 7). mRNA expression of calpain-4 was increased 2.5-fold in the Ob⁺Ab⁺ ASCs compared to the other three groups. RNA expression of calpain-6 and calpain-7 was decreased in the Ob⁺ groups by 2.3-fold and 2.5-fold, respectively. In addition, Ob⁺Ab⁺ ASCs showed significant reduction by 3.5-fold in calpastatin mRNA expression (Figure 4; Figure 7).

2.4.5 Expression and activity of MMPs and calpains vary among the four groups

Western blots or zymograms were performed on the protease or protease inhibitors that displayed varying RNA expression among the four groups. To confirm the protein expression of MMP-2 and MMP-9, zymography was performed with conditioned media serving as a baseline control. MMP-2 and MMP-9 activity levels were increased in Ob⁺ groups as compared to Ob⁻ groups (Figure 8). Western Blotting was performed for MMP-12, MMP-15, MMP-21, MMP-23, MMP-28, TIMP-4, calpain-4, calpain-6, calpain-7, calpain-10, calpastatin, with beta-actin serving as a control. The expression of MMP-14 was assessed as well because previous studies have suggested MMP-14 as the sole molecule responsible for the invasion of bone marrow derived MSC (BMSCs) [223, 225]. Western blot analysis showed no differences in protein expression of MMP-12, MMP-14, MMP-21, MMP-23, MMP-28, TIMP-4, calpain-6, calpain-7, or calpain-10. Western Blot confirmed the decrease in calpastatin expression by 4.0-fold in Ob⁺Ab⁺ ASCs relative to other ASCs (Figure 9). Increased expression in MMP-15 and calpain-4 by 2.2- and 2.0-fold, respectively, was observed in Ob⁺Ab⁺ ASCs when compared with the other ASC groups (Figure 9).
2.4.6 Assessing variability of MMP-15, calpain-4, and calpastatin expression

In order to assess the variability between donor samples within groups and between groups, an additional 3 donors were obtained for each ASC group, resulting in a total of 24 donor samples. ASCs were primed in MDA-MB-231 conditioned media prior to the isolation of protein and, where indicated, were pooled according to donor’s obesity status and depot site, prior to blotting with MMP-15, calpain-4, or calpastatin antibody. Quantification of Western blots with densitometry demonstrated no statistically significant variability within the groups, while statistically significant differences were observed between the groups as demonstrated with the analysis of the pooled samples (Figure 10).

2.4.7 Reduced calpastatin and enhanced calpain-4 expression increase invasion

To confirm the functional role of calpastatin and calpain-4 in invasion, ASCs were transfected with calpastatin or calpain-4 shRNA (Figure 11). Knockdown efficiency was assessed with Western blot analysis after antibiotic selection was complete. Calpastatin knockdowns in Ob^-Ab^-, Ob^-Ab^+, and Ob^+Ab^- ASCs showed a reduction in protein expression of calpastatin by 78%, 82%, and 80%, respectively. Calpastatin knockdown in Ob^+Ab^- ASCs was comparable to nontransfected ASCs, suggesting knockdown efficiency of calpastatin in Ob^+Ab^- was low, possibly due to low levels of endogenous calpastatin in these cells. Protein expression of calpain-4 was decreased in all four groups after shRNA transfection and antibiotic selection, with the greatest decrease of 87% observed in Ob^-Ab^+ ASCs.
Functionally, Ob′Ab−, Ob′Ab+, and Ob′Ab+ ASCs transfected with calpastatin shRNA demonstrated increased invasion towards conditioned media compared to shRNA negative control cells or naïve untransfected cells (Figure 12). Invasion of Ob′Ab+ ASCs transfected with calpastatin shRNA showed a similar invasive phenotype as the naïve untransfected cells or shRNA negative control Ob′Ab+ ASCs (Figure 12). No significant difference in invasion was observed between the four groups of ASCs after calpastatin shRNA transfection. These experiments confirm that low calpastatin expression correlates with enhanced invasion. Calpain-4 knockdowns in Ob′Ab+ ASCs diminished the invasion by 5-fold and negated the enhanced invasion observed in Ob′Ab+ ASCs exposed to breast cancer cell conditioned media. These observations indicate that the simultaneous reduction in calpastatin and increase in calpain-4 in Ob′Ab+ ASCs contribute to the cellular invasion.

2.4.8 Inhibition of MMP-15 shRNA reduced invasion of ASCs

To determine the role of MMP-15 on ASC invasion, ASCs were transfected with MMP-15 shRNA followed by antibiotic selection. MMP-14 shRNA was also assessed because previous studies have suggested that MMP-14 may be responsible for BMSC invasion. Knockdown efficiency was assessed through Western blot analysis. Protein expression of MMP-14 and MMP-15 was decreased in all four groups after shRNA transfection and antibiotic selection (Figure 11). Since high levels of endogenous MMP-15 were found in Ob′Ab+ ASCs, the greatest decrease in expression after shRNA knockdown was observed in Ob′Ab+ ASCs. Functionally, inhibition of MMP-14 resulted in a 2-fold decrease in all four groups, indicating that all ASCs can utilize, but do not
Figure 8. Zymographic analyses of MMP-2 and MMP-9. ASCs were cultured in CCM until 70% confluent and grown in breast cancer conditioned media for 24 hours. (A) Zymographic analysis of gelatinase secretion from ASCs. A total of 20 µg of culture supernatants were analyzed. Breast cancer conditioned media was used as a control to demonstrate basal levels of protein content prior to ASC exposure. (B) Quantification of MMP-2 and MMP-9 activity with densitometry of the zymography gels. Bars, ± SD.
Figure 9. Western blot analyses of MMPs, TIMPs, calpains, and calpastatin in different ASC population. ASCs were cultured in CCM until 70% confluent and grown in breast cancer conditioned media for 24 hours. (A) Western blot detection of ASC lysate. About 20 µg of protein was separated by SDS-PAGE under reducing conditions, blotted, and probed with indicated antibodies. (B) Quantification of MMPs, TIMPs, calpains, and calpastatin was assessed with densitometry of Western blots. Bars, ± SD. *$P < 0.05$
Figure 10. Western blot analyses of ASCs isolated from 24 donors for MMP-15, calpain-4, and calpastatin expression. Protein lysate was isolated from ASCs isolated from 24 donors and primed with breast cancer conditioned media. Where indicated, samples isolated from donors with the same obesity status or depot site were pooled together for analysis. A total of 20 µg of protein was separated by SDS-PAGE under reducing conditions, blotted, and probed for MMP-15, calpain-4, and calpastatin. Bars, ± SD. *P < 0.05
Figure 11. shRNA constructs effectively target genes of interest. ASCs were transfected with an shRNA construct targeting a non-human gene (shRNA Neg Ctrl) or an shRNA construct targeting the gene of interest (shRNA) followed by antibiotic selection. (A) ASCs expressing the shRNA vectors were assessed for their efficiency of gene inhibition by Western blot analysis. About 20 µg of cell lysate isolated from transfected ASCs grown in conditioned media for 24 hours were separated by SDS-PAGE under reducing conditions, blotted, and probed with the indicated antibodies. (B) Quantification of protease expression was assessed with densitometry of Western blots. Bars, ± SD. *P < 0.05
require MMP-14 to invade (Figure 12).

After MMP-14 shRNA knockdown, Ob⁺Ab⁺ ASCs retained their enhanced invasion, suggesting that MMP-14 is not responsible for the enhanced invasion of Ob⁺Ab⁺. In contrast, inhibition of MMP-15 demonstrated reduced invasion in Ob⁺Ab⁺ ASCs by 2-fold, although no significant changes were observed in the invasion of Ob⁺Ab⁻, Ob⁻Ab⁺, or Ob⁻Ab⁻ ASCs. ShRNA knockdown of MMP-15 abolished the enhanced invasion of Ob⁺Ab⁺ ASCs in response to breast cancer cell conditioned media, suggesting that these cells utilize an alternative pathway with MMP-15 as their central player.

2.4.9 Invasion of ASCs in vivo

To evaluate ASC invasion in a complex ECM model system that inherently contains various chemokines and chemoattractants, an in vivo chick CAM model was used. The CAM contains a type I collagen-rich ECM barrier commonly used to study invasive processes [221, 224]. The shRNA negative control of Ob⁺Ab⁺ ASCs demonstrated enhanced invasion compared to control Ob⁺Ab⁻, Ob⁻Ab⁺, or Ob⁻Ab⁻ ASCs (Figure 13). While MMP-14 knockdown in ASCs significantly reduced the invasion of Ob⁺Ab⁺, Ob⁻Ab⁺, and Ob⁻Ab⁻ ASCs, Ob⁺Ab⁺ ASCs maintained their more invasive phenotype compared to the other groups. In contrast, knockdowns of MMP-15 and calpain-4 in Ob⁺Ab⁺ ASCs reduced the invasive phenotype of these cells to the same invasiveness as other ASCs, while knockdowns of MMP-15 and calpain-4 in Ob⁺Ab⁻, Ob⁻Ab⁺, and Ob⁻Ab⁻ ASCs had no effect on the invasion of these cells, suggesting the use of MMP-15 and calpain-4 by Ob⁺Ab⁺ ASCs but not by Ob⁺Ab⁻, Ob⁻Ab⁺, or Ob⁻Ab⁻ ASCs.
In comparison, knockdowns of calpastatin in Ob⁺Ab⁺ ASCs demonstrated no effect on the invasion of these cells, while knockdowns of calpastatin in Ob⁺Ab⁻, Ob⁻Ab⁺, and Ob⁻Ab⁻ ASCs demonstrated enhanced invasion. Interestingly, although levels of invasion in Ob⁺Ab⁻, Ob⁻Ab⁺, and Ob⁻Ab⁻ ASCs were increased with calpastatin shRNA, naïve Ob⁺Ab⁺ ASCs remained more invasive, indicating that an additional molecule may be required for invasion.

2.5 Discussion

ASCs have been shown to be recruited to damaged tissue and inflammation, which are hallmarks of cancer [217, 226]. However, the invasive potential of ASCs based on obesity status and depot site of origin have not been previously investigated. Herein, we have combined the use of Matrigel and the chick CAM models to interrogate ASC invasion through the perforation of basement membrane barriers as well as intravasate vascular networks in vivo. This study is among the first to show that ASCs isolated from the subcutaneous abdominal adipose tissue of obese patients, Ob⁺Ab⁺ ASCs, have enhanced invasion due to increased expression of MMP-15 and calpain-4 and decreased expression of calpastatin. These findings are consistent with recent reports documenting increased levels of colony forming unit mesenchymal progenitor cells recovered from the circulation of obese relative to lean subjects and further increases in this cell population in patients with colorectal cancer [203, 227].

MMP-15 knockdown in Ob⁺Ab⁺ ASCs demonstrated decreased invasion. Other studies have shown that cancer cells expressing MMP-15 play a role in degrading the
Figure 12. ASC invasion influenced by shRNA knockdown. ASCs were transfected with an shRNA construct targeting a non-human gene (shRNA Neg Ctrl) or an shRNA construct targeting the gene of interest (shRNA) followed by antibiotic selection. The shRNA transfected ASCs were seeded in the upper compartment of the transwell inserts with 8-µm pore membrane filters pre-coated with growth factor-reduced Matrigel and incubated for 24 hours. Data was normalized by dividing the values obtained with serum-free media or MDA-MB-231 conditioned media as a chemoattractant by the values obtained for the serum-free media (set as 1). Values are means of three independent experiments. *, P < 0.05; **, P < 0.01.
Figure 13. The role of MMP-15, calpain-4, and calpastatin in ASC invasion in the CAM model. ASCs transfected with an shRNA construct targeting a non-human gene (shRNA Neg Ctrl) or an shRNA construct targeting the gene of interest (shRNA) underwent antibiotic selection. (A) ASCs were labeled with Cell Tracker Green (10µM) and seeded atop the CAM of 10-day-old chick embryos. After 3 days, CAM cross-sections were stained with DAPI and visualized by fluorescent microscopy. Dashed lines outline the upper CAM surface such that cells below the dashed line demarcate invading ASCs. Bar represents 10 µM. (B) CAM invasion is quantified as the number of ASCs that cross the CAM surface and average depth of the leading front of the invading cells. Values are means of three independent fields. Bars, ± SD. *P < 0.01, **P < 0.001.
venular basement membranes during angiogenesis, and invasive cells express higher levels of MMP-15 compared to non-invasive cells [228, 229]. Additional studies have demonstrated that hypoxic conditions can lead to the dysregulation of MMP-15, resulting in an invasive phenotype [230]. In obesity, the rapid proliferation and hypertrophy of cells results in excessive accumulation of adipose tissue and hypoxia due to inadequate blood supply to maintain the tissue [231]. Therefore, it is possible that Ob$^+$Ab$^+$ ASCs, due to their local hypoxic conditions, acquire an invasive phenotype through the dysregulation of MMP-15.

It should be noted that previous work conducted with MMP-14 and MMP-15 has been conducted with BMSCs rather than with ASCs. The discrepancy seen between previously published data and our data may be explained by the inherent differences between BMSCs and ASCs. Previous studies investigating the protease(s) involved in BMSC invasion identified MMP-14 as a major molecule contributing to ECM degradation and BMSC invasion [223, 225]. The current study has shown that ASCs also use MMP-14 for invasion, but MMP-14 knockdown did not diminish the enhanced invasion observed in Ob$^+$Ab$^+$ ASCs, suggesting MMP-14 alone is not responsible for the enhanced invasion observed in these ASCs. Quantitative comparisons between these two different cell types have revealed differences at the transcriptional and proteomic levels as well as functional differences in their differentiation processes [232, 233].

The current study demonstrates that the calpain-calpastatin system is involved in the invasion of ASCs. In resting cells, calpastatin inhibits calpains, which are calcium dependent cysteine proteases [234]. However, upon the activation of calpains \textit{in vivo}, calpains mediate the degradation of calpastatin, resulting in increased protein synthesis of
The aggregation of calpastatin and its sub-cellular redistribution also serve as mechanisms that facilitate calpain activation by compartmentalizing the calpastatin [237]. The interplay between calpain and calpastatin provide an interesting area of investigation because of their involvement in cellular invasion [238]. In breast cancer, decreased calpastatin expression has been shown to be involved in lymphovascular invasion [239]. Calpain-4 over-expression has been linked with enhanced invasiveness of hepatocellular carcinoma cells and siRNA-mediated knockdown expression of calpain-4 significantly inhibited the motility and invasive phenotype of these cells [240]. Additional studies using calpain-4 knockout fibroblast revealed decreased cell migration rates, altered actin cytoskeleton organization, and proteolytic cleavage of focal adhesion kinase (FAK), paxillin, spectrin, cortactin, and talin 1 [241]. These studies support the involvement of the calpain-calpastatin system in cellular invasion mechanisms, and our present study confirmed the role of calpastatin and calpain-4 specifically on ASC invasion.

While the studies described here utilized ASCs isolated from subcutaneous adipose tissue expanded in vitro, another interesting avenue for further investigation will be to focus on the biologic properties and invasive potential of non-cultured ASCs isolated directly from the stromal vascular fraction. Previous studies have utilized FACS sorted or magnetic bead purified ASCs to isolate progenitor cells from adipose tissue [242, 243], but further investigation is necessary to determine potential differences in the invasive potential of non-cultured ASCs with respect to depot site of origin and BMI status of the subjects. Furthermore, while our study focused on the biology of ASCs isolated from subcutaneous adipose tissue, the study of ASCs from visceral adipose tissue
may provide further insight into the influence of obesity on the ASC biology. Studies comparing ASCs isolated from visceral and subcutaneous adipose tissue indicated differences in proliferation capacity and adipogenic potential, while no significant differences were observed in differentiation capacity, morphological properties, and immunophenotypic properties [244-246]. Further comparison studies will be necessary to determine molecular differences in these ASCs as they relate to obesity.

Taken together, the results from our analysis combined with data from other investigators clearly describe the key proteases involved in ASC invasion through ECM barriers. Collectively, data from these studies suggest that the invasive capacity of ASCs is based on inherent variation between depots of subcutaneous adipose tissue [247, 248]. Additional studies will be needed to understand the causes of and mechanism(s) driving obesity-induced changes between subcutaneous adipose tissue depots.
Chapter 3: Obese ASCs Influence Breast Cancer Tumorigenesis

Obesity associated alterations in the biology of adipose stem cells mediate enhanced tumorigenesis by estrogen dependent pathways

Amy L. Strong, Thomas A. Strong, Lyndsay V. Rhodes, Julie A. Semon, Xiujuan Zhang, Zhenzhen Shi, Shijia Zhang, Jeffrey M. Gimble, Matthew E. Burow, Bruce A. Bunnell

Accepted for Publication in Breast Cancer Research

3.1 Abstract

Obesity has been associated with increased incidence and mortality of breast cancer. While the precise correlation between obesity and breast cancer remains to be determined, recent studies suggest that adipose tissue and adipose stem/stromal cells (ASCs) influence breast cancer tumorigenesis and tumor progression. Breast cancer cell lines were co-cultured with ASCs (n=24), categorized based on tissue site of origin and body mass index (BMI), and assessed for enhanced proliferation, alterations in gene expression profile with PCR arrays, and enhanced tumorigenesis in immunocompromised mice. The gene expression profile of ASCs was assessed with PCR arrays and qRT-PCR and confirmed with Western blot analysis. Inhibitory studies were conducted by delivering estrogen antagonist ICI182,780, leptin neutralizing antibody, or aromatase inhibitor letrozole and assessing breast cancer cell proliferation. To assess the role of
leptin in human breast cancers, Oncomine and Kaplan Meier plot analyses were conducted. ASCs derived from the abdominal subcutaneous adipose tissue of obese subjects (BMI > 30) enhanced breast cancer cell proliferation *in vitro* and tumorigenicity *in vivo*. These findings were correlated with changes in the gene expression profile of breast cancer cells after co-culturing with ASCs, particularly in estrogen receptor-alpha (ESR1) and progesterone receptor (PGR) expression. Analysis of the gene expression profile of the four groups of ASCs revealed obesity induced alterations in several key genes, including leptin (LEP). Blocking estrogen signaling with ICI182,780, leptin neutralizing antibody, or letrozole diminished the impact of ASCs derived from obese subjects. Women diagnosed with estrogen receptor/progesterone receptor positive (ER⁺/PR⁺) breast cancers that also expressed high levels of leptin had poorer prognosis than women with low leptin expression. ASCs isolated from the abdomen of obese subjects demonstrated increased expression of leptin, through estrogen stimulation, which increased breast cancer cell proliferation. The results from this study demonstrate that abdominal obesity induces significant changes in the biological properties of ASCs and that these alterations enhance ER⁺/PR⁺ breast cancer tumorigenesis through estrogen dependent pathways.

### 3.2 Introduction

Breast cancer is the most frequently diagnosed cancer in women and the second leading cause of cancer deaths in the United States. While many risk factors increase the incidence of breast cancer, obesity is among one of the most important risk factors for breast cancer in postmenopausal women as it not only increases the incidence of breast
cancer but also the mortality rate due to poor prognosis and outcome [208, 249-251]. Obesity has been shown to affect prognosis through multiple mechanisms, including enhanced metastasis rate and drug resistance [252, 253]. Furthermore, recent studies have demonstrated a stronger correlation between abdominal obesity and breast cancer [254, 255].

While the precise link between obesity and breast cancer remains to be determined, previous studies have described the activation of adipose stromal cells (ASCs) in the presence of breast cancer to contribute to its pathogenesis [256, 257]. ASCs are mesenchymal lineage stem cells that are recruited to the tumor or sites of inflammation and are essential components that establish the tumor microenvironment [202, 258, 259]. This recruitment enhances tumor growth through the secretion of an abundance of growth factors from ASCs, such as IL-6, CCL5, and PDGR, which have been shown to contribute to both the breast cancer tumorigenesis and the metastasis of breast cancer cells [260, 261].

While previous studies have determined that ASCs play an integral role in the progression of breast tumors, the impact of obesity and abdominal obesity on the relationship between cancer and ASCs has not been investigated. Studies have revealed that ASCs isolated from subjects with vastly different body mass indices display differences in their secretory profile, angiogenic potential, and invasive capacity [262, 263]. Recent work by Zhang et al. demonstrated increased numbers of ASC in obese mice relative to lean mice, and this increase in ASC number enhances vascularization and proliferation of malignant cells [202]. The results from these studies suggest that the local microenvironment from which these ASCs are isolated can influence their gene
expression profiles. In addition, the site of origin of the adipose tissue from which the ASCs are derived may alter essential cellular signaling pathways that may directly influence breast cancer tumorigenesis.

This study investigated the impact of ASCs, isolated from different subcutaneous adipose depots in subjects with increasing BMI, on the growth, gene expression profile, and tumorigenesis of breast cancer cells. The results from this study demonstrated that ASCs isolated from abdominal depots of obese subjects (BMI > 30) enhanced breast cancer cell growth and tumorigenesis via an estrogen mediated response.

3.3 Materials and Methods

3.3.1 Human Subjects

All protocols were reviewed and approved by the Pennington Biomedical Research Center Institutional Review Board and all human participants provided written informed consent (PBRC #23040). Human ASCs were obtained from 24 Caucasian females (4 groups, 6 donors per group) undergoing elective liposuction procedures, as previously described [3, 218]. Briefly, ASCs were isolated from processed lipoaspirates from subcutaneous abdominal adipose tissue of obese (Ob\(^+\)Ab\(^+\)) or non-obese (Ob\(^-\)Ab\(^+\)) subjects and from non-abdominal subcutaneous adipose depots of obese (Ob\(^+\)Ab\(^-\)) and non-obese (Ob\(^-\)Ab\(^-\)) subjects. Liposuction aspirates were incubated in 0.1% type I collagenase (Sigma) and 1% powered bovine serum albumin (BSA, fraction V; Sigma) dissolved in 100ml of phosphate buffered saline (PBS) supplemented with 2mM calcium chloride. Mixture was placed in a 37°C shaking water bath at 75 rpm for 60 min and then centrifuged to remove oil, fat, primary adipocytes, and collagenase solution, leaving
behind a pellet of cells. Cells were resuspended in complete culture media (CCM), which consisted of α-Minimal Essential Medium (αMEM; GIBCO; Grand Island, NY), 20% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA), 100 units per ml penicillin/100 µg/mL streptomycin (P/S; GIBCO), and 2 mM L-glutamine (GIBCO), and plated on 150 cm² culture dishes. Fresh CCM was added every 2-3 days until cells achieved 80-90% confluence, at which time cells were harvested with 0.25% trypsin / 1mM EDTA (GIBCO) and cryopreserved prior to experimental use. Non-abdominal subcutaneous adipose tissue was isolated from the hip, knee, thigh, ankle, flank, upper torso, scapula, forearm, arm, and back. The mean BMI for each of the four donor groups was as follows: Ob⁺Ab⁺ (32.7 ± 3.7), Ob⁺Ab⁻ (31.1 ± 0.6), Ob⁻Ab⁺ (22.7 ± 1.9) and Ob⁻Ab⁻ (22.5 ± 1.2). The mean age of the subjects for each group of donors was as follows: Ob⁺Ab⁺ (42.5 ± 8.9), Ob⁺Ab⁻ (44.0 ± 12.4), Ob⁻Ab⁺ (38.8 ± 7.0) and Ob⁻Ab⁻ (52.4 ± 18.0). No statistical significance in age was observed between the donor groups.

3.3.2 Cell Culture

ASCs. Frozen vials of ASCs were thawed and cultured on 150 cm² culture dishes (Nunc, Rochester, NY) in 25 ml CCM and incubated at 37°C with 5% humidified CO₂. After 24 hours, viable cells were harvested with 0.25% trypsin / 1mM EDTA and replated at 100 cells/cm² in CCM. Media was changed every 2-3 days. For all experiments, sub-confluent cells (≤70% confluent) between passages 2-6 were used.

To characterize the cells, ASCs were induced to undergo osteogenic and adipogenic differentiation. For osteogenic differentiation, ASCs were cultured in 6-well plates in CCM until 70% confluent and media was replaced with fresh media containing
osteogenic supplements, consisting of 50 µM ascorbate 2-phosphate (Sigma), 10 mM β-glycerol phosphate (Sigma), and 10 nM dexamethasone. After three weeks cells were fixed in 10% formalin for 1 hour at 4°C and stained for 10 minutes with 40 mM Alizarin Red (pH 4.1) to visualize calcium deposition in the extracellular matrix. Images were acquired at 4x magnification on Nikon Eclipse TE200 (Melville, NY) with Nikon Digital Camera DXM1200F using the Nikon ACT-1 software version 2.7. For adipogenic differentiation, ASCs were cultured in 6-well plates in CCM until 70% confluent, and media was replaced with fresh media containing adipogenic supplements, consisting of 0.5 µM dexamethasone (Sigma), 0.5 mM isobuutylmethylxanthine (Sigma), and 50 µM indomethacin (Sigma). After three weeks, cells were fixed in 10% formalin for 1 hour at 4°C, stained for 10–15 minutes at room temperature with Oil Red O (Sigma) to detect neutral lipid vacuoles, and images were acquired at 10x magnification on Nikon Eclipse TE200 with Nikon Digital Camera DXM1200F using Nikon ACT-1 software version 2.7.

To determine the ability to form colony forming units (CFU), ASCs at passage 3 were plated at a density of 100 cells on a 10 cm² plate in CCM and incubated for 14 days. Plates were rinsed three times with PBS, and 10 mL of 3% crystal violet (Sigma) was added for 30 minutes at room temperature. Plates were washed three times with PBS and once with tap water. Each experiment was performed in triplicate.

Analysis by flow cytometry of the cell surface marker profile was conducted by harvesting ASCs with 0.25% trypsin / 1 mM EDTA for 3-4 minutes at 37°C. A total of 3 x 10⁵ cells were concentrated by centrifugation at 500 x g for 5 min, suspended in 50 µl PBS and labeled with the primary antibodies. The following primary antibodies were used: Anti-CD45-PeCy7, anti-CD11b-PeCy5, anti-CD166-PE, anti-CD105-PE, anti-
CD90-PeCy5, anti-CD34-PE, isotype-control FITC human IgG1 and isotype-control PE human IgG2a were purchased from Beckman Coulter (Indianapolis, IN). Anti-CD44-APC was purchased from BD Biosciences (San Jose, CA). The samples were incubated for 30 minutes at room temperature and washed three times with PBS. The samples were then analyzed with Gallios Flow Cytometer (Beckman Coulter, Brea, CA) running Kaluza software (Beckman Coulter). To assay cells by forward and side scatter of light, FACScan was standardized with microbeads (Dynosphere uniform microspheres; Bangs Laboratories Inc.; Thermo Scientific; Waltham, MA). At least 10,000 events were analyzed and compared with isotype controls.

**Breast cancer cell lines.** MCF7 and MDA-MB-231 cells were obtained from American Type Culture Collection (Manassas, VA) and cultured in Dulbecco’s Modified Eagle’s Medium (DMEM; GIBCO), supplemented with 10% FBS and P/S. Cells were grown at 37°C with 5% humidified CO2, fed every 2-3 days, and split 1:4 to 1:6 when they reached 90% confluency.

### 3.3.3 Synthesis of GFP breast cancer cells

To produce retroviruses, 293T cells were transfected by means of a modified calcium chloride transfection protocol when cells reached 90-95% confluent. The following amount of DNA was used to transfect cells on a 10cm plate: 10 µg pMSCVneo-GFP vector, 10 µg pVPACK-Gp-dl packaging plasmid, and 10 µg pCI-VSV-G envelope-encoding plasmid. Twenty-four hours after transfection, cells were washed with PBS, replaced with fresh media, and collected after 48 hours. To transduce MCF7 or MDA-MB-231 cells, conditioned media containing retrovirus was added to MCF7 or
MDA-MB-231 cells at 70% confluency. MCF7 cells were selected with 1 mg/ml of Genticin (Invitrogen), while MDA-MB-231 cells were selected with 500 µg/ml of Genticin for 2 week and GFP expression was verified with flow cytometry.

3.3.4 Synthesis of DsRed ASCs

To produce retroviruses, 293T cells were transfect by means of a modified calcium chloride transfection protocol when cells reached 90-95% confluent. The following amount of DNA was used to transfect cells on a 10cm plate: 10 µg pMSCVneo-DsRed vector, 10 µg pVPACK-Gp-dl packaging plasmid, and 10 µg pCI-VSV-G envelope-encoding plasmid. Twenty-four hours after transfection, cells were washed with PBS, replaced with fresh media, and collected after 48 hours. To transduce ASCs, conditioned media containing retrovirus was added to cells at 70% confluency. ASCs cells were selected with 500 µg/ml of Genticin for 2 week and DsRed expression was verified with flow cytometry.

3.3.5 Breast cancer cell and ASC co-culture

MCF7 cells or MDA-MB-231 cells were co-cultured with Ob-Ab- (n=6), Ob-Ab+ (n=6), Ob+Ab- (n=6), or Ob+Ab+ (n=6) ASCs (1:1 ratio) at 200 cells/cm² in DMEM supplemented with 10% FBS and P/S. After 7 days, cells were harvested, washed, and analyzed by flow cytometry. The percentage of GFP positive cells (MCF7 cells or MDA-MB-231) was determined with Gallios Flow Cytometer running Kaluza software (Figure 1). Where indicated, MCF7 cells were co-cultured with Ob-Ab-, Ob-Ab+, Ob+Ab-, or Ob+Ab+ ASCs (1:1 ratio) grown in CCM made with charcoal dextran stripped-FBS (CDS-
Figure 1. Sorting GFP⁺ MCF7 cells from DsRed⁺ ASCs. MCF7 cells were transduced with retroviruses carrying neomycin resistance and GFP, and ASCs were transduced with retroviruses carrying neomycin resistance and DsRed. MCF7 cells and ASCs were co-cultured for 7 days and sorted based on fluorescence. Representative dot plot is shown. Where indicated, GFP⁺ breast cancer cells were sorted for RNA extraction.
FBS) with or without supplemental estrogen (E\(_2\); 10 nM), leptin neutralizing antibody (R&D Systems; Minneapolis, MN), or letrozole (Sigma). For RNA isolation, MCF7 cells were sorted after co-culture with the Becton-Dickinson FACSVantage SE Cell Sorter with DiVa option (BD, Franklin Lakes, NJ) and analyzed with the DiVa software v5.02 (BD).

### 3.3.6 ASC Conditioned Media

ASCs, pooled from six donors per group, were plated on at 150 cm\(^2\) culture dish at 100 cells/cm\(^2\). After overnight culture, media was replaced with serum free αMEM medium. After 7 days, conditioned media was collected and filtered to remove debris. ASC conditioned media from each group was plated on top of MCF7 cells set up in triplicates. After 7 days, the total number of MCF7 cells were counted with a hemocytometer. Three independent experiments were conducted, each in triplicates.

### 3.3.7 RT\(^2\) Profiler\(^{TM}\) PCR Arrays

*Breast Cancer PCR Arrays.* Total cellular RNA was extracted from FACS purified MCF7 cells after co-culture with a pool of ASCs (n=6 per group) or MCF7 control cells (not co-cultured) using RNeasy Mini Kit (Qiagen, Valencia, CA) and treated with DNase I digestion (Qiagen) according to manufacturer’s instructions. One μg of RNA was converted to cDNA with the RT\(^2\) First Strand Kit (SABiosciences, Frederick, MD) according to manufacturer’s protocol. Gene expression profiling was performed using the Breast Cancer RT\(^2\) Profiler PCR Array (SA Biosciences) and RT\(^2\) qPCR Master Mix (SABiosciences). PCR amplification was performed in a Bio-Rad CFX96 Real-Time
System (Bio-Rad, Hercules, CA). The reaction conditions were as follows: 95°C for 10 min, 40 cycles of 95°C for 15 sec and 60°C for 1 min, followed by a dissociation curve. At the completion of the reaction, Cт values were determined, and ΔΔ Cт and fold change were determined using the RT² Profiler PCR Array Data Analysis web portal (SABiosciences). Genes whose mRNA levels increased or decreased more than 2-fold in MCF7 cells after co-culture with ASCs relative to MCF7 cells without co-culture were considered differentially expressed (P < 0.05).

**Obesity PCR Arrays.** Ob´Ab´ (n=6), Ob´Ab+ (n=6), Ob+Ab´ (n=6), or Ob+Ab+ (n=6) ASCs were expanded in CCM and collected for RNA extraction using RNeasy Mini Kit, and the total cellular RNA was treated with DNase I per manufacturer’s instructions. One µg of RNA was converted to cDNA with the RT² First Strand Kit according to manufacturer’s protocol. Gene expression profiling was performed using the Obesity RT² Profiler PCR Array (SABiosciences) and RT² qPCR Master Mix. Reaction settings and analysis was conducted as described above. Genes whose mRNA levels increased or decreased more than 2-fold (P < 0.05) in MCF7 cells after co-culture with ASCs relative to MCF7 cells without co-culture were considered differentially expressed.

### 3.3.8 Western Blot

MCF7 cells and FACS sorted MCF7 cells after co-cultured with ASC donors (n=6 per group) were incubated in phosphatase and protease inhibitors (Pierce, Thermo Scientific, Rockford, IL), lysed with RIPA buffer (Pierce), and centrifuged. Cell lysate was also obtained from Ob´Ab´ (n=6), Ob´Ab+ (n=6), Ob+Ab´ (n=6), or Ob+Ab+ (n=6) ASCs cultured in CCM made with charcoal dextrose stripped FBS (Atlanta Biologicals,
Lawrenceville, GA). Where indicated, ASCs were treated with 10 nM 17β-estradiol (E2, Sigma, St. Louis, MO) and/or 100 nM ICI182,780 (Sigma), and cell lysate was obtained. A total of 20 μg of protein was fractionated on 4-12% SDS-polyacrylamide gels (Invitrogen) and transferred to nitrocellulose membranes (Invitrogen). The blots were blocked with block Noise Canceling Reagents (Millipore, Billerica, MA) and probed using primary antibodies incubated overnight at 4°C, washed with phosphate-buffered solution with 0.01% TWEEN-20 (PBST), followed by a secondary antibody conjugated to horseradish peroxidase (HRP), washed with PBST and visualized with chemiluminescence reagent (Invitrogen) on an ImageQuant LAS 4000 (GE Healthcare Life Science; Piscataway, NJ).

Antibodies against cyclin dependent kinase inhibitor 2A (CDKN2A), estrogen receptor-alpha (ESR1), and progesterone receptor (PGR) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-secreted frizzled-related protein 1 (anti-SFRP1), anti-mouse-horseradish peroxidase (HRP), anti-rabbit-HRP antibodies were purchased from Abcam (Cambridge, MA). Anti-glutathione S-transferase P (anti-GSTP1) was purchased from Cell Signaling Technologies (Danvers, MA), anti-actin was purchased from Sigma, and anti-leptin was purchased from R&D systems.

3.3.9 In Vivo Tumorigenicity Assay

All procedures involving animals were conducted in compliance with State and Federal law, standards of the US Department of Health and Human Services, and guidelines established by Tulane University Institutional Animal Care and Use Committee (IACUC). All protocols were approved by the Tulane IACUC.
SCID/beige (CB17.Cg-Prkdc<sup>scid</sup>Lyst<sup>bg-J</sup>/Crl) immunocompromised female ovariectomized mice (5 weeks old) were obtained from Charles River Laboratories (Wilmington, MA). Mice were divided into treatment groups of five animals, with or without estrogen: MCF7 only, MCF7 plus Ob<sup>−</sup>Ab<sup>−</sup> ASCs (n = 6 donors), MCF7 plus Ob<sup>−</sup>Ab<sup>+</sup> ASCs (n = 6 donors), MCF7 plus Ob<sup>+</sup>Ab<sup>−</sup> ASCs (n = 6 donors), and MCF7 plus Ob<sup>+</sup>Ab<sup>+</sup> ASCs (n = 6 donors). Where indicated, estradiol pellets (0.72mg, 60-day release, Innovative Research of America, Sarasota, FL) were implanted subcutaneously in the lateral area of neck.

MCF7 cells (10<sup>6</sup>) alone or MCF7 cells (10<sup>6</sup>) in combination with ASCs (10<sup>6</sup>) suspended in a total volume of 50 µl of sterile PBS were mixed with 100 µl of reduced growth factor Matrigel (BD Biosciences, Bedford, MA). Cells were injected subcutaneously into the 5<sup>th</sup> mammary fat pad on both sides. All procedures in animals were carried out under anesthesia using a mixture of isoflurane and oxygen delivered by mask.

Tumor size was measured every 3 days using digital calipers and calculated as previously described [264]. At necropsy, animals were euthanized by cervical dislocation after exposure to CO<sub>2</sub>. Tumors were removed and frozen in liquid nitrogen or fixed in 10% neutral buffered formalin and paraffin embedded for further analysis.

3.3.10 Immunohistochemistry

Formalin-fixed, paraffin-embedded (FFPE) tumor sections were deparaffinized, rehydrated in a graded solution of Sub-X solutions, stained with hematoxylin and eosin or quenched with 0.3% H<sub>2</sub>O<sub>2</sub> (Sigma), rinsed with PBST, blocked with 1% BSA and stained
with primary antibodies against Ki-67 (Abcam) or human progesterone receptor (PGR; DAKO North America, Inc., Carpentaria, CA) overnight at 4°C. Each tumor section was subsequently washed in PBST, incubated with appropriate HRP conjugated secondary antibody for 1 hour at room temperature, and washed with PBST. For colorimetric staining, slides were then incubated in 3,3'-Diaminobenzidine (DAB; Vector Laboratories), washed with PBST, counterstained with hematoxylin, and rinsed with deionized water. Slides were sealed with Permount Mounting Medium (Sigma). For apoptosis analysis, the TACS-XL In situ Apoptosis Detection Kit (R&D Systems) was used according to manufacturer’s instructions. After staining, tumor sections were counterstained and sealed as mentioned above. Images were acquired at 10x and 40x. Quantification of the percentage of positivity was assessed using ImageScope (Aperio, Vista, CA) and determined by the percentage of positive pixels divided by the total number of pixels in a given section.

3.3.11 Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

Ob⁻Ab⁻ (n = 6 donors), Ob⁻Ab⁺ (n = 6 donors), Ob⁺Ab⁻ (n = 6 donors), or Ob⁺Ab⁺ (n = 6 donors) ASCs cultured in CCM were collected for total cellular RNA extraction using RNeasy Mini Kit. Where indicated, ASCs were cultured in CCM containing charcoal dextrose-stripped FBS, with or without supplementation with 10 nM E₂ and/or 100 nM ICI182,780. RNA was then purified with DNase I digestion (Invitrogen), and reverse transcribed using the SuperScript VILO cDNA synthesis kit (Invitrogen). Quantitative real-time PCR was performed using the EXPRESS SYBR GreenER qPCR SuperMix Kit (Invitrogen) according to the manufacturer’s instructions. The following
primer set sequence for leptin (forward 5’-gaagaccacatccacacag-3’, reverse 5’-agctcagccagacctcata-3’) and aromatase (forward 5’-cagagccagaggttctgg-3’, reverse 5’-acactagcagttctttgg-3’) were used. β-actin (forward 5’-caccttcatagctgc-3’ and reverse 3’-cttctagagctgc-5’) was used as an internal reference point. At the completion of the reaction, ΔΔCt was calculated to quantify mRNA expression.

3.3.12 Oncomine Analysis

A set of 440 normal breast tissue and invasive ductal carcinoma (IDC) deposited by The Cancer Genome Atlas (TCGA) was analyzed using the Oncomine Research Edition to assess leptin expression. Details of the standardized normalization techniques and statistical calculations can be found on the Oncomine website (http://www.oncomine.com).

3.3.13 KM Plot Analysis

To determine the five year relapse free survival of patients diagnosed with breast cancer based on leptin expression, an online survival analysis tool was utilized and can be found on the Kaplan-Meier Plotter website (http://www.kmplot.com). Details of the standardized normalization techniques and characterization of high or low expression have been previously described [265].

3.3.14 Statistical Analysis

All values are presented as means ± standard deviation (SD). The statistical differences among two or more groups were determined by ANOVA, followed by post-
hoc Dunnet multiple comparison tests versus the respective control group. The statistical differences between two groups were performed by Student’s t-test. Statistical significant was set at $P < 0.05$. Analysis was performed using Prism (Graphpad Software, San Diego, CA).

3.4 Results

3.4.1 Characterization of ASCs

ASCs were isolated from lipoaspirates of obese (Ob+) and non-obese (Ob-) 78adipose tissue (Ab+) or non-abdominal subcutaneous depots (Ab-). Each ASC donor (n=24), irrespective of the ASC group the donor was categorized into, was analyzed for the expression of cell surface markers and were positive for CD44, CD90, CD105, and CD166 and negative for CD34, CD45, and CD11b determined with flow cytometry (Figure 2). Each group of ASCs was able to generate colony-forming units and undergo osteogenesis and adipogenesis (Figure 2). No differences were observed among the four groups for ASC differentiation or self-renewal capacity as defined by colony forming units.

3.4.2 ASCs isolated from obese subjects enhance the proliferation of MCF7 cells in vitro

To investigate the effect of the donor’s BMI status and depot site on ASC interaction with breast cancer cells, MCF7 cells or MDA-MB-231 cells were directly co-cultured in a 1:1 ratio with ASCs from non-abdominal sources of non-obese subjects (Ob-Ab-), abdominal source of non-obese subjects (Ob-Ab+), non-abdominal sources of obese
Figure 2. Characterization of ASCs isolated from donors based on obesity status and deposit site. (A) ASCs (n=24) in each of the four groups were stained with antibodies against the indicated antigens and analyzed by flow cytometry. Represented cell surface marker profile for each group are shown. Histograms are shown as colored lines and the respective isotype controls are in gray. (B) CFUs were seeded at low density and incubated in CCM for 14 days. Cells were fixed and stained with crystal violet. Images were captured with a digital camera. Representative image for each group are shown. (C) ASCs were grown until 70% confluent in CCM and then switched to differentiation media. After 21 days, cells were fixed and stained with Alizarin Red for osteogenesis and Oil Red O for adipogenesis. Representative images for each group are shown. Original magnification for osteogenesis is 4x and for adipogenesis is 10x for all panels. Scale bars represents 100 µm.
subjects (Ob\(^+\)Ab\(^-\)), or abdominal sources of obese subjects (Ob\(^+\)Ab\(^+\)). Irrespective of depot site of origin or BMI, ASCs increased the proliferation of MDA-MB-231 cells \((P < 0.01)\), with no statistically significant difference between the ASC groups (Figure 3a). However, ASCs isolated from obese subjects increased the proliferation of MCF7 cells: 1.5-fold (from 1.1 x 10\(^6\) to 1.6 x 10\(^6\) MCF7 cells) after co-culture with Ob\(^+\)Ab\(^-\) ASCs \((P < 0.05)\) and 2.0-fold (from 1.1 x 10\(^6\) cells to 2.2 x 10\(^6\) cells) after co-culture with Ob\(^+\)Ab\(^+\) cells \((P < 0.01\); Figure 3a). MCF7 cells co-cultured with ASCs isolated from non-obese subjects failed to increase the cell number.

To determine whether ASCs can influence MCF7 cell growth indirectly, conditioned media was collected from all four ASC groups and added to MCF7 cells. The total number of MCF7 cells was assessed after 7 days. MCF7 cells grown in ASC conditioned media from obese subjects increased their proliferation by 1.6-fold (from 0.8 x 10\(^6\) to 1.3 x 10\(^6\) MCF7 cells after co-culture with Ob\(^+\)Ab\(^-\) ASCs) and 1.9-fold (from 0.8 x 10\(^6\) cells to 1.5 x 10\(^6\) cells after co-culture with Ob\(^+\)Ab\(^+\) cells), respectively \((P < 0.01\); Figure 3b); while no statistically significant increase was observed when MCF7 cells were exposed to ASC CM from non-obese donors.

\[\] 3.4.3 Donor’s obesity status and depot site of the ASCs influence their effect on the gene expression profile of MCF7 cells

To determine whether the ASCs could induce changes in the gene expression of MCF7 cells, a breast cancer PCR array containing 84 genes known to contribute to breast cancer tumorigenesis and progression was utilized. Of the 84 genes assessed, 13 genes
Figure 3. Direct co-culture of breast cancer cells with ASCs result in increased proliferation in vitro. (A) After 7 day of co-culture (CC) with 6 ASC donors per group, quantification of MCF7 cells and MDA-MB-231 (231) cells was based on the percentage of GFP+ cells in the population multiplied by the total number of cells in each condition. (B) To determine the influence of ASCs on MCF7 cells indirectly, conditioned media (CM) from pooled ASCs (n=6 donor per group) were collected and added to MCF7 cells. After 7 days, the total number of MCF7 cells was counted. Values reported are the mean of three independent experiments, each performed in triplicate. Bars, ± SD. *, P < 0.05; #, P < 0.01
Table 1. mRNA expression of MCF7 cells after exposure to ASCs

<table>
<thead>
<tr>
<th>Functional gene grouping</th>
<th>Gene</th>
<th>MCF7 gene expression after CC with</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Ob<em>Ab</em></td>
</tr>
<tr>
<td>Cell Cycle</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CDKN2A</td>
<td>1.41</td>
</tr>
<tr>
<td></td>
<td>CCND2</td>
<td>1.41</td>
</tr>
<tr>
<td></td>
<td>PTEN</td>
<td>1.05</td>
</tr>
<tr>
<td>Apoptosis</td>
<td>GSTP1</td>
<td>9.79</td>
</tr>
<tr>
<td></td>
<td>BAD</td>
<td>2.21*</td>
</tr>
<tr>
<td></td>
<td>SFRP1</td>
<td>1.40</td>
</tr>
<tr>
<td>Angiogenesis</td>
<td>SERPINE1</td>
<td>15.72*</td>
</tr>
<tr>
<td></td>
<td>IL8</td>
<td>3.47*</td>
</tr>
<tr>
<td></td>
<td>CDH13</td>
<td>3.65*</td>
</tr>
<tr>
<td></td>
<td>PLAU</td>
<td>1.99</td>
</tr>
<tr>
<td></td>
<td>SLIT2</td>
<td>3.15</td>
</tr>
<tr>
<td>Signal Transduction</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hedgehog</td>
<td>SNAI2</td>
<td>4.47*</td>
</tr>
<tr>
<td>Notch</td>
<td>BIRC5</td>
<td>3.17*</td>
</tr>
<tr>
<td>Steroid Receptor Mediated</td>
<td>PGR</td>
<td>4.15*</td>
</tr>
<tr>
<td>Steroid Receptor-Mediated</td>
<td>ESR1</td>
<td>3.97*</td>
</tr>
<tr>
<td>Glucocorticoid</td>
<td>IGFBP3</td>
<td>15.47*</td>
</tr>
<tr>
<td></td>
<td>NME1</td>
<td>2.86*</td>
</tr>
<tr>
<td>Metastasis</td>
<td>MMP2</td>
<td>12.22*</td>
</tr>
<tr>
<td></td>
<td>MMP9</td>
<td>3.52*</td>
</tr>
<tr>
<td></td>
<td>ID1</td>
<td>1.95*</td>
</tr>
<tr>
<td></td>
<td>TWIST1</td>
<td>1.45</td>
</tr>
<tr>
<td></td>
<td>PTGS2</td>
<td>1.34</td>
</tr>
<tr>
<td>Adhesion</td>
<td>THBS1</td>
<td>1.05</td>
</tr>
<tr>
<td></td>
<td>CSF1</td>
<td>1.99</td>
</tr>
<tr>
<td></td>
<td>ADAM23</td>
<td>1.42</td>
</tr>
<tr>
<td>Xenobiotic Transport</td>
<td>ABCG2</td>
<td>12.56*</td>
</tr>
</tbody>
</table>

Data is shown as fold change relative to MCF7 cells without previous co-culture with ASCs.

* P < 0.05, ** P < 0.01
Figure 4. Cluster diagram of relative gene expression of MCF7 cells co-culturing with ASCs characterized by obesity status and depot site of origin. Expression is relative to MCF7 cells without exposure to ASCs.
were significantly altered in MCF7 cells by direct co-culture with ASCs, irrespective of the categorical ASC group \((P < 0.05; \text{Table 1})\). Alterations in the expression of apoptotic, angiogenic, signal transduction, glucocorticoid, metastasis, and xenobiotic transportation genes were observed. These genes included: BAD, SERPINE1, IL6, CDH13, SNAI2, BIRC5, PGR, ESR1, IGFBP3, NME1, MMP2, MMP9, and ABCG2 (Table 1). However, the Ob\(^+\)Ab\(^+\) ASCs altered the expression of an additional 14 unique genes in MCF7 cells \((P < 0.05; \text{Table 1})\). Alterations in the expression of cell cycle, apoptotic, angiogenesis, metastasis, and adhesion genes were observed. These genes included: CDKN2A, CCND2, PTEN, SFRP1, PLA2, SLIT2, TWIST1, PTGS2, THBS1, CSF1, and ADAM23. The complete comparative analysis of changes in MCF7 gene expression profile after co-culture with ASCs can be found in Figure 4.

### 3.4.4 Increased expression of CDKN2A, GSTP1, SFRP1, ESR1 and PGR in MCF7 cells after co-culture with ASCs

Western blot analysis was performed to confirm the altered gene expression related to cell cycle control, apoptosis, and steroid receptors identified in the MCF7 cells on the PCR array with fold changes greater than 5-fold. Cell cycle regulator CDKN2A, apoptotic gene GSTP1 and SFRP1, and the steroid receptors ESR1 and PGR were analyzed. Robust increases in the levels of protein expression for CDKN2A, GSTP1, SFRP1, ESR1, and PGR were observed in MCF7 cells after co-culture with ASCs, irrespective of the ASC group \((P < 0.05; \text{Figure 5})\). However, co-culture with Ob\(^+\)Ab\(^+\) ASCs induced the most significant fold increase in the expression of CDKN2A (from 0.4
to 0.9), GSTP1 (from 0.3 to 0.9), SFRP1 (from 0.1 to 0.3), ESR1 (from 1.1 to 1.7) and PGR (from 1.4 to 3.2) \((P < 0.05)\).

### 3.4.5 Estrogen enhances the ASC-induced MCF7 cell proliferation in vitro

Due to the marked increase in ESR1 and PGR expression in MCF7 cells, the effect of estrogen on the ASC-induced MCF7 cell proliferation was assessed. In the absence of estrogen, MCF7 cells demonstrated no significant change in cell proliferation after co-culture with ASCs grown in CCM made with CDS-FBS \((P > 0.05; \text{Figure 6a})\), indicating that the depletion of estrogen and other growth factors commonly found in FBS eliminated the stimuli that enhanced cellular proliferation. The addition of estrogen increased MCF7 proliferation 1.4-fold \((\text{from } 2.3 \times 10^5 \text{ cells to } 3.1 \times 10^5 \text{ MCF7 cells})\) after 7 days. Estrogen induced proliferation was greatly increased when MCF7 cells were co-cultured with ASCs isolated from obese subjects, increasing the proliferation of MCF7 1.7-fold after co-culture with \(\text{Ob}^+\text{Ab}^-\) ASCs \((\text{from } 3.1 \times 10^5 \text{ cells to } 5.3 \times 10^5 \text{ MCF7 cells})\) and 1.9-fold after co-culture with \(\text{Ob}^+\text{Ab}^+\) ASCs \((\text{from } 3.1 \times 10^5 \text{ cells to } 6.0 \times 10^5 \text{ MCF7 cells})\) \((P < 0.05; \text{Figure 6b})\). While co-culturing MCF7 cells with ASCs isolated from non-obese subjects increased the proliferation of the MCF7 cells, these results were not statistically significant \((\text{Figure 6b})\).

### 3.4.6 Tumor volume of MCF7 and ASC xenografts is related to the obesity status and depot source of the ASCs

To assess the role of ASCs in tumorigenesis, MCF7 cells and ASCs were mixed and injected into the mammary fat pad of ovariectomized immunocompromised
SCID/beige mice, and tumor volumes were measured regularly over 36 days. In the absence of estrogen, MCF7 xenografts were $140 \pm 9.8 \text{ mm}^3$, while co-mixed xenografts (MCF7 and ASCs) were between $84.0 - 107.5 \text{ mm}^3$, with no statistically significant difference observed between the four groups of co-mixed xenografts (Figure 7). These results suggest that in the absence of estrogen, ASCs do not exert any influence on breast cancer tumorigenesis.

In the presence of estrogen, xenografts formed with MCF7 cells became both easily palpable and visible by day 36 compared to xenografts in the absence of estrogen ($P < 0.05$; Figure 8a). The co-mixed xenografts (MCF7 and ASCs) were significantly larger than the MCF7 only xenografts ($P < 0.01$; Figure 6a), suggesting that estrogen may be mediating its effects by activating the ASCs. Tumor volumes of xenografts formed with ASCs isolated from non-obese subjects were similar in size, irrespective of depot site of origin Ob$^{-}$Ab$^{+}$ ASCs (TV = $816.5 \pm 36.7 \text{ mm}^3$) or Ob$^{+}$Ab$^{-}$ ASCs (TV = $774.4 \pm 38.2 \text{ mm}^3$) (Figure 6a). However, xenografts formed with MCF7 cells and Ob$^{+}$Ab$^{+}$ ASCs (tumor volume (TV) = $1779.6 \pm 49.0 \text{ mm}^3$) were significantly larger in size than xenografts formed with Ob$^{+}$Ab$^{-}$ ASC (TV = $1230.0 \pm 33.5 \text{ mm}^3$) ($P < 0.05$; Figure 8a). In the presence of estrogen, irrespective of MCF7 cell or co-mixed xenografts, increased cellularity was observed, without distinction between the groups (Figure 8b).

In order to assess proliferation and apoptosis, tumor sections were stained with Ki-67 and TUNEL. In the presence of estrogen, co-mixed tumors consisting of ASCs isolated from obese subjects, Ob$^{+}$Ab$^{-}$ ASCs and Ob$^{+}$Ab$^{+}$ ASCs, demonstrated higher proliferation rates than MCF7 only xenografts. However, the co-mixed tumors formed with MCF7 cells and Ob$^{+}$Ab$^{+}$ ASCs demonstrated significantly more expression of Ki-67
Figure 5. Changes in the expression of cell cycle regulators and steroid receptors of MCF7 cells. MCF7 cells were grown alone or in the presence of ASCs (n=6 donors per group) grouped by the donor’s obesity status and depot site of ASCs for 7 days. Cell lysate from sorted GFP^+ MCF7 cells was collected for Western blot analysis of CDKN2A, GSTP1, SFRP1, ESR1, and PGR. Blots were then stripped and probed for actin as a control.
Figure 6. Estrogen enhances the effect of ASCs on MCF7 proliferation in vitro.
ASCs (n=6 donors per group) were co-cultured (CC) with MCF7 cells in media supplemented with charcoal dextran stripped fetal bovine serum. (A) After 7 days, quantification of MCF7 cells was based on the percentage GFP+ cells in the population multiplied by the total number of cells in the condition. (B) ASCs were grown in 10 nM estrogen (E2) replenished every 3 days, and after 7 days, quantification of MCF7 cells was based on the percentage of GFP+ cells in the population multiplied by the total number of cells in each condition. Values reported are the mean of three independent experiments, each performed in triplicate. Bars, ± SD. *, P < 0.05.
Figure 7. Tumorigenesis of MCF7 cells when co-mixed with the 4 categorical ASC group in the absence of estrogen. (A) Tumor volume of MCF7 cells alone or co-mixed cells injected into the mammary fat pad in the absence of estrogen. (B) Representative images of immunohistochemistry staining for human Ki-67, TUNEL, and PGR staining in tumor sections. (C) Quantification of Ki-67, TUNEL, and PGR staining with ImageScope represented as the percentage of positive pixels over total number of pixels per tumor section. All images were acquired at 10x and 40x. Scale bar represents 50 µm. Bars, ± SD. *, $P < 0.05$. 
Figure 8. Donor’s obesity status and depot site of ASCs differentiates its influence on tumorigenicity in vivo. MCF7 cells alone or MCF7 cells and ASCs were co-mixed in a 1:1 ratio in a total volume of 50 µl of sterile PBS and mixed with 100 µl of reduced growth factor Matrigel. Cells were injected into the 5th mammary fat pad of 6-week old female ovariectomized SCID/beige mice (n = 5 mice per group). Estrogen was delivered subcutaneously in the neck as a 0.72 mg 60-day time-release estrogen pellet. Tumor volume was measured every 3 days for a total of 36 days. (A) Tumor volume of tumors formed with MCF7 cells alone or co-mixed cells (MCF7 and ASCs) injected into the mammary fat pad with or without estrogen pellet (E2). (B) Representative images of H&E, Ki-67, TUNEL, and PGR staining of tumor sections from tumor sections taken at 10x and 40x. Scale bars both represent 50 µm. (C) Quantification of Ki-67, TUNEL, and PGR staining with ImageScope represented as the percentage of positive pixels over total number of pixels per tumor section. Values reported are means of ten tumor sections. Bars, ± SD. *, P < 0.05 between MCF7 and other groups. †, P < 0.01 between MCF7 with estrogen and other groups, ‡, P < 0.001 between MCF7 and other groups.
(51.5% Ki-67 positive; \( P < 0.05 \); Figure 8b; Figure 8c). The detection of apoptotic cells demonstrated a lower frequency of apoptosis events in co-mixed tumors formed with MCF7 and \( \text{Ob}^+\text{Ab}^+ \) ASCs with 6.5% positive compared to 17.4% positive in MCF7 xenografts \( (P < 0.05; \) Figure 8b; Figure 6c).

3.4.7 Enhanced progesterone receptor expression in xenografts formed with \( \text{Ob}^+\text{Ab}^+ \) ASCs

Due to the increased tumor volume and decreased levels of apoptosis observed in co-mixed xenografts formed with MCF7 cells and \( \text{Ob}^+\text{Ab}^+ \) ASCs in the presence of estrogen, the possibility of estrogen receptor (ER)-mediated signaling was further explored. Tumor sections were stained for progesterone receptor (PGR), as PGR expression is mediated by ER signaling and its increased expression correlates with ER activation. The xenografts formed in the absence of estrogen demonstrated no PGR staining (Figure 7; Figure 8b). In the presence of estrogen, PGR expression increased in all xenografts and was highest in the MCF7 cell/\( \text{Ob}^+\text{Ab}^+ \) ASCs xenografts (20.9% PGR positivity) compared to 11.7% PGR positivity in MCF7 only xenografts \( (P < 0.05; \) Figure 8b; Figure 8c).

3.4.8 Gene expression profiles differ between ASCs based on obesity status and depot source

In order to explore quantifiable differences in gene expression between the groups of ASCs, a PCR array with genes known to play a role in obesity was utilized. The results demonstrated that the mRNA expression levels of 8 genes were altered between the \( \text{Ob}^- \)
Ab⁺ ASCs, Ob⁺Ab⁻ ASCs, and Ob⁺Ab⁺ ASCs when compared to Ob⁻Ab⁻ ASCs: leptin (LEP), leptin receptor (LEPR), sortilin 1 (SORT1), thyrotropin-releasing hormone (TRH), melanin-concentrating hormone 1 (MCHR1), peroxisome proliferator-activated receptor-gamma (PPAR-gamma), peroxisome proliferator-activated receptor gamma coactivator 1-α (PPARGC1A), and thyroid hormone receptor-β (THRB) \( P < 0.05 \); Figure 9; Table 2). Furthermore, the Ob⁺Ab⁺ ASCs demonstrated more robust changes in the expression of both LEP (201.5-fold increase) and TRH (50.44 fold decrease; \( P < 0.01 \); Figure 9; Table 2). The full comparative analysis of the gene expression profile of the four groups of ASCs can be found in Figure 9 and Table 2.

3.4.9 Estrogen stimulates leptin expression in ASCs which then enhances MCF7 proliferation

With the increased expression of leptin in Ob⁺Ab⁺ ASCs, the potential ER-mediated response through leptin was explored by exposing ASCs cultured in CDS-FBS to estrogen and/or ICI182,780, a steroidal estrogen antagonist. The removal of endogenous estrogen in the FBS resulted in no statistical alterations of leptin expression between the groups (Figure 10a), with a 3.1-fold increase in Ob⁻Ab⁻ ASCs, 4.4-fold increase in Ob⁻Ab⁺ ASCs, 3.5-fold increase in Ob⁺Ab⁻ ASCs, and 5.8-fold increase in Ob⁺Ab⁺ ASCs. However in the presence of estrogen, the expression of leptin in the ASCs increased from 11.1- (Ob⁻Ab⁻ ASCs), 37.2- (Ob⁻Ab⁺ ASCs), 18.7- (Ob⁺Ab⁻ ASCs), and 128.8-fold (Ob⁺Ab⁺ ASCs) \( P < 0.05 \); Figure 10a). While all of the groups of ASCs demonstrated an increase in leptin expression, the Ob⁻Ab⁻ ASCs demonstrated the greatest fold increase (10-fold) in leptin expression compared to the other groups \( P <
These results suggest robust activation of Ob$^+$Ab$^+$ ASCs by estrogen. The treatment of the cells with ICI182,780, in the presence estrogen reduced leptin expression in each group to pretreatment levels.

To confirm that the levels of leptin protein increased in the ASCs, cells were cultured in CDS-FBS with or without supplementation of estrogen and probed for leptin expression by Western blot. Leptin levels in each ASC group increased in the presence of estrogen (Figure 10b). In order to prove that these results were not donor dependent, each individual ASC donor was treated with estrogen and probed for leptin expression. While some donor variability was observed between the donors within a group, leptin always increased and the levels of variability were not statistically significant. Moreover, all Ob$^+$Ab$^+$ ASC donors demonstrated the greatest increase in leptin expression compared to the other ASC donors ($P < 0.01$; Figure 10c).

To determine the role of leptin in proliferation of breast cancer cells, MCF7 cells were co-cultured with ASCs in the presence of a neutralizing antibody to leptin. After 7 days, in the presence of the neutralizing antibody and estrogen, MCF7 cells did not increase in proliferation, irrespective of the ASC group (Figure 11a). These results indicate that ASCs induce MCF7 proliferation, at least in part, through an estrogen-mediated activation of leptin in ASCs.

3.4.10 Enhanced ASC aromatase expression and activity increases MCF7 cells

In order to assess local estrogen synthesis, ASCs were grown in CCM and aromatase mRNA expression was assessed. ASCs isolated from obese donors demonstrated enhanced aromatase expression, as Ob$^-$Ab$^-$, Ob$^-$Ab$^+$, Ob$^+$Ab$^-$, and Ob$^+$Ab$^+$
Figure 9. Cluster diagram of relative gene expression of ASCs characterized by obesity status and depot site of origin. Expression is relative to Ob'Ab' ASCs.
Table 2. Fold change in mRNA expression of ASCs based on obesity status and depot site of origin

<table>
<thead>
<tr>
<th>Functional Grouping</th>
<th>Hormone and Receptor Grouping</th>
<th>Gene</th>
<th>Ob Ab⁺</th>
<th>Ob⁺Ab⁺</th>
<th>Ob⁻Ab⁻</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anorectic</td>
<td>Adipocyte-derived</td>
<td>LEP</td>
<td>9.47⁺</td>
<td>7.72⁺</td>
<td>201.47⁺⁺⁺⁺</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LEPR</td>
<td>-1.23⁺</td>
<td>1.35⁺</td>
<td>3.34⁺</td>
</tr>
<tr>
<td></td>
<td>Neuro</td>
<td>SORT1</td>
<td>1.49⁺</td>
<td>2.37⁺</td>
<td>1.92⁺</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TRH</td>
<td>-4.25⁺</td>
<td>-2.52⁺</td>
<td>-50.44⁺⁺⁺⁺</td>
</tr>
<tr>
<td>Orexigenic</td>
<td>Neuro</td>
<td>MCHR1</td>
<td>1.30⁺</td>
<td>-1.50⁺</td>
<td>-2.29⁺</td>
</tr>
<tr>
<td>Energy Expenditure</td>
<td>Adipocyte-derived</td>
<td>PPARG</td>
<td>1.55⁺</td>
<td>1.37⁺</td>
<td>1.40⁺</td>
</tr>
<tr>
<td></td>
<td>CNS-derived</td>
<td>PPARGC1A</td>
<td>-2.48⁺</td>
<td>-2.19⁺</td>
<td>-2.16⁺</td>
</tr>
<tr>
<td></td>
<td></td>
<td>THR1</td>
<td>-2.21⁺</td>
<td>1.49⁺</td>
<td>-1.84⁺</td>
</tr>
</tbody>
</table>

⁺ Data is shown as fold change relative to Ob⁺Ab⁻ ASCs.
⁺⁺⁺⁺ P < 0.05, 𝑎 P < 0.01
Figure 10. Estrogen exposure influences leptin expression in ASCs. (A) ASCs (n=6 donors per group) were cultured in media supplemented with charcoal dextran stripped FBS (CDS-FBS) supplemented with (E₂) or ICI182,780. Cells were collected at 70% confluence and total RNA was isolated for real-time PCR analysis of leptin expression. (B) Cell lysate was collected from n=6 ASC donors cultured in CDS-FBS with or without E₂ at 70% confluency and subjected to Western blot analysis with 20 µg of protein and separated by SDS-page under reducing conditions, blotted, and probed with antibodies to leptin or actin. (C) Cell lysate was collected from 6 different ASC donors and either pooled or analyzed as individual donors (D#), cultured in CDS-FBS with or without estrogen. A total of 20 µg of protein was separated by SDS-page under reducing conditions, blotted, and probed with leptin and actin antibodies. Bars, ± SD. *, P < 0.05; #, P < 0.005.
Figure 11. Leptin neutralizing antibody and letrozole negate enhanced proliferation in obese ASCs. (A) ASCs (n=6 donors per group) were co-cultured with MCF7 cells and treated with vehicle (DMSO) neutralizing leptin antibody (Lep nAb), estrogen (E$_2$) or neutralizing leptin antibody and estrogen (Lep nAb + E$_2$) in complete culture media (CCM) containing CDS-FBS. After 7 days, the number of MCF7 cells was calculated based on the percentage of GFP$^+$ MCF7 cells by the total number of cells in the population. (B) ASCs (n=6 donors per group) were cultured in CCM and collected at 70% confluence and total RNA was isolated for real-time PCR analysis of aromatase expression. (C) ASCs (n=6 donors per group) were co-cultured with MCF7 cells and treated with letrozole (10 nM) in CCM. After 7 days, the number of MCF7 cells was determined by the percentage of GFP$^+$ cells multiplied by the total number of cells. Percent change was determined by the relative change in the number of MCF7 cells after letrozole treatment compared to their non-treated controls. Values reported are the mean of three independent experiments, each performed in triplicate. Bars, ± SD. *, $P < 0.05$; **, $P < 0.01$; ###, $P < 0.005$. 
Figure 12. Levels of leptin expression correlate with decreased survival in ER\(^+\)/PR\(^+\) breast cancers. Kaplan Meier analysis of the probability of five-year relapse-free survival of women diagnosed with (A) ER\(^+\)/PR\(^+\) or (B) ER\(^-\)/PR\(^-\) breast cancers based on leptin expression.
ASCs demonstrated 11.6-, 13.7-, 19.7- and 25.4-fold increase relative to MCF7 cells, respectively \((P < 0.05; \text{Figure 11b})\). Furthermore, ASCs isolated from obese donors demonstrated greater aromatase expression compared to non-obese donors \((P < 0.01)\). Furthermore, delivery of aromatase inhibitors reduced the enhanced proliferation of MCF7 cells due to the co-culture with ASCs. MCF7 cells co-cultured with ASCs from obese subjects demonstrated the most significant reduction, with the most significant decrease in \(\text{Ob}^+\text{Ab}^-\) ASCs (-66.3%) and \(\text{Ob}^+\text{Ab}^+\) ASCs (-131.4%) \((P < 0.01; \text{Figure 9c})\).

3.4.11 High leptin levels correlate with poor relapse-free survival in \(\text{ER}^+/\text{PR}^+\) breast cancer

In order to assess expression of leptin in human breast cancer samples, leptin expression was analyzed using the TCGA cDNA microarray data set of breast cancers deposited in Oncomine. Compared to normal breast tissue \((n = 73)\), invasive ductal breast carcinoma (IBC; \(n = 367\)) demonstrated a 2.0-fold increase in expression \((P = 0.005)\). To determine whether leptin expression related to prognosis and the hormone status of the breast cancer, further analysis was conducted with the Kaplan-Meier (KM) Plotter. KM plots demonstrated that women diagnosed with \(\text{ER}^+/\text{PR}^+\) breast cancers whose tumor also expressed high levels of leptin \((n = 25)\) demonstrated poorer prognosis with increased mortality rates as compared to \(\text{ER}^+\text{PR}^-\) breast cancers with lower levels of leptin \((n = 73; P = 0.038; \text{Figure 12a})\). In contrast, analysis of leptin expression in women diagnosed with \(\text{ER}^+\text{PR}^-\) breast cancer did not demonstrate a correlation between leptin and relapse-free survival outcomes \((P = 0.15; \text{Figure 12b})\). Together, this suggests that invasive breast
carcinoma overexpresses leptin compared to normal breast tissue and that leptin levels may also be a potential prognostic factor for ER$^+/PR^+$ invasive breast carcinomas.

3.5 Discussion

The results of this study demonstrate that site of origin and BMI alter the biologic properties of subcutaneous human ASCs and their role in cancer tumorigenesis. ASCs were categorized based on depot source and obesity status of the donor subject. ASCs isolated from obese subjects led to the greatest increase in the proliferation and tumorigenicity of MCF7 cells \textit{in vitro} and \textit{in vivo} via an estrogen-activated response mediated through leptin.

Previous studies have explored the endocrine role of adipocytes on breast cancer cell proliferation and metastasis [266]; however, only a few studies have examined the effects of ASCs on breast cancer cell lines. Muehleberg et al. demonstrated that ASCs increased the proliferation of a murine breast cancer cell line and enhanced the invasion and metastasis of cancer cells [260]. Utilizing ASCs isolated from the breast and from abdominal adipose tissue, Walter et al. demonstrated that the secretion of IL-6 from ASCs enhanced the migration and invasion of breast cancer cells [257]. The results from this study provide further support for the role of ASCs in the tumor microenvironment. The data from this study demonstrated that ASCs effectively alter the gene expression profile of breast cancer cells. More specifically, ASCs isolated from obese subjects enhanced breast cancer cell proliferation and tumor volume and ASCs isolated from the abdomen of obese subjects (BMI > 30) further altered the expression of several additional genes. This data provides additional support for the role of obesity, especially abdominal
obesity, in affecting breast cancer prognosis. At present, the mechanism(s) by which abdominal obesity induces changes in the ASCs is not known. Rehrer et al. demonstrated differences in the subcutaneous adipose tissue isolated from the hip and flank compared to adipose tissue isolated from the abdomen, providing support for differences in adipose tissue based on depot site [267]. More specifically, genes involved in the biochemical metabolism were expressed at higher levels in the abdomen compared to the hip [267]. Additional studies into the microenvironment of the adipose tissue in obese subjects, prior to isolation, would provide substantial insight into the influence of obesity on ASC biology.

Analysis of the gene expression profiles of the four ASC groups revealed significant differences in leptin expression and further determined that the enhanced leptin levels were the direct result of exposure to estrogen. The analysis of the four groups of ASCs, categorized based on depot site and obesity status of the subject, provides support for the potential of ASCs to alter breast cancer cells. More specifically, while leptin expression was increased in ASCs isolated from the abdomen of non-obese subjects and from non-abdominal sources of obese subjects, a more robust increase in leptin expression was observed in ASCs isolated from the abdomen of obese subjects when the cells are exposed to estrogen. Leptin has been shown to play a vital role in the progression of breast cancer through the activation of several signaling cascades. Delivery of leptin to breast cancer cells enhances proliferation rate through the activation of STAT3 and ERK1/2 signaling pathway and diminishes apoptosis through a significant reduction in p53 expression and Bax production [268, 269]. Several studies suggest that leptin also exhibits estrogen-producing activity by enhancing aromatase expression and
enhances the sensitivity of breast cancer cells to estrogen through the upregulation of estrogen receptor alpha [270, 271]. In addition, the inhibition of leptin-signaling results in diminished tumor growth and progression. Animal studies demonstrate that subcutaneous injection of leptin receptor antagonist peptide delayed the development and slowed the growth of breast cancer tumors, suggesting the involvement of leptin in tumor latency and growth [272].

While ASCs isolated from the abdomen of obese subjects demonstrated an increase in leptin expression, the cells failed to elicit an effect on breast cancer cell proliferation or tumor growth. After exposure to estrogen the ASCs increased in leptin expression and breast cancer cell proliferation and tumor growth, suggesting that a threshold of expression must be achieved before leptin can effectively activate breast cancer cell proliferation. These findings indicate that one of the primary mechanism(s) by which ASCs influence breast cancer is via estrogen-mediated pathways. While this study did not focus on the origins of the estrogen, it has been shown that the adipose tissue of obese subjects produce significantly more estrogen through enhanced aromatase activity [273]. As such, enhanced estrogen production in the adipose tissue of obese subjects could potentially stimulate an altered ASC phenotype to secrete an abundance of leptin to alter the gene expression profile of breast cancer cells.

The analysis of the gene expression profiles of breast cancer cells after co-culture with ASCs indicate that the ASCs can activate signaling cascades that enhance proliferation, reduce apoptosis, stimulate angiogenesis, and increase metastatic rate of breast cancer cells [257]. The direct co-culture studies revealed the upregulation of CDKN2A, a cell cycle regulator, and GSTP1, a gene responsible for the detoxification of
drugs, which have been shown to be upregulated in multi-drug resistant breast cancer [274]. More specifically, Kars et al. demonstrated enhanced GSTP1 expression and CDKN2A expression among their paclitaxel and vincristine resistant MCF7 cell lines [274]. These results may suggest that co-culturing ASCs isolated from the abdomen of obese subjects may induce a multi-drug resistant MCF7 phenotype, but additional studies are necessary.

Although the studies described here utilized MCF7 and MDA-MB-231 cell lines, further analysis with additional ER$^+$ breast cancer cell lines may provide insight into the full capacity of ASCs to impact different types of breast cancer. Further studies to evaluate the role of additional adipokines in the conditioned media as well as potential contribution of cell-cell interactions are necessary to fully understand the mechanism by which ASCs influence breast cancer tumorigenesis and progression. Nevertheless, our study provides insight into the variability among donors and within donors. While previous studies have demonstrated significant variation in the growth properties, osteogenic capacity, and adipogenic capacity of bone marrow-derived stem cells and adipose stromal cells, respectively, the cause of the variability between donors and between different aspirates within the same donor have not been previously identified [275, 276]. Herein, our study provides insights into the potential for the depot site of origin as well as donor’s obesity status to contribute to donor-to-donor variability and variability observed within the same donor.

The link between high leptin expression and prognosis for breast cancer patients was determined to be significant in ER$^+$/PR$^+$ breast cancer. The analysis of leptin expression in primary breast cancer samples demonstrated significant differences in
normal breast tissue compared to invasive ductal carcinoma [277, 278]. It should be noted that these publically available resources do not separate the tumor stroma from the cancer cells, and as such increased leptin expression could correlate with increased expression of leptin in the tumor stroma, where the ASCs are localized. More detailed analysis of primary breast cancer samples is needed to assess the cell type expressing the leptin. However, the assessment of the ASCs from obese and non-obese subjects as well as their depot site suggest that more profound differences could be observed, if obesity status could be taken into account. Since the patient population analyzed in these two resources did not differentiate patients based on obesity status, it would be expected that the results presented here would reveal only a limited scope on the effects of leptin on survival rate. Taken together, the assessment of the effect of ASCs on breast cancer suggests that the tumor microenvironment consisting of these ASCs could dictate the outcomes in obese patients. Moreover, these findings suggest that leptin may be a novel therapeutic target for breast cancer treatment in obese patients.

3.6 Conclusion

This study demonstrates that ASCs are conditioned by their local microenvironment in obese subjects as both the donor’s obesity status and the site of deposition contribute to the stimulatory effects of ASCs on breast cancer cell growth. Furthermore, the positive effects of estrogen on leptin expression in ASCs suggest a potential mechanism by which these ASCs are elicited to influence breast cancer cell proliferation and suggest a new avenue to be explored for breast cancer treatment in obese patients.
PART III: OBESE ASCS ENHANCE ESTROGEN RECEPTOR POSITIVE BREAST CANCER EMT AND METASTASIS

Leptin Produced by Obese ASCs Enhances Proliferation and Metastasis of Estrogen Receptor Positive Breast Cancers

Amy L. Strong, Jason F. Ohlstein, Brandi A. Biagas, Lyndsay V. Rhodes, Dorothy T. Pei, Alan Tucker, Claire Llamas, Annie C. Bowles, Maria F. Dutreil, Shijia Zhang, Jeffrey M. Gimble, Matthew E. Burow, Bruce A. Bunnell

Manuscript submitted to Breast Cancer Research

4.1 Abstract

Introduction: The steady increase in the incidence of obesity among adults in the United States has been paralleled with higher levels of obesity-associated breast cancer. While recent studies have suggested that adipose stromal/stem cells (ASCs) isolated from obese women enhance tumorigenicity, the mechanism(s) by which this occurs remains undefined. Epidemiological evidence suggests that increased adiposity results in increased leptin secretion from the adipose tissue, which has been shown to increased cancer cell proliferation. Previously, our group has demonstrated that ASCs isolated from obese women (obASCs) also expressed higher levels of leptin relative to ASC isolated from lean women (lnASC) and that this obASC-derived leptin may account for enhanced
breast cancer cell growth. The current study investigates the impact of inhibiting leptin expression in lnASCs and obASCs on breast cancer cell (BCC) growth and progression. Methods: Estrogen receptor positive (ER⁺) BCCs were co-cultured with leptin shRNA lnASCs or leptin shRNA obASCs and changes in the proliferation, migration, invasion, and gene expression of BCCs were investigated. To assess the direct impact of leptin inhibition in obASCs on BCC proliferation, MCF7 cells were injected alone or mixed with control shRNA obASCs or leptin shRNA obASCs into SCID/beige mice. Results: ER⁺ BCCs were responsive to obASCs during direct co-culture, whereas lnASCs were unable to increase ER⁺ BCC growth. shRNA silencing of leptin in obASCs negated the enhanced proliferative effects of obASC on BCCs following direct co-culture. Consistent with phenotypic changes, BCCs co-cultured with obASCs demonstrated enhanced expression of epithelial-to-mesenchymal transition (EMT) and metastasis genes (SERPINE1, MMP-2, and IL-6), while BCCs co-cultured with leptin shRNA obASCs did not display similar levels of gene induction. Knockdown of leptin significantly reduced tumor volume and persistence of ASCs within the tumor. Furthermore, knockdown of leptin decreased the number of metastatic lesions from the primary tumor site to the lung and liver. These results correlated with reduced expression of both SERPINE1 and MMP-2 in tumors formed with MCF7 cells mixed with leptin shRNA obASCs, when compared to tumors formed with MCF7 cells mixed with control shRNA obASCs. Conclusion: This study provides mechanistic insight as to how obesity enhances the proliferation and metastasis of breast cancer cells; specifically, obASC-derived leptin contributes to the aggressiveness of breast cancer in obese women.
4.2 Introduction

Obesity is defined by the accumulation of excessive adipose tissue that can contribute to physical and psychosocial impairment. The prevalence of obesity in the world, particularly in the United States, has increased over the past four decades, with one-third of adults in the United States meeting the criteria for obesity [279]. As a result, there has been an increase in the incidence of obesity-associated cancers [280-282]. More specifically, recent studies suggest that obesity increases the incidence of breast cancer [283, 284].

Epidemiological studies investigating the role of obesity in breast cancer suggest that obesity increases the incidence of metastatic breast tumors, results in higher rates of incidence of re-occurrence, and increases mortality. Haakinson et al. found that obese patients are diagnosed with larger primary tumors and had an increased incidence of lymph node metastases [285]. Furthermore, in postmenopausal breast cancer patients, up to 50% of deaths have been attributed to obesity [286]. While the link between obesity and breast cancer has been well documented from epidemiologic analyses, the molecular mechanisms underlying this correlation are not fully defined.

An analysis of the interplay between breast cancer and obesity provides some insights into the underlying pathophysiology. During breast cancer development and progression, a complex multi-step cascade converts normal breast epithelial cells into malignant cells [287-289]. One of the key steps involves the interaction between the epithelial cells and the stromal microenvironment, which contains adipose stromal/stem cells (ASCs) [290]. Studies have shown that obesity significantly increases the number of ASCs within the adipose tissue. This ASC hyperplasia has been shown to support both
angiogenesis and adipogenesis and to alter the gene expression profile of ASCs such that they enhance cancer growth[202, 291, 292]. Recently, our group has demonstrated that ASCs isolated from obese patients (BMI ≥ 30; obASCs) enhance the tumorigenicity of MCF7 breast cancer cells; as well as alter their gene expression profile [291]. Additionally, the data showed that the obASCs expressed significantly higher levels of leptin compared to ASCs isolated from lean patients (BMI ≤ 25; lnASCs). However, the overexpression of leptin in obASCs and the impact it has on increasing the aggressiveness of tumor cell biology *in vitro* and *in vivo* has not been investigated.

The role of leptin produced by obASCs on BCCs was investigated in this study by inhibiting the expression of leptin using an shRNA knockdown strategy. The obASCs preferentially increased the proliferation, migration, and invasion of several ER+ BCC lines, including MCF7, ZR75, and T47D, during direct co-culture. Reducing the levels of leptin in obASCs negated their effects on BCCs. Consistent with phenotypic changes, inhibiting leptin expression in obASCs negated alterations to the gene expression profile of BCC after co-culture. Furthermore, reducing leptin levels in obASCs also resulted in a reduction in tumor volume and fewer metastatic lesions in the lung and liver of SCID/beige mice. These results implicate obASC-derived leptin as a key mechanism that alters BCC growth and supports changes to the biology of BCCs into a more aggressive phenotype. The results here suggest that the inhibition of leptin secreted by obASCs may result in reduced tumor volume and metastasis to distant organs, reducing the burden of obesity-associated breast cancers.

4.3 Material and Methods
4.3.1 Human Subjects

Human ASCs were obtained from 12 Caucasian females (2 groups, 6 donors per group) undergoing elective liposuction procedures, as previously described [3]. All protocols were reviewed and approved by the Pennington Biomedical Research Center Institutional Review Board, and all human participants provided written informed consent. Briefly, ASCs were isolated from processed lipoaspirates from the subcutaneous abdominal adipose tissue of lean or obese patients. Liposuction aspirates were incubated in 0.1% type I collagenase (Sigma) and 1% powdered bovine serum albumin (BSA, fraction V; Sigma) dissolved in 100ml of phosphate buffered saline (PBS) supplemented with 2mM calcium chloride. The mixture was placed in a 37°C shaking water bath or incubator at 75 rpm for 60 min and then centrifuged to remove oil, fat, primary adipocytes, collagenase solution and cellular debris. The resulting cell pellet was re-suspended in stromal medium (SM), which consisted of Dulbecco’s Modified Eagles Medium (DMEM)/F12 (Hyclone, Logan UT), 10% fetal bovine serum (Hyclone, Logan UT), 1% antibiotic/antimycotic (Fisher Scientific, Houston TX), and plated in 175 cm² flasks. Fresh SM was added every 2-3 days until cells achieved 80-90% confluence, at which time cells were harvested with 0.25% trypsin / 1mM EDTA (GIBCO) and cryopreserved prior to experimental use. The mean BMI for the lnASC group was 22.7 ± 1.9, while the mean BMI for the obASCs was 32.7 ± 3.7. The mean age of the subjects for each group of donors was as follows: lnASCs (38.8 ± 7.0) and obASCs (42.5 ± 8.9). No statistical significance in age was observed between the donor groups.

4.3.2 Cell Culture
ASCs. Frozen vials of ASCs were thawed and cultured on 150 cm$^2$ culture dishes (Nunc, Rochester, NY) in 25 ml CCM and incubated at 37°C with 5% humidified CO$_2$. After 24 hours, viable cells were harvested with 0.25% trypsin / 1mM EDTA and re-plated at 100-200 cells/cm$^2$ in complete culture medium (CCM), which consisted of α-Minimal Essential Medium (αMEM; GIBCO; Grand Island, NY), 20% fetal bovine serum (FBS; Atlanta Biologicals, Lawrenceville, GA), 100 units per ml penicillin/100 µg/mL streptomycin (P/S; GIBCO), and 2 mM L-glutamine (GIBCO). Medium was changed every 2-3 days. For all experiments, sub-confluent cells ($\leq$70% confluent) between passages 2-6 were used. Characterization of stem cells were previously performed and published [291].

Breast cancer cell (BCC) lines. MCF7 (HTB-22), ZR75 (CRL-1500), T47D (HTB-133), MDA-MB-231 (HTB-26), MDA-MB-157 (HTB-24), and BT549 (HTB-122) cells were obtained directly from American Type Culture Collection (ATCC; Manassas, VA) and used for fewer than 6 months after resuscitation. Cell line authentication was conducted by ATCC via short tandem repeat profiling. Cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM; GIBCO), supplemented with 10% FBS and P/S. Cells were grown at 37°C with 5% humidified CO$_2$, fed every 2-3 days, and split 1:4 to 1:6, when the cells reached 90% confluent.

4.3.3 Synthesis of GFP$^+$ BCCs

To produce lentivirus, 293T cells were transfected through a modified calcium chloride transfection protocol when cells reached 70-75% confluent. For each transfection, 10 µg of packaging plasmid, enveloping encoding plasmid, and transfer
plasmid containing GFP and neomycin resistance or dsRed and neomycin resistance were used. After 48 hours, medium was harvested and used to transduce cancer cells. To transduce ASCs, conditioned medium containing virus with dsRed and neomycin resistance was added to ASCs at 70% confluence. MCF7, ZR75, T47D, MDA-MB-231, MDA-MB-157, BT-549, and ASCs were selected with 500 µg/ml of Geniticin (Invitrogen; Carlsbad, CA) for 2 weeks and GFP expression or dsRed expression was verified with flow cytometry. All cancer cells and ASCs used for experimentation expressed GFP or dsRed unless otherwise specified.

4.3.4 Conditioned Media

ASCs, pooled from six donors per group, were plated on a 150 cm² cell culture dish at 100 cells/cm². After overnight culture, medium was replaced with serum free αMEM. After 7 days, conditioned media (CM) was collected and filtered to remove cellular debris. The total number of ASCs was also counted to verify equal number of cells after 7 days. ASC CM from lnASCs and obASCs was plated on top of BCCs set up in triplicates. After 7 days, the total number of MCF7 cells was counted. Where indicated, CM was collected from control shRNA lnASCs, leptin shRNA lnASCs, control shRNA obASCs, and leptin shRNA obASCs.

4.3.5 Stable Transfection of shRNA

Short hairpin RNA (shRNA) constructs targeting leptin and an shRNA construct targeting a non-human gene serving as a negative control were purchased from SABiosciences (Frederick, MD). The GFP sequence in the shRNA construct was
removed and replaced with dsRed and neomycin resistance, producing a dsRed, neomycin resistant leptin shRNA construct and a dsRed, neomycin resistant negative control shRNA construct. The lnASCs (n=6 donors) and obASCs (n=6 donors) were transfected with a dsRed, neomycin resistant leptin shRNA construct or a dsRed, neomycin resistant negative control shRNA construct using the Neon Transfection System (Invitrogen), using 1400 V for the pulse voltage, 10 ms for the pulse width, and 3 pulses. Cells were allowed to recover, expanded, underwent antibiotic selection for two weeks, and sorted by flow cytometry to verify dsRed expression. Four groups of cells (n=6 donor/group) were produced: control shRNA lnASCs, leptin shRNA lnASCs, control shRNA obASCs, and leptin shRNA obASCs.

4.3.6 Alamar Blue Cell Proliferation Assay

Alamar blue cell proliferation assay was conducted according to manufacturer’s instructions. Briefly, 100 cells from each donor (lnASCs, control shRNA lnASCs, leptin shRNA lnASCs, obASCs, control shRNA obASCs, or leptin shRNA obASCs) were plated in a 96 well plate in triplicates. After cells adhered overnight, the medium was removed, the wells were washed three times in PBS, and the cells were incubated in 10% alamarBlue reagent (Invitrogen). After 18 hours, the fluorescence intensity was measured at an excitation wavelength of 540 nm and an emission wavelength of 580 nm using a fluorescence plate reader. Cells were assessed on days 1, 3, and 7.

4.3.6 RNA Isolation Followed by Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR)
Subconfluent cultures of control shRNA lnASCs (n=6 donors), leptin shRNA lnASCs (n=6 donors), control shRNA obASCs (n=6 donors), and leptin shRNA obASCs (n=6 donors) were analyzed. RNA was extracted from ASCs using TRIzol reagent (Invitrogen), purified with RNeasy columns (Qiagen), and digested with DNase I (Invitrogen). A total of 1 µg of cellular RNA was used for cDNA synthesis with SuperScript VILO cDNA synthesis kit (Invitrogen). Quantitative real-time PCR was performed using the EXPRESS SYBR GreenER qPCR SuperMix Kit (Invitrogen) according to the manufacturer’s instructions. The following primer set sequence for leptin (forward 5’-gaagaccacatccacacacg-3’, reverse 5’-agctcagccagacccatcta-3’) and β-actin (forward 5’-caccttctacaatgagctgc-3’ and reverse 3’-ctctctgatgctcgacgga-5’) were used. At the completion of the reaction, ΔΔCt was calculated to quantify mRNA expression.

4.3.8 Characterization of ASCs

ASCs were characterized as previously described [293]. Briefly, ASCs were induced to undergo osteogenic or adipogenic differentiation. For osteogenic differentiation, ASCs were cultured in 6-well plates in CCM until 70% confluent and medium was replaced with fresh medium containing osteogenic supplements, consisting of 50 µM ascorbate 2-phosphate (Sigma), 10 mM β-glycerol phosphate (Sigma), and 10 nM dexamethasone. After three weeks, cells were fixed in 10% formalin for 1 hour at 4°C and stained for 10 minutes with 40 mM Alizarin Red (pH 4.1) to visualize calcium deposition in the extracellular matrix. Images were acquired at 4x magnification on Nikon Eclipse TE200 (Melville, NY) with Nikon Digital Camera DXM1200F using the Nikon ACT-1 software version 2.7. For adipogenic differentiation, ASCs were cultured
in 6-well plates in CCM until 70% confluent, and medium was replaced with fresh medium containing adipogenic supplements, consisting of 0.5 µM dexamethasone (Sigma), 0.5 mM isobutylmethylxanthine (Sigma), and 50 µM indomethacin (Sigma). After three weeks, cells were fixed in 10% formalin for 1 hour at 4°C, stained for 10–15 minutes at room temperature with Oil Red O (Sigma) to detect neutral lipid vacuoles, and images were acquired at 10x magnification.

To determine the ability to form colony-forming units (CFU), ASCs were plated at a density of 100 cells on a 10 cm² plate in CCM and incubated for 14 days. Plates were rinsed three times with PBS, and 10 ml of 3% crystal violet (Sigma) was added for 30 minutes at room temperature. Plates were washed three times with PBS and once with tap water.

Analysis by flow cytometry of the cell surface marker profile was conducted by harvesting ASCs with 0.25% trypsin / 1mM EDTA for 3-4 minutes at 37°C. A total of 3 x 10⁵ cells were concentrated by centrifugation at 500 x g for 5 min, suspended in 50 µl PBS and labeled with the primary antibodies. The following primary antibodies were used: Anti-CD45-PeCy7, anti-CD11b-PeCy5, anti-CD166-PE, anti-CD105-PE, anti-CD90-PeCy5, anti-CD34-PE, isotype-control FITC human IgG1 and isotype-control PE human IgG2a were purchased from Beckman Coulter (Indianapolis, IN). Anti-CD44-APC was purchased from BD Biosciences (San Jose, CA). The samples were incubated for 30 minutes at room temperature, washed with PBS, and analyzed with Galios Flow Cytometer (Beckman Coulter, Brea, CA) running Kaluza software (Beckman Coulter). To assay cells by forward and side scatter of light, FACScan was standardized with microbeads (Dynosphere uniform microspheres; Bangs Laboratories Inc.; Thermo
Scientific; Waltham, MA). At least 10,000 events were analyzed and compared with isotype controls.

4.3.9 BCC and ASC Co-culture

BCCs were co-cultured with InASCs (n=6 donors) or obASCs (n=6 donors) at 200 cells/cm² in a 1:1 ratio in DMEM supplemented with 10% FBS and P/S. After 7 days, cells were harvested, washed, and analyzed by flow cytometry. The percentage of GFP⁺ cells (BCCs) was determined with Galios Flow Cytometer running Kaluza software and calculated based on the total number of cells. Where indicated, control shRNA InASCs (n=6 donors), leptin shRNA InASCs (n=6 donors), control shRNA obASCs (n=6 donors), and leptin shRNA obASCs (n=6 donors) were co-cultured with MCF7, ZR75, or T47D for 7 days. The percentage of GFP⁺ cells (BCCs) was determined with Galios Flow Cytometer running Kaluza software and calculated based on the total number of cells. For RNA isolation, BCCs were sorted after co-culture with the Becton-Dickinson FACSVantage SE Cell Sorter with DiVa option (BD, Franklin Lakes, NJ) and analyzed with the DiVa software v5.02 (BD).

4.3.10 Transwell Migration and Invasion Assays

Migration assays were performed in transwell inserts with 8-µm pore membrane filters, while invasion assays were performed with 8-µm transwell inserts pre-coated with a growth factor-reduced Matrigel layer to mimic basement membranes (BD Biosciences). BCC cells were cultured alone or co-cultured with control InASCs, leptin shRNA InASCs, control obASCs, or leptin shRNA obASCs for 7 days in a 1:1 ratio. BCCs were
purified with fluorescence-activated cell sorting (FACS), and $1.25 \times 10^4$ BCCs suspended in 50 µl were added to the apical chamber. A total of 200 µl of chemoattractant (10% FBS) was added to the basal chamber and incubated for 4 hours or 24 hours for migration or invasion, respectively. After the allotted time, the lower side of the transwell insert was carefully washed with cold PBS and non-migrating or non-invading cells remaining on the top chamber were removed with a cotton tip applicator. Migrating and invading cells were stained with Calcein-AM (2 µg/ml; Invitrogen; Grand Island, NY) and measured on a fluorescent plate reader (FLUOstar optima; BMG Labtech Inc.; Durham, NC). Data was normalized to the respective BCCs without previous exposure to ASCs.

4.3.11 RNA Isolation Followed by Custom RT² Profiler™ PCR Arrays

To assess cells with the PCR array, total cellular RNA was extracted using RNeasy Mini Kit from FACS purified BCCs cultured alone or after co-culture with a pool of control shRNA InASCs, leptin shRNA InASCs, control shRNA obASCs, or leptin shRNA obASCs with an n=6 donor per group (Qiagen, Valencia, CA). RNA was treated with DNase I (Qiagen) according to manufacturer’s instructions. One µg of RNA was converted to cDNA with the RT² First Strand Kit (SABiosciences, Frederick, MD) according to the manufacturer’s protocol. Where indicated, total cellular RNA was extracted from tumors using RNeasy Mini Kit, treated with DNase I, and converted to cDNA with the RT² First Strand Kit according to the manufacturer’s instructions. Gene expression profiling was performed using a Custom Breast Cancer RT² Profiler PCR Array (SA Biosciences) and RT² qPCR Master Mix (SABiosciences). The Custom Breast Cancer RT² Profiler PCR Array was manufactured to detect the expression of the
following genes: SERPINE1, IGFBP3, GSTP1, MMP-2, SNAI2, IL-6, PGR, TWIST1, PTGS2, SFRP1, THBS1, CDKN2A, PLAU, CSF1, and ACTB. PCR amplification was performed in a Bio-Rad CFX96 Real-Time System (Hercules, CA). The reaction conditions were as follows: 95°C for 10 min, 40 cycles of 95°C for 15 sec and 60°C for 1 min, followed by a dissociation curve. At the completion of the reaction, C_t values were determined, and ΔΔ C_t and fold change were determined using the RT^2 Profiler PCR Array Data Analysis web portal (SABiosciences). Genes with mRNA levels increased or decreased by more than 2-fold were considered significantly differentially expressed (P < 0.05).

4.3.12 In Vivo Tumorigenicity Assay

SCID/beige (CB17.Cg-Prkdc<sup>scid</sup>Lyst<sup>bg</sup>−/Crl) immunocompromised female ovariectomized mice (5 weeks old) were obtained from Charles River Laboratories (Wilmington, MA). To assess whether leptin impacts tumorigenicity, mice were divided into 3 groups (n=5 mice/group): MCF7 cells only, MCF7 cells plus control shRNA obASCs (n = 6 donors), and MCF7 plus leptin shRNA obASCs (n = 6 donors). Estradiol pellets (0.72mg, 60-day release, Innovative Research of America, Sarasota, FL) were implanted subcutaneously in the lateral area of the neck. Cell implants were prepared with MCF7 cells (10<sup>6</sup>) alone or MCF7 cells (10<sup>6</sup>) in combination with ASCs (10<sup>6</sup>) suspended in a total volume of 150 µl (1 part sterile PBS and 2 parts reduced growth factor Matrigel; BD Biosciences, Bedford, MA). Cells were injected subcutaneously into the 5<sup>th</sup> mammary fat pad on both sides. All procedures in animals were carried out under anesthesia using a mixture of isoflurane and oxygen delivered continuously by mask.
After 36 days, animals were euthanized by cervical dislocation after exposure to CO₂. Organs were removed, weighed, digitally imaged, and fixed in 10% neutral buffered formalin. Where indicated, additional mice were divided into 3 groups (n=5 mice/group): MCF7 only, MCF7 plus lnASCs, and MCF7 plus obASCs to assess for potential metastasis by lnASCs or obASCs. Lungs and livers were also harvested and fixed in 10% neutral buffered formalin for histological analyses.

All procedures involving animals were conducted in compliance with State and Federal law, standards of the US Department of Health and Human Services, and guidelines established by Tulane University Institutional Animal Care and Use Committee (IACUC). All animal protocols were approved by the Tulane University IACUC.

4.3.13 Flow Cytometry

Flow cytometry was conducted on the tumors to assess for GFP expressing MCF7 cells and dsRed expressing ASCs. Tumors were dissociated with collagenase/hyaluronidase (Stem Cell Technologies, Vancouver, BC, Canada) for 16 hours at 37°C. After enzymatic dissociation, the reaction was neutralized with pre-warmed medium consisted of 10% FBS. Cells were centrifuged at 350xg for 10 minutes, counted, and resuspended in PBS. The samples were then analyzed with Galios Flow Cytometer (Beckman Coulter, Brea, CA) running Kaluza software (Beckman Coulter).

4.3.14 Immunohistochemistry
Formalin-fixed, paraffin-embedded (FFPE) tumor, lung, and liver sections were de-paraffinized, rehydrated in Sub-X (Leica, Buffalo Grove, IL) and graded solutions of ethanol, and stained with hematoxylin and eosin. FFPE tumor sections were de-paraffinized, rehydrated in Sub-X and graded solutions of ethanol, quenched with 0.3% H$_2$O$_2$ (Sigma), rinsed with Tris-NaCl-Tween buffer (TNT), which consisted of 0.1 M Tris-HCl (pH 7.5; Sigma), 0.15M NaCl (Sigma), and 0.05% Tween-20 (Invitrogen). Tumor sections were then blocked with 1% BSA, and stained with primary antibodies obtained from Abcam against GFP, dsRed, SERPINE1, or MMP-2 overnight at 4°C. Each tumor section was subsequently washed in TNT buffer. Tissue sections were incubated with appropriate HRP conjugated secondary antibody (Abcam) for 1 hour at room temperature, and washed with TNT buffer. For colorimetric staining, slides were then incubated in 3,3'-Diaminobenzidine (DAB; Vector Laboratories), washed with TNT, counterstained with hematoxylin (Thermo Scientific), and rinsed with deionized water. Slides were dehydrated in graded solutions of ethanol, followed by SubX in the final step, and sealed with Permount Mounting Medium (Sigma). After staining, images were acquired at 10x and 40x magnification with the ScanScope CS2 (Aperio, Vista, CA).

4.3.15 Protein Isolation and Western Blot

Protein lysates were isolated with RIPA buffer (Pierce; Thermo Scientific) from primary tumors formed with MCF7 cells, MCF7 cells mixed with control shRNA obASCs, or MCF7 cells mixed with leptin shRNA obASCs. Tumors were homogenized in RIPA buffer for 5 minutes, and the cell lysate was clarified by centrifugation at 15,000xg for 15 min. Protein concentration was determined by the BCA Protein Assay.
Lysate (20 µg) was resolved on 4-12% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Invitrogen). Blots were blocked with block Noise Canceling Reagents (Millipore). Blots were then incubated with anti-leptin antibody (R&D Systems; Minneapolis, MN) overnight at 4°C and washed with PBS with Tween 20 three times before incubated with species-specific IgG conjugated to HRP for 1 hour at room temperature. Antigen-antibody complexes were visualized after incubation in chemiluminescence reagent (Invitrogen). Blots were imaged on an ImageQuant LAS 4000 (GE Healthcare Life Sciences; Piscataway, NJ) and quantitative analysis of Western blots was performed with densitometry.

4.3.16 Metastasis Assessment

Metastatic lesions were quantified by determining the area occupied by the lesion divided by the total area of the tissue section. The percentage the tissue occupied by metastatic cells in the liver and lung were averaged together for each mouse (n=5 mice/group) and represented as the metastatic index.

4.3.17 Statistical Analysis

All values are presented as means ± standard deviation (SD) or means ± standard error of the mean (SEM). The statistical differences among three or more groups were determined by ANOVA, followed by post-hoc Dunnet multiple comparison tests versus the respective control group. Statistical significance was set at $P < 0.05$. Analysis was performed using Prism (Graphpad Software, San Diego, CA).
4.4 Results

4.4.1 ER\textsuperscript{+} BCCs selectively respond to obese ASCs

The impact of ASCs on BCC growth was investigated using a co-culture assay. BCC lines MCF7, ZR75, T47D, MDA-MB-231, MDA-MB-157, and BT549 were cultured alone or at a 1:1 ratio with either \textit{ln}ASCs or \textit{ob}ASCs for 7 days. Prior to co-culture, BCCs were transduced with lentivirus expressing GFP in order to isolate BCCs expressing the fluorochrome following the co-culture period. \textit{ob}ASCs increased the proliferation of ER\textsuperscript{+} BCCs: 2.0±0.2-fold in MCF7 cells, 2.2±0.1-fold in ZR75, and 1.9±0.1-fold in T47D (\textit{P} < 0.05; Figure 1A). While \textit{ln}ASCs increased the proliferation of ER\textsuperscript{+} BCCs, the effect was not as robust or statistically significant (Figure 1A). In contrast, both \textit{ln}ASCs and \textit{ob}ASCs increased the proliferation of triple-negative breast cancer (TNBC) cell lines. \textit{ln}ASCs increased MDA-MB-231, MDA-MB-157, and BT-549 cells by 1.5±0.2-fold, 1.5±0.1-fold, and 1.6±0.1-fold, respectively (\textit{P} < 0.05; Figure 1B), and \textit{ob}ASCs increased BCCs by 1.6±0.1-fold in MDA-MB-231, 1.5±0.0-fold in MDA-MB-157, and 1.6±0.1-fold in BT-549 (\textit{P} < 0.05; Figure 1B). These results suggest that ASCs increase the proliferation of all BCCs; however, \textit{ob}ASCs markedly increase the proliferation of ER\textsuperscript{+} BCCs over \textit{ln}ASCs.

Previously, our group demonstrated that \textit{ob}ASCs produce high levels of the adipokine leptin. To investigate whether the effects of leptin were through increase aromatase activity, since aromatase is a downstream target of leptin, we determined the level of aromatase expression in \textit{ln}ASCs and \textit{ob}ASCs and the effects of aromatase inhibitor on our system. The mRNA expression of aromatase was 12.1-fold and 30.4-fold higher in \textit{ln}ASCs and \textit{ob}ASCs, respectively, compared to MCF7 cells (\textit{P}\textless0.05; Figure 2).
There was no significant difference in aromatase expression in cancer cells (Figure 2). To assess the functional activity of aromatase in our system, we collected conditioned media from ASCs treated with vehicle or letrozole. CM collected from vehicle-treated obASCs induced proliferation of MCF7 cells (by 53.4%), ZR75 cells (by 83.9%) and T47D cells (by 65.2%; \( P<0.05 \); Figure 3). Upon treatment with CM collected from lnASCs and obASCs treated with letrozole, a reduction in cancer cell number was observed; however, cancer cells exposed to CM from obASCs treated with letrozole were still able to induced BCC proliferation. These results suggest that while aromatase produced by obASCs may contribute to increase proliferation of cancer cells, alternative pathways activated by leptin may be driving the effects of obASCs on BCCs proliferation.

4.4.2 obASCs expressing leptin shRNA have diminished capacity to enhance BCC proliferation

The role that leptin plays in the biology of obASCs and their impact on BCCs was investigated through the stable transfection of shRNA constructs targeting leptin into lnASCs and obASCs. These cells were used to determine the impact of silencing leptin on BCC proliferation. Leptin knockdown lnASCs and obASCs were initially collected to demonstrate a reduction of leptin expression and functional assays were performed to determine changes in stem cell properties. Knockdown efficiency was assessed with real-time RT-PCR after purification of transfected cells with FACS. lnASCs showed a reduction in leptin expression by 63.8%, from 1.04-fold in control shRNA lnASCs to 0.37-fold in leptin shRNA lnASCs, and obASCs demonstrated a reduction in leptin expression by 99.4%, from 147.81-fold in control shRNA obASCs to 0.85-fold in leptin
shRNA obASCs, respectively \((P < 0.05; \text{Figure 4})\). No statistically significant difference was observed in the transcript level of leptin between leptin shRNA \(lnASCs\) and leptin shRNA obASCs. To characterize the cells, \(lnASCs\) or obASCs transfected with the leptin shRNA or control shRNA were differentiated in osteogenic differentiation medium or adipogenic differentiation medium, plated to determine colony forming unit capacity, and analyzed for the expression of cell surface markers. Transfected ASCs did not differ from non-transfected ASCs and maintained the ability to generate colony forming units and differentiate into bone and fat (Figure 4). The cells also retained expression of CD44, CD90, CD105, and CD166 (while negative for the lymphohematopoietic markers CD34 and CD45 and monocyte marker CD11b; Figure 4) similar to untransfected ASCs. The proliferation rate of transfected ASCs did not differ from non-transfected ASCs (Figure 4), and no significant difference was observed between the proliferation rate of \(lnASCs\) and obASCs transfected with control shRNAs or leptin shRNAs (Figure 4).

The direct role of leptin secreted from obASCs on BCC proliferation was investigated by co-culturing either control shRNA \(lnASCs\), leptin shRNA \(lnASCs\), control shRNA obASCs, or leptin shRNA obASCs with BCCs at a 1:1 ratio. The proliferation of BCCs was compared to BCCs cultured alone. MCF7, ZR75, and T47D cells demonstrated an increase in the total cell number following co-culture with control shRNA obASCs compared to BCCs cultured alone or co-cultured with control \(lnASCs\) \((P < 0.05; \text{Figure 5})\). The obASCs transfected with the leptin shRNA were unable to induce proliferation of BCCs, in comparison to the obASC expressing the control shRNA. Leptin shRNA obASCs reduced the extent of proliferation in MCF7, ZR75, and T47D from 1.3E6 cells to 0.5E6 cells (2.2-fold decrease), 1.2E6 cells to 0.3E6 cells (4.0-fold decrease).
decrease), and 1.3E6 cells to 0.9E6 cells (1.4-fold decrease), respectively, comparing BCCs exposed to control shRNA obASCs to leptin shRNA obASCs ($P < 0.05$; Figure 5). To assess whether CM collected from ASCs was able to induce BCC proliferation, CM was collected from control shRNA lnASCs, leptin shRNA lnASCs, control shRNA obASCs, and leptin shRNA obASCs. BCCs incubated in control shRNA obASCs induced BCC proliferation by 1.4-fold in MCF7 cells, 1.8-fold in ZR75, and 1.6-fold in T47D; however BCCs incubated in control shRNA lnASCs, leptin shRNA lnASCs, and leptin shRNA obASCs were unable to induce BCC proliferation. These results implicate obASC-derived leptin as a key factor leading to enhanced proliferation of BCCs.

4.4.3 obASCs enhance BCC migration and invasion

BCCs were directly co-cultured for 7 days with lnASCs or obASCs transfected with control shRNA or leptin shRNA and collected by detection of GFP expression using FACS. Sorted cells were re-suspended in serum-free medium and plated on top of a transwell and assessed for migration after 4 hours or plated on top of a Matrigel coated transwell and assessed for invasion after 24 hours. The BCCs collected after co-culture demonstrated enhanced migration towards chemoattractants by at least 2.7-fold relative to non-co-cultured BCCs, following direct co-culture of BCCs with lnASCs or obASCs (Figure 6A). Silencing of leptin slightly diminished the capacity of both lnASCs and obASCs, to affect BCC migration; however, these results were not statistically significant (Figure 6A). The invasive capacity of BCCs was enhanced after direct exposure to lnASCs and obASCs. The impact of obASCs on BCC invasion was more robust than the effect of lnASC on BCC invasion. lnASCs enhanced the invasion of MCF7, ZR75, and
Figure 1. *obASCs enhance BCC proliferation.* (A) Estrogen receptor positive and (B) triple negative GFP+ BCCs were co-cultured with *lnASCs* (n=6 donors) or *obASCs* (n=6 donors) for 7 days and FACS sorted based on GFP expression. FACS-sorted BCCs were counted and data is shown relative to the number of cells cultured alone. Bar ± SD. *, P < 0.05, **, P < 0.01, ***, P < 0.001, relative to no co-culture; #, P < 0.05, ##, P < 0.01 between BCCs co-cultured with *lnASCs* and *obASCs*. 
Figure 2. Letrozole was unable to inhibit the effects of obASCs. (A) RNA was collected from MCF7 cells, T47D cells, ZR75 cells, ctrl shRNA lnASCs, and ctrl shRNA obASCs and analyzed for aromatase expression by qRT-PCR. Data was normalized to the β-actin expression. *, $P < 0.05$; ***, $P < 0.001$ relative to MCF7 cells. ΦΦΦ, $P < 0.001$ relative to lnASCs and obASCs. (B) Conditioned media (CM) was collected from control shRNA lnASCs or control shRNA obASCs treated with vehicle or letrozole. BCCs were cultured in the CM for 7 days and the number of GFP$^+$ BCCs was counted. *, $P < 0.05$; **, $P < 0.01$ relative to unconditioned cells.
Figure 3. Leptin in conditioned media is essential for the obASC-driven BCC proliferation. Conditioned media (CM) was collected from control shRNA lnASCs, leptin shRNA lnASCs, control shRNA obASCs, and leptin shRNA obASCs. BCCs were cultured in the CM for 7 days and the number of GFP⁺ BCCs was counted. *, $P < 0.05$; **, $P < 0.01$ relative to unconditioned cells.
Figure 4. *lnASCs* and *obASCs* were stably transfected with ctrl-shRNA and lep-shRNA. *lnASCs* (n=6 donors) and *obASCs* (n=6 donors) were transfected with a ctrl-shRNA construct targeting a non-human gene or a lep-shRNA construct targeting leptin. Cells underwent antibiotic selection, followed by FACS sorting. ASCs stably transfected with the shRNA vectors were assessed with (A) real-time polymerase chain reaction to quantitative leptin expression at the RNA level (B) Stably transfected ASCs were characterized by self-renewal capacity demonstrated by colony forming unit assay. Cells seeded at low density were assessed after 14 days by staining with crystal violet. (C) Stably transfected ASCs were plated in 6 well dishes and exposed to bone differentiation medium and fat differentiation medium to determine their ability to undergo adipogenic and osteogenic differentiation. After 21 days, cells were fixed and stained with Oil Red O or Alizarin Red for adipogenic differentiation and osteogenic differentiation, respectively. Representative images of ASCs acquired at 10x (adipogenic differentiation) and 4x (osteogenic differentiation) are shown. (D) Stably transfected cells were assessed by flow cytometry for the expression of indicated cell surface markers. (E) The proliferation rate of *lnASCs* and *obASCs* were compared with stably transfected control shRNA *lnASCs* and control shRNA *obASCs*. (F) The proliferative rate of stably transfected control shRNA and leptin shRNA *lnASCs* and *obASCs* were compared. Bar ± SD. ***, P < 0.001 between ctrl-shRNA *lnASC* and ctrl-shRNA *obASC*, ###, P < 0.001 between ctrl-shRNA *obASCs* and lep-shRNA *obASCs*. 
Figure 5. Leptin is essential for the obASC-driven BCC proliferation. InASCs and obASCs transfected with either a ctrl-shRNA or lep-shRNA were co-cultured with BCCs. After 7 days, the GFP\(^+\) BCCs were FACS-sorted and counted. Bar ± SD. *, \( P < 0.05 \), ***, \( P < 0.001 \), relative to no co-culture; ###, \( P < 0.01 \), ####, \( P < 0.001 \) between BCCs co-cultured with ctrl-shRNA InASCs and ctrl-shRNA obASCs; ΦΦΦ, \( P < 0.001 \) between BCCs co-cultured with ctrl-shRNA obASCs and lep-shRNA obASCs;
Figure 6. obASC-derived leptin increases invasion of BCC cells. BCC cells were co-cultured with lnASCs or obASCs stably transfected with a ctrl-shRNA or lep-shRNA for seven days. GFP⁺ BCC cells were isolated with FACS and plated in the top chamber of (A) an uncoated 8-µm pore membrane and assessed after 4 hours for migratory potential or (B) a Matrigel coated 8-µm pore membrane and assessed after 24 hours for invasive capacity. Medium containing FBS was plated in the lower chamber and served as a chemoattractant. Data is shown relative to cells without prior exposure to lnASCs or obASCs. Bar ± SD. *, $P < 0.05$, ***, $P < 0.001$ relative to BCCs cultured alone; ##, $P < 0.01$ between BCCs after co-culture with ctrl-shRNA lnASCs and ctrl-shRNA obASCs; ⦿, $P < 0.05$, ⦿⦿, $P < 0.01$ between BCCs after co-culture with ctrl-shRNA obASCs and lep-shRNA-obASCs.
T47D cells by 4.1±1.1-fold, 3.9±0.8-fold, and 3.8±1.0-fold, respectively, while obASCs increased the invasive potential of MCF7, ZR75, and T47D cells by 10.2±2.3-fold, 9.2±1.1-fold, and 11.0±1.2-fold, respectively (P < 0.05; Figure 6B). Inhibiting leptin expression in obASCs significantly decreased the levels of BCC invasion, while reducing leptin expression in lnASCs did not have the same impact. Leptin inhibition in obASCs reduced MCF7, ZR75, and T47D cell invasion by 27.5%, 29.3%, and 28.1%, respectively (P < 0.05; Figure 6B). These results suggest that while obASCs have a greater capacity to enhance the migration and invasion of BCCs compared to lnASCs, obASC-derived leptin only plays an integral role in BCC invasion.

4.4.4 Leptin shRNA obASCs reduce expression of proliferation and EMT genes

The mechanism(s) by which ASC-derived leptin affects BCC signaling pathways was investigated using a custom breast cancer PCR array to determine changes in the expression of previously identified cell cycle, apoptotic, and angiogenic genes. Previously, our group showed alterations in these genes in the MCF7 cells after co-culture with obese ASCs [291]. Of the cell cycle, apoptotic, and angiogenic genes assessed (CDKN2A, GSTP1, SFRP1, PLAU, THBS1, CSF), none were significantly increased in the three BCC lines following direct co-culture with obASCs (Table 1, Table 2, Table 3).

In contrast, investigation of EMT and metastatic genes previously identified by us to be altered in MCF7 cells after co-culturing with obASCs showed uniform changes in all three BCC lines. Of the genes assessed, BCCs overexpressed SERPINE1, IL-6, and MMP-2 following direct co-culture with obASCs. Furthermore, inhibition of leptin in
obASCs diminished the expression of these genes in BCCs (relative to obese ASCs; \( P < 0.05 \); Figure 7, Table 1, Table 2, Table 3). No statistically significant differences in gene expression were observed in BCCs after co-culture with control shRNA lnASCs or leptin shRNA lnASCs (Figure 7, Table 1, Table 2, Table 3). SERPINE1 expression was significantly decreased in MCF7, ZR75, and T47D after leptin knockdown by 393.9-fold, 21.7-fold, and 12.6-fold, respectively (\( P < 0.05 \); Figure 7, Table 1, Table 2, Table 3). Additionally, leptin inhibition in obASCs limited the induction of IL-6 expression in BCC by the obASCs: 2.3-fold decrease in MCF7 cells, 2.0-fold decrease in ZR75, and 1.5-fold decrease in T47D (\( P < 0.05 \); Figure 7, Table 1, Table 2, Table 3). Moreover, inhibition of leptin in obASCs resulted in a 27.4-fold, 179.2-fold, and 843.5-fold decrease in MMP-2 expression in MCF7, ZR75, and T47D cells, respectively (\( P < 0.05 \); Table 1, Table 2, Table 3). Together, these results indicate that obASCs increase the mRNA expression of key metastatic genes in ER\textsuperscript{+} BCC lines and suggest that obASCs may play a significant role in EMT and metastasis.

4.4.5 Leptin shRNA obASCs result in diminished tumorigenicity and expression of EMT genes

In order to investigate the effects of leptin silencing on tumorigenesis, MCF7 cells were implanted alone or mixed with control shRNA obASCs or leptin shRNA obASCs into the mammary fat pad of SCID/beige mice. After 36 days, tumors formed with MCF7 cells mixed with leptin shRNA obASCs were significantly smaller (1115.1±36.7 mm\(^3\) and 1.22±0.03 g) compared to tumors formed with MCF7 cells mixed with control shRNA obASCs (2141.5±29.5 mm\(^3\) and 1.92±0.03 g) (\( P < 0.001 \); Figure 8A-C).
Histological assessment of the tumors confirmed that the bulk of the tumor was composed of GFP expressing MCF7 cells (Figure 8D). Furthermore, immunohistochemistry confirmed the presence of dsRed expressing ASCs in tumors formed with control shRNA obASCs mixed with MCF7 cells. In contrast, dsRed expression was not visible in tumors formed with MCF7 alone or mixed with leptin shRNA obASCs (Figure 8D).

To assess the mechanism by which obASC-derived leptin affects BCC signaling in vivo, RNA was isolated from tumors and assessed with the custom breast cancer PCR array (Table 4). Assessment of the tumors showed increased expression of SERPINE1, MMP-2, and IL-6 in tumors formed with control shRNA obASCs mixed with leptin ($P < 0.05$; Figure 9A; Table 4). Inhibition of leptin in obASCs negated the gene induction of SERPINE1 and MMP-2 caused by obASCs in tumors. Western blot analysis was conducted on tumor samples to validate the expression of SERPINE1 and MMP-2 proteins. Tumors formed with MCF7 cells mixed with control shRNA obASCs demonstrated a 3.0-fold ($\pm 0.5$) induction of SERPINE1 while tumors formed with MCF7 cells mixed with leptin shRNA obASCs demonstrated a 1.6-fold ($\pm 0.1$) increase in SERPINE1, relative to MCF7 cells unexposed to ASCs (Figure 9C). Likewise, tumors formed with MCF7 cells mixed with control shRNA obASCs demonstrated a 4.5-fold ($\pm 0.2$) increase in MMP-2. Meanwhile, tumors formed with MCF7 cells mixed with leptin shRNA obASCs showed no difference from MCF7 cells without prior exposure to ASCs (Figure 9C). Confirmation by immunohistochemistry demonstrated increased expression of SERPINE1 and MMP-2 in tumors formed with MCF7 cells and control shRNA ASCs compared to tumors formed with MCF7 cells and leptin shRNA ASCs.
Figure 7. Leptin inhibition reduces the expression of key regulatory genes involved in invasion and metastasis. BCC cells were co-cultured with lnASCs or obASCs stably transfected with a ctrl-shRNA or lep-shRNA. After 7 days, co-cultured were FACS sorted and BCCs were analyzed by qRT-PCR. Bar ± SD. *, $P < 0.05$, ***, $P < 0.001$ relative to BCCs cultured alone; ###, $P < 0.01$ between BCCs after co-culture with ctrl-shRNA lnASCs and ctrl-shRNA obASCs; ΦΦΦ, $P < 0.001$ between BCCs after co-culture with ctrl-shRNA obASCs and lep-shRNA-obASCs.
Table 1. mRNA expression of MCF7 cells after exposure to leptin knockdown ASCs. Data is shown as fold change relative to respective breast cancer cell line without previous co-culture with ASCs. *, P < 0.05; #, P < 0.01; ¥, P < 0.001

<table>
<thead>
<tr>
<th>Function</th>
<th>Gene Name</th>
<th>ctrl-shRNA ASCs</th>
<th>lep-shRNA ASCs</th>
<th>ctrl-shRNA ASCs</th>
<th>lep-shRNA ASCs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>lnASCs</td>
<td>lnASCs</td>
<td>obASCs</td>
<td>obASCs</td>
</tr>
<tr>
<td>Cell Cycle and Apoptosis</td>
<td>CDKN2A</td>
<td>3.6</td>
<td>4.8</td>
<td>22.2*</td>
<td>11.6*</td>
</tr>
<tr>
<td></td>
<td>GSTP1</td>
<td>19.2*</td>
<td>20.7*</td>
<td>284.5¥</td>
<td>15.4*</td>
</tr>
<tr>
<td></td>
<td>SFRP1</td>
<td>10.2*</td>
<td>1.0</td>
<td>74.4¥</td>
<td>19.9*</td>
</tr>
<tr>
<td>Angiogenesis</td>
<td>PLAU</td>
<td>1.4</td>
<td>1.3</td>
<td>5.9¥</td>
<td>2.1</td>
</tr>
<tr>
<td></td>
<td>THBS1</td>
<td>16.4*</td>
<td>13.8*</td>
<td>20.9*</td>
<td>15.5*</td>
</tr>
<tr>
<td></td>
<td>CSF</td>
<td>16.4¥</td>
<td>0.3</td>
<td>20.9¥</td>
<td>15.5</td>
</tr>
<tr>
<td>EMT and Metastasis</td>
<td>SERPINE1</td>
<td>26.2*</td>
<td>29.9*</td>
<td>3624.0¥</td>
<td>9.2*</td>
</tr>
<tr>
<td></td>
<td>MMP2</td>
<td>124.9</td>
<td>109.3</td>
<td>2895.0¥</td>
<td>105.7</td>
</tr>
<tr>
<td></td>
<td>IL-6</td>
<td>3.2</td>
<td>5.5</td>
<td>17.6¥</td>
<td>7.6</td>
</tr>
<tr>
<td></td>
<td>TWIST1</td>
<td>2.1</td>
<td>0.8</td>
<td>91.2¥</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td>PTGS2</td>
<td>3.2</td>
<td>9.5</td>
<td>81.0¥</td>
<td>54.5¥</td>
</tr>
<tr>
<td></td>
<td>SNAI2</td>
<td>2.2</td>
<td>4.2</td>
<td>517.0¥</td>
<td>4.2</td>
</tr>
</tbody>
</table>
Table 2. mRNA expression of ZR75 cells after exposure to leptin knockdown ASCs. Data is shown as fold change relative to respective breast cancer cell line without previous co-culture with ASCs. *, P < 0.05; #, P < 0.01; ¥, P < 0.001

<table>
<thead>
<tr>
<th>Function</th>
<th>Gene Name</th>
<th>ctrl-shRNA lnASCs</th>
<th>lep-shRNA lnASCs</th>
<th>ctrl-shRNA obASCs</th>
<th>lep-shRNA obASCs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell Cycle and Apoptosis</td>
<td>CDKN2A</td>
<td>2.4</td>
<td>1.9</td>
<td>10.2*</td>
<td>2.3</td>
</tr>
<tr>
<td></td>
<td>GSTP1</td>
<td>3.3</td>
<td>3.1</td>
<td>0.8</td>
<td>4.0</td>
</tr>
<tr>
<td></td>
<td>SFRP1</td>
<td>8.3*</td>
<td>3.1</td>
<td>50.3*</td>
<td>14.2*</td>
</tr>
<tr>
<td>Angiogenesis</td>
<td>PLAU</td>
<td>1.4</td>
<td>1.9</td>
<td>2.3*</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>THBS1</td>
<td>1.2</td>
<td>1.6</td>
<td>2.3*</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>CSF</td>
<td>1.4</td>
<td>1.4</td>
<td>2.3</td>
<td>1.9</td>
</tr>
<tr>
<td>EMT and Metastasis</td>
<td>SERPINE1</td>
<td>14.9*</td>
<td>12.0*</td>
<td>204.0*</td>
<td>9.4*</td>
</tr>
<tr>
<td></td>
<td>MMP2</td>
<td>2.1</td>
<td>3.0</td>
<td>1845.9*</td>
<td>10.3</td>
</tr>
<tr>
<td></td>
<td>IL-6</td>
<td>4.2</td>
<td>3.3</td>
<td>20.3*</td>
<td>10.2*</td>
</tr>
<tr>
<td></td>
<td>TWIST1</td>
<td>3.5</td>
<td>2.6</td>
<td>15.0*</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td>PTGS2</td>
<td>1.3</td>
<td>1.1</td>
<td>2.3</td>
<td>2.1</td>
</tr>
<tr>
<td></td>
<td>SNAI2</td>
<td>1.3</td>
<td>2.1</td>
<td>10.3</td>
<td>5.1</td>
</tr>
</tbody>
</table>
Table 3. mRNA expression of T47D cells after exposure to leptin knockdown ASCs.
Data is shown as fold change relative to respective breast cancer cell line without previous co-culture with ASCs. *, P < 0.05; #, P < 0.01; ¥, P < 0.001

<table>
<thead>
<tr>
<th>Function</th>
<th>Gene Name</th>
<th>ctrl-shRNA lnASCs</th>
<th>lep-shRNA lnASCs</th>
<th>ctrl-shRNA obASCs</th>
<th>lep-shRNA obASCs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell Cycle and Apoptosis</td>
<td>CDKN2A</td>
<td>2.8</td>
<td>2.7</td>
<td>1.5</td>
<td>1.9</td>
</tr>
<tr>
<td></td>
<td>GSTP1</td>
<td>1.7</td>
<td>1.4</td>
<td>15.6*</td>
<td>30.4*</td>
</tr>
<tr>
<td></td>
<td>SFRP1</td>
<td>4.0</td>
<td>0.6</td>
<td>0.7</td>
<td>0.7</td>
</tr>
<tr>
<td>Angiogenesis</td>
<td>PLAU</td>
<td>0.4</td>
<td>0.3</td>
<td>0.5</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>THBS1</td>
<td>0.3</td>
<td>0.3</td>
<td>0.5</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>CSF</td>
<td>0.3</td>
<td>0.3</td>
<td>0.5</td>
<td>0.3</td>
</tr>
<tr>
<td>EMT and Metastasis</td>
<td>SERPINE1</td>
<td>15.8*</td>
<td>15.3*</td>
<td>222.0*</td>
<td>17.6*</td>
</tr>
<tr>
<td></td>
<td>MMP2</td>
<td>3.8</td>
<td>2.9</td>
<td>2193.0*</td>
<td>2.6</td>
</tr>
<tr>
<td></td>
<td>IL-6</td>
<td>2.2</td>
<td>2.2</td>
<td>14.0*</td>
<td>9.0*</td>
</tr>
<tr>
<td></td>
<td>TWIST1</td>
<td>5.6</td>
<td>4.2</td>
<td>19.6*</td>
<td>2.3</td>
</tr>
<tr>
<td></td>
<td>PTGS2</td>
<td>0.0</td>
<td>0.1</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>SNAI2</td>
<td>0.3</td>
<td>0.6</td>
<td>0.4</td>
<td>0.3</td>
</tr>
</tbody>
</table>
Figure 8. Leptin inhibition in obASCs reduces tumor volume. MCF7 cells were prepared alone or co-injected with ctrl-shRNA obASCs or lep-shRNA obASCs (1:1 ratio) into the mammary fat pad of SCID/beige mice (n=5 mice/group). (A) Tumors volume was assessed every 3-4 days. (B) Representative tumors from each mouse (n=5/group). Scale bar represents 1cm. (C) Tumor weight on day 36. (D) Representative images of H&E, GFP staining, and dsRed staining of tumor sections visualized at 10x and 40x (inset) magnification. Scale bar represents 50µm. Bar ± SEM. *, P < 0.001 relative to MCF7 xenografts; Φ, P < 0.001 between MCF7 plus ctrl-shRNA obASC xenografts and MCF7 plus lep-shRNA obASC xenografts.
4.4.6 **Leptin inhibition reduces obASCs-induced metastasis of BCCs**

Since *obASCs* enhance the expression of EMT and metastatic genes *in vitro*, the role *obASCs* play in EMT and metastasis of BCCs was assessed *in vivo*. Immunocompromised mice were implanted with MCF7 cells alone, MCF7 cells mixed with *lnASCs*, or with *obASCs* for 36 days. Metastatic lesions in the lung and liver were visualized by H&E staining and quantified. Mice implanted with MCF7 cells alone demonstrated no metastatic lesions, whereas mice injected with MCF7 cells mixed with *lnASCs* (0.8 ± 0.07%) and mice injected with MCF7 cells mixed with *obASCs* (2.0 ± 0.09%) had significantly greater number of metastatic lesions (*P* < 0.05; Figure 10A-B; Figure 11). These results indicate that while both *lnASCs* and *obASCs* enhanced metastasis of MCF7 cells, *obASCs* more efficiently induced the metastasis to the lung and liver, compared to *lnASCs*.

The effects of inhibiting *obASC*-derived leptin on EMT and metastasis was investigated *in vivo*. SCID/beige mice were implanted with MCF7 cells alone, MCF7 cells mixed with control shRNA *obASCs*, or MCF7 cells mixed with leptin shRNA *obASCs* for 36 days. Metastasis to the lung and liver was visualized by H&E staining and quantified. Mice injected with MCF7 cells mixed with control shRNA *obASCs* (2.0 ± 0.20%) had significantly more metastasis compared to mice injected with MCF7 cells mixed with leptin shRNA *obASCs* (0.5 ± 0.04%)(*P* < 0.05; Figure 10C-D; Figure 11). These results suggest that *obASCs*-derived leptin drives metastasis of MCF7 cells in the lung and liver.
4.5 Discussion

The incidence of obesity has been steadily increasing over the past few decades. Obese and overweight individuals now account for more than two-thirds of the adult population in the United States [294]. While there are many co-morbidities associated with obesity, a clear epidemiological association between obesity and the prevalence of numerous cancers has been established; one being breast cancer [147]. While the relative risk amongst studies varies from 1.5 to 2.5, there is consensus that there is an increased relative risk of breast cancer development in women with a BMI greater than 30 [295-297].

Recent studies investigating the role of adipose tissue, in particular the excessive accumulation of adipose tissue in obesity, on breast cancer implicate ASCs as a significant contributory factor to disease development [298]. ASCs are stromal/stem progenitor cells of mesenchymal origin and have been shown to travel through the blood to distant tumor sites where they differentiate into vascular pericytes or secrete growth factors that support the tumor microenvironment [299, 300]. Additional studies suggest that ASCs originating from remote fat depots have the potential to traffic to the tumor and promote tumor progression through the secretion of proteases and pro-angiogenic factors [202, 263]. In the present study, the impact of obASCs on several BCC lines was investigated. The data presented here indicates that obASCs enhance the proliferation of ER$^+$ BCCs and the preferential impact of obASC on ER$^+$ BCC lines suggest that ER$^+$ BCCs are able to respond to factors produced by the obASCs, which are not produced by
In ASCs. Furthermore, the data suggests that TNBC may not express receptors or that their signaling pathways do not respond to the factors produced by obASCs. Previously, ASCs have been shown to be a source of the leptin in the adipose tissue, and this leptin produced by the ASCs has been shown to stimulate BCC proliferation [291]. Leptin, a growth factor produced within adipose tissue, primarily functions to maintain energy balance. It also plays an important role in cell growth and differentiation under normal physiological conditions [301]. Previous studies have also indicated that a leptin-leptin receptor signaling axis may cross talk with the ER and enhance tumorigenesis and metastasis [302-305]. These findings are consistent with our current report of obASCs and their preferential role in increasing ER⁺ BCCs but not ER⁻ BCCs.

Obesity has been shown to result in hyperleptinemia [306]. Hyperleptinemia leads to an increase in breast cancer cell proliferation, migration, and invasion, which gives rise to more aggressive and metastatic tumor cells [187, 268, 307-313]. Several laboratories have confirmed that exogenously delivered leptin increases BCC proliferation at different concentrations (100-1600 ng/ml) [268, 307-313]. Previously, studies have shown that the mechanism by which leptin promotes the survival of cancer cells is through the activation of multiple signaling pathways, such as those involving mitogen-activated protein kinase (MAPK), Janus kinase 2-signal transducer and activator of transcription 3 (JAK2-STAT3) and phosphatidylinositol 3-kinase-protein kinase B (PI3K-AKT) [268, 308, 314-316]. However, additional studies are necessary to determine the sources of leptin in the adipose tissue and whether leptin secreted by the obASCs utilizes similar signal transduction pathways to promote the survival of cancer cells.
Table 4. mRNA expression of xenografts formed with MCF7 cells and leptin knockdown ASCs. Data is shown as fold change relative to respective breast cancer cell line without previous co-culture with ASCs. *, P < 0.05; #, P < 0.05; ¥, P < 0.05

<table>
<thead>
<tr>
<th>Function</th>
<th>Gene Name</th>
<th>ctrl-shRNA obASCs</th>
<th>lep-shRNA obASCs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Xenografts of MCF7 cells co-injected with</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>CDKN2A 1.7</td>
<td>1.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GSTP1 1.2</td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SFRP1 0.8</td>
<td>1.3</td>
</tr>
<tr>
<td>Cell Cycle and Apoptosis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>PLAU 2.0</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>THBS1 3.0</td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CSF 10.4 ¥</td>
<td>3.4 #</td>
</tr>
<tr>
<td>Angiogenesis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>SERPINE1 5.6 ¥</td>
<td>2.8 #</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MMP2 2.8 ¥</td>
<td>1.4 #</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IL-6 3.3</td>
<td>3.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TWIST1 0.3</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PTGS2 4.5</td>
<td>3.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SNAI2 13.8 #</td>
<td>9.0 *</td>
</tr>
<tr>
<td>EMT and Metastasis</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 9. *ob*ASC-derived leptin induces SERPINE1 and MMP2 expression in MCF7 xenografts. MCF7 cells were prepared alone or co-injected with ctrl-shRNA *ob*ASCs or lep-shRNA *ob*ASCs into the mammary fat pad of SCID/beige mice. After 36 days, tumors were harvested for analysis. (A) Total cellular RNA was isolated from tumors and analyzed by qRT-PCR. (B) Western blot analysis of tumor lysate. A total of 20 µg of protein was separated by SDS-PAGE under reducing conditions, blotted, and probed with indicated antibodies. Blots were stripped and probed with actin antibody for normalization. (C) Densitometry analysis of SERPINE1 and MMP2 bands, normalized to actin. (D) Representative images of SERPINE1 staining and MMP-2 staining of tumor sections visualized at 10x and 40x (inset) magnification. Scale bar represents 50µm. Bar ± SD. *, *P* < 0.05, **, *P* < 0.01, ***, *P* < 0.0001 relative to MCF7 xenografts; ***, *P* < 0.05, ***, *P* < 0.01, ***, *P* < 0.0001 between MCF7 plus ctrl-shRNA *ob*ASC xenografts and MCF7 plus lep-shRNA *ob*ASC xenografts.
Figure 10. Leptin is essential for the obASC-driven metastasis MCF7 cells in vivo. (A) MCF7 cells were prepared alone or co-injected with lnASCs or obASCs (1:1 ratio) or (C) co-injected with ctrl-shRNA obASCs or lep-shRNA obASCs into the mammary fat pad of SCID/beige mice (n=5 mice/group). Primary tumors were allowed expand for 36 days, and the lung and liver were harvested for histological analysis of metastasis. Metastasis index was determined by the percentage of the liver and lung occupied by metastatic lesions. (B, D) Representative images of liver and lung sections. Liver sections were visualized at 40x and lung sections were visualized at 10x and 40x (inset). Scale bars represent 50µm. Bar ± SD. ***, P < 0.0001 relative to MCF7 xenografts; ###, P < 0.0001 between MCF7 plus lnASC xenografts and MCF7 plus obASC xenografts; ΦΦΦ, P < 0.0001 between MCF7 plus ctrl-shRNA obASC xenografts and MCF7 plus lep-shRNA obASC xenografts. Arrows highlight metastatic lesions within the liver or lung.
Figure 11. *obASCs enhance metastasis of BCCs.* (A) MCF7 cells were prepared alone or co-injected with *lnASCs* or *obASCs* (1:1 ratio) or (B) co-injected with ctrl-shRNA *obASCs* or lep-shRNA *obASCs* into the mammary fat pad of SCID/beige mice (n=5 mice/group). After 36 days, tissues were harvested for histological analysis of metastasis. Representative histological images liver sections with and without highlighted metastatic cells are shown. Images were acquired at 2x magnification.
The mechanism by which obASC-derived leptin promotes alterations to the biology of BCCs was investigated by inhibiting leptin expression by stably transfecting lnASCs and obASCs with a leptin shRNA construct. The data suggests that obASC-derived leptin enhances several central processes such as proliferation and metastasis of cancer cells that ultimately enhance the aggressiveness of breast cancer cells. However, the precise mechanism by which obASC-derived leptin enhances proliferation remains to be determined. Our assessment of several key proliferative factors demonstrated increased expression of CDKN2A and SFRP1 in MCF7 and ZR75 cells following co-culture with obASCs. Inhibiting leptin expression negated the increased expression of CDKN2A and SFRP1 in MCF7 and ZR75 cells even after co-culture, suggesting that the expression of these two genes are leptin-mediated; however, these results did not translate into the T47D cells, which indicate obASC is not signaling through CDKN2A and SFRP1 to increase the proliferation of T47D cells. Therefore, additional analysis utilizing RNA-sequencing or other global approaches to assess gene expression may be warranted in order to assess the broader impact of obASCs and obASC-derived leptin on the proliferation of all ER+ BCCs.

With respect to metastasis, obASC-derived leptin enhances metastasis and appears to signal primarily through SERPINE1 and MMP-2. SERPINE1, a serine protease inhibitor, limits the activity of matrix metalloproteases in the extracellular matrix microenvironment [317]. Paradoxically, SERPINE1 is also involved in other molecular interactions, including binding to the extracellular matrix protein vitronectin and endocytosis receptors of the low-density lipoprotein receptor (LRP) family. Binding of SERPINE1 to vitronectin results in detachment of the tumor cell from the ECM,
leading to enhanced mobility of the cells [318]. Likewise, MMP-2 expression correlates with increased metastasis and poorer clinical prognosis [319, 320]. Furthermore, the cells expressing MMP-2 are also important and indicative of the aggressiveness of the breast cancer [321]. Elevated expression of MMP-2 in cancer cells was related to smaller tumors, while expression of MMP-2 by stromal cells was associated with increased aggressiveness [321]. The elevated expression of these two metastatic factors correlated with the increased incidence of metastatic lesions in the lung and liver of mice injected with a mixture of obASCs and cancer cells, compared to mice that received cancer cells alone or cancer cells mixed with lean ASCs. Furthermore, the expression of SERPINE1 and MMP2 was reduced in tumors formed with cancer cells and leptin shRNA obASCs, relative to tumors formed with cancer cells and control shRNA obASCs. These results suggest that leptin affects the overexpression of these key metastatic factors within the tumor.

In summary, these results indicate that obASCs not only contribute to the primary tumor growth, but these cells also enhance metastasis through a leptin-mediated pathway(s) involving SERPINE1 and MMP-2. The data suggest that the ASCs, which are in close proximity to the cancer cells within a tumor, are a novel avenue by which obesity influences the biology of cancer cells. Additional studies investigating the cells that contribute to the tumor microenvironment and their interaction with the cancer cells in obese patients are necessary to provide additional targets that will delineate the development and progress of breast cancer in these patients. With respect to leptin, the results of these studies would suggest that in obese individuals leptin is a primary molecule driving changes in the BCC that result in enhanced tumorigenicity and
metastasis. The results presented here suggest that the development of strategies to block
or inhibit leptin production or secretion by \textit{ob}ASCs in obese patients may reduce
proliferation of BCC and their metastasis to distant organs.
CHAPTER 5: DISCUSSION

5.1 Obesity alters ASC Biology

Obesity is characterized by chronic, low-grade inflammation, resulting in the secretion of pro-inflammatory cytokines and adipokines, such as IL-6, TNF-α, leptin, and PAI-1, within the adipose tissue microenvironment. These cytokines and adipokines activate downstream pathways, such as JAK2-STAT3, PI3K-Akt-GSK3, ERK1/2, and uPAR-uPA-PAI-1, which alters the stem cell niche within the adipose tissue [189-191]. Studies have demonstrated that obesity decreases the number of ASCs per gram of adipose tissue and that these ASCs have diminished potential to differentiate into adipogenic and osteogenic lineages [202-206]. These results confirm the potential for the obesity-driven inflammatory microenvironment to alter ASC biology.

Since ASCs are important components of the tumor microenvironment, understanding the obesity-associated alterations to ASC biology will aid in determining their role in increasing the incidence of breast cancer among obese women. Previous studies have shown that factors secreted by cancer cells promote the recruitment of ASCs through SDF-1-CXCR4 axis, whereby cancer cells secrete factors that activate ASCs to secret SDF-1, which then binds to CXCR4 on breast cancer cells and induces cellular proliferation (AKT, ERK1/2, JAK2-STAT3), migration (FAK) and metastasis (E-cadherin) [82-85]. Studies have also investigated the role of obesity on the trafficking of
ASCs towards cancer cells. Utilizing tumor-bearing mice, mice that were fed a high fat diet demonstrated greater number of circulating ASCs [202]. However, it is unclear from previous studies whether this increase is due to increased invasiveness of ASCs or whether it is caused by increased abundance of ASCs associated with obesity. The work conducted in Chapter 2 demonstrates the enhanced invasion of obese ASCs towards cancer stimuli. ASCs were isolated from obese and non-obese women and from abdominal and non-abdominal sources. It was shown that abdominally isolated obese ASCs were able to home towards breast cancer stimuli significantly more than non-abdominal ASCs or lean ASCs [263]. The increased invasive potential of these ASCs was attributed to increased MMP-15 and calpain-4 expression and decreased calpastatin expression in these cells. Knockdown experiments confirmed the role of these proteases and protease inhibitors in an in vitro Matrigel transwell invasion system as well as an in vivo CAM model. Previous studies have shown that hypoxia can induce MMP-15 expression, which enhances invasion of cells [230]. In obesity, the rapid growth of adipocytes results in a hypoxic environment; therefore, the hypoxia induced upregulation of MMP-15 could result in the increased invasiveness of ASCs [228, 229].

Both calpain-4 and calpastatin was implicated in the enhanced invasion of ASCs and is consistent with previously published work. Studies conducted in hepatocellular carcinoma demonstrated enhanced invasion of the cells in the presence of increased calpain-4 expression [240]. Studies utilizing fibroblasts demonstrated that calpain-4 knockdown cells displayed decreased cell migration, through decreased cleavage of FAK [241]. In contrast to calpain-4, calpastatin is a protease inhibitor, and its expression is inversely associated with invasion. Breast cancer samples with decreased calpastatin
expression has been shown to be involved in lymphovascular invasion [239]. Our analysis of primary breast cancers (Appendix A) demonstrated that triple negative breast cancers, which are more aggressive and invasive by nature, display lower levels of calpastatin compared to less malignant forms of breast cancer. The highest level of calpastatin expression was in normal adjacent breast cancer tissue, where increased calpastatin expression was observed mainly in the stroma. Analysis of breast cancer samples based on obesity status will help delineate whether increased MMP-15 and calpain-4 expression along with decreased calpastatin expression is specific to the breast cancers in obese women. Additional analysis of calpastatin expression in additional tumor types (Appendix B) suggest that calpastatin may play a significant role in more than breast cancers and that calpastatin may play a significant role in ovarian and colon cancers. These cancers have also been linked to obesity. Collectively, it seems likely that obesity increases not only the number of ASCs but also the invasiveness of ASCs, allowing these cells to circulate through the blood or locally to incorporate into the cancer microenvironment. Whether these proteases also play a role in other cancer systems will require additional studies.

Once ASCs traffic to the tumor microenvironment, the role that obesity plays on their involvement in the microenvironment was investigated. In the absence of obesity, studies have shown that ASCs and BMSC increase the proliferation of breast cancer cells [256, 257, 260, 261]. Muehleberg et al. demonstrated that ASCs increased the proliferation of a murine breast cancer cell line [260]. In obesity, the studies reported in Chapter 3 would suggest that ASCs acquire a more supportive phenotype that allows for the growth of breast cancer cells. ASCs isolated from the obese women (obese ASCs)
enhanced the proliferation of breast cancer cells, compared to ASCs isolated from lean women. Interestingly, this phenomenon was isolated to ER\(^+\) breast cancer cells, suggesting that ASCs might be activating an ER pathway. Furthermore, it was also shown that the role of obese ASCs on breast cancer cells was abolished in the absence of estrogens, suggesting that estrogens are necessary to activate obese ASCs. These obese ASCs were shown to express higher levels of leptin, and the expression of leptin in obese ASCs was further increased in the presence of estrogen, suggesting an estrogen-mediated leptin-response. The enhanced leptin in obese ASCs, in turn resulted in the increased expression of aromatase, which leads to the production of more estrogens and has the potential to activate ER\(^+\) cells [270, 271, 273]. Animal studies demonstrate that subcutaneous delivery of leptin receptor antagonist peptide slowed the growth of breast cancer tumors, suggesting the involvement of leptin in tumor growth [272]. In our study, the effects of obese ASCs was also shown to impact the expression of several key regulatory genes involved in cell cycle, apoptosis, angiogenesis, EMT, and metastasis genes [291]. These genes were either not altered or the effect was blunted when co-cultured with ASCs from lean individuals (lean ASCs). Taken together, the data described in Chapter 3 provides a mechanism by which obesity alters ASC biology and that effect on tumorigenesis of breast cancer cells.

To further isolate the source of leptin in the ASC-BCC interaction, leptin knockdown lean and obese ASCs were co-cultured with BCC cells. In Chapter 4, it was determined that leptin knockdown obese ASCs were unable to induce proliferation of ER\(^+\) BCCs (MCF7, ZR75, and T47D). However, naïve obese ASCs or obese ASCs transduced with negative control shRNAs were able to increase the proliferation of
BCCs. These results suggest that the source of leptin is as important as its effect on breast cancer cell proliferation. Further investigations into the mechanism by which leptin acts on the breast cancer cells led to the discovery that cell cycle regulator CDKN2A was increased significantly with the co-culture of obese ASCs, but the levels of expression returned to baseline after the co-culture with leptin knockdown obese ASCs. Furthermore, EMT and metastasis markers, PAI-1, IL-6, and MMP-2 were markedly increased following the co-culture with obese ASCs and the effects was blunted in leptin knockdown obese ASCs. The expressions of these molecular markers in breast cancer are associated with poorer prognosis due to increased invasiveness and metastasis of BCCs into distant organs [318-320, 322, 323]. In vitro invasion assays and in vivo models demonstrated that co-culturing obese ASCs with BCC cells resulted in the enhanced invasion and metastasis of BCC cells, yet the effects were eliminated with leptin knockout obese ASCs. Together, these results suggest that obesity-associated alterations in leptin expression alter BCC proliferation, invasion, and metastasis.

These studies provide a foundation for future studies to investigate ASC as the link between obesity and breast cancer. While it has been postulated that obesity affects breast cancer through inflammatory processes, the studies presented here support the finding that the pro-inflammatory adipokine leptin induces significant alterations on BCCs. In fact, the alterations are associated with invasion and metastasis, which could explain the larger tumor volume, increased incidence of metastasis, and increased reoccurrence rate among obese women. These factors all contribute to higher mortality rates in obese women compared to lean women.
5.2 Obese ASCs increase metastasis and reoccurrence of breast cancer

In order for metastasis to take place, breast cancer cells must undergo epithelial-to-mesenchymal transition (EMT), acquiring genetic mutations driven by increasing genomic instability and epigenetic changes. It is likely that the stroma is contributing to this transition, and obesity is enhancing the transformation towards a more aggressive phenotype through a ‘metastasis-permissive’ environment in the primary tumor niche. For instance, breast cancer has been shown to frequently express leptin receptors, and chronic inflammation associated with obesity may contribute to mammary tumor invasion through increased leptin production [187, 324]. The studies presented here would suggest that the obese ASCs provide the necessary leptin to bind to leptin receptor on breast cancer cells and activate downstream targets that enhance invasion and metastasis.

While mobilization of the cancer cells is necessary, it is equally important that the secondary site is suitable for implantation. Cancer cells trafficking through tissue and the circulation undergo many challenges, such as shear stress and immune detection [287]. These challenges reduces the number of surviving cancer cells outside of the primary tumor. As such, the metastasis site must provide a hospitable microenvironment for the trafficking cancer cell [325]. It is possible that obesity alters the stromal cells that make up the microenvironment of the secondary site, whereby enhancing the engraftment of cancer cells into distant organs. In this case, obesity would also indirectly increase metastasis.

Reoccurrence is also more common in obese women than in lean women. It is also possible that dormant cancer cells that lack the supportive environment due to the
removal of the primary tumor for surgery may be reactivated by obesity-associated cytokines and adipokines. It has previously been shown that chronic inflammation increases the reoccurrence of breast cancer [326]. Thus, obesity, which is characterized by chronic low-grade inflammation, could provide the essential growth factors to reactivate remaining dormant cells or micrometastasis, resulting in reoccurrence of the disease.

5.3 ASC as a therapeutic target for breast cancer

Metastatic cells acquire many genetic mutations prior to acquisitioning the ability to circulate to distant organs, and these mutations oftentimes results in a heterogeneous cancer population. Therefore, targeting metastatic cancerous cells have been challenging. Utilizing ASCs to target cancer cells may be a more plausible therapeutic target for breast cancer. As cancer cells require a supportive microenvironment to flourish, targeting the ASCs that make up this tumor microenvironment may limit the progression of the disease.

5.4 Implications on stem cell therapy

Lastly, while our analysis focused on the impact of obesity on ASCs to enhance breast cancer tumorigenesis, it is possible that the alterations associated with obesity on ASC function extends beyond enhancing tumorigenesis and metastasis. It is possible that obese ASCs may not be suitable candidates for cell-based therapies for immunosuppression or regeneration. Altered gene expression profiles of obese ASCs would suggest that these cells have different capacities to response to environmental
factors. Similar implications have been drawn between ASCs isolated from young and old subjects. ASCs isolated from old subjects have been shown to be less effective at treating autoimmune disease [327, 328]. Similarly, animal studies have shown that ASCs isolated from inflammatory disease models (e.g. EAE and diabetes) have less therapeutic efficacy compared to ASCs isolated from wild-type mice [329, 330]. Therefore, ASCs isolated from obese subjects may be less therapeutically suitable for the treatment of diseases than ASCs isolated from lean, healthy subjects.
To assess the role of CAST in primary breast cancer, a tissue array of 150 core biopsies of breast tissue and breast cancer, including normal adjacent breast tissue and invasive ductal carcinoma, was purchased from Biomax (BR1503b, Rockville, MD). Tissues were deparaffinized, rehydrated with Histochoice (Sigma) and a graded solution of ethanol solutions, quenched with 0.3% H$_2$O$_2$ (Sigma), rinsed with PBST, blocked with 1% BSA and stained with primary antibodies against CAST (Abcam) overnight at 4°C. Each tumor section was subsequently washed in PBST, incubated with appropriate HRP conjugated secondary antibody for 1 hour at room temperature, and washed with PBST. Slides were then incubated in 3,3'-Diaminobenzidine (DAB; Vector Laboratories), washed with PBST, counterstained with hematoxylin, and rinsed with deionized water. Slides were these dehydrated and sealed with Permount mounting medium (Sigma). Images were acquired on the ScanScope (Aperio; Vista, CA) and quantified with ImageScope software (Aperio) running the positive pixel count macro. (A) Representative images of tissue sections stained with H&E. Images are acquired at 10x magnification, and enlarged images were acquired at 40x magnification. (B) Representative images of tissue sections stained with CAST antibody. Images are acquired at 10x magnification, and enlarged images were acquired at 40x magnification. (C) Quantification of the stain was based on the percentage of CAST positive cells. While adjacent breast tissue displayed the most abundant CAST, particularly in the stroma, tumors defined as triple negative breast cancer (TNBC) displayed the least amount of CAST, and ER$^+$/PR$^+$ breast cancer fell in between the two extremes. These results suggest that low CAST expression in TNBC could be the cause of the enhanced tumorigenesis in this subset of cancers.
To investigate the expression of CAST in primary tumors, Oncomine analysis of The Cancer Genome Atlas (TCGA) database was used to compare normal and cancerous tissue. Data is presented relative to its respective normal controls. Decreased CAST expression, relative to non-cancerous tissue, was detected in several cancer types. These results suggest that decreased CAST may play a significant role in increased invasion of several cancers, and obesity may play a significant role in these cancers as well.
REFERENCES


144. Adult Obesity Facts, P.A. Division of Nutrition, and Obesity, National Center for Chronic Disease Prevention and Health Promotion, Editor. 2013, CDC. Centers for Disease Control and Prevention: Atlanta.


Amy Lin Strong was born on January 31, 1985 in Guangzhou China. She grew up in Lawrenceville, Georgia with her parents and brother. She attended Emory University from 2003 to 2007, and majored in Biology and Neuroscience and Behavioral Biology. While at Emory University, she participated in various activities that led her to pursue a career in medicine. After receiving her Bachelor of Science degree from Emory University in 2007, she attended Tufts University, where she completed her dual Master’s degree in Biomedical Sciences and Public Health. While completing the requirements for her Master’s degree in Biomedical Sciences, she participated in a research project that sparked her interest in science. During her time in Boston, she also met Michael, her future husband. In 2009, she and Michael entered the M.D. program at Tulane University and both participated in the Debakey Scholars program. The Debakey Scholars program allowed her to gain additional research experience under the supervision of Dr. Bruce Bunnell in the Center for Stem Cell Research and Regenerative Medicine. In 2011, she and Michael both entered into the joint M.D./Ph.D. Program at Tulane University and began her thesis work with Dr. Bruce Bunnell that same year. The following year, Amy married Michael. Amy plans to continue research in the biomedical field and hopes to practice translational medicine to utilize laboratory findings to advance patient care.