Investigating the Role of the PGF2 Alpha/Calcineurin-Signaling Pathway in the Regulation of Adipogenesis

Damodaran Annamalai
Loyola University Chicago, damodaran06@gmail.com

Recommended Citation
Annamalai, Damodaran, "Investigating the Role of the PGF2 Alpha/Calcineurin-Signaling Pathway in the Regulation of Adipogenesis" (2014). Dissertations. Paper 885.
http://ecommons.luc.edu/luc_diss/885

This Dissertation is brought to you for free and open access by the Theses and Dissertations at Loyola eCommons. It has been accepted for inclusion in Dissertations by an authorized administrator of Loyola eCommons. For more information, please contact ecommons@luc.edu.

This work is licensed under a Creative Commons Attribution-Noncommercial-No Derivative Works 3.0 License.
Copyright © 2014 Damodaran Annamalai
LOYOLA UNIVERSITY CHICAGO

INVESTIGATING THE ROLE OF

THE PGF2α/CALCINEURIN-SIGNALING PATHWAY

IN THE REGULATION OF ADIPOGENESIS

A DISSERTATION SUBMITTED TO

THE FACULTY OF THE GRADUATE SCHOOL

IN CANDIDANCY FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

PROGRAM IN MOLECULAR PHARMACOLOGY

AND THERAPEUTICS

BY

DAMODARAN ANNAMALAI

CHICAGO, ILLINOIS

MAY 2014
ACKNOWLEDGEMENTS

I am grateful to my mentor Dr. Neil Clipstone for his continuous support, encouragement and excellent mentorship throughout the course of my graduate education. I am thankful to him for accepting me into his lab, when I was in a difficult situation and helping me to complete PhD. He was always available not only to answer my scientific questions, but was also available to patiently listen and provide helpful suggestions for my personal issues as well. He always encouraged and supported my interest to combine my veterinary skills with research experience and to become a laboratory animal veterinarian. It is a great honor to work under his guidance and I look forward to his mentorship throughout my career.

I would like to thank my dissertation committee members, Dr. Tarun Patel, Dr. Mitchell F. Denning, Dr. Adriano Marchese and Dr. Basabi Rana for their time, support and directions during my committee meetings to shape this dissertation.

I thank Dawn Dike for her help and support during my rotation and during the initial period of my research work in Dr. Clipstone’s lab. I am thankful to Lorraine Grimsby, Leona Moore, Ellen Goodman, Donna Karl and Janet Flores for their help and taking care of administrative hurdles.

I am grateful for my friends Veluswamy & Priya, Velmurugan & Sathya, Ravi Sundaresan & Prabha, Saminathan & Vanji, Shankar & Suguna, Prassanna & Subha,
Bharath & Theja and Anand who made my life enjoyable during my stay in Chicago. My special thanks goes to Dr. Sethubathi who helped me in all the possible ways.

Finally, I want to thank my parents, Annamalai and Kuppmmal and my in-laws Jayaraman and Kalaiarasi for their love and support. I am grateful for my wife, Lavanya and my daughter, Govardhini for their support, affection and their patience to adjust with my student life.
To my beloved parents

Annamalai & Kuppammal
# TABLE OF CONTENTS

ACKNOWLEDGEMENTS iii

LIST OF TABLES x

LIST OF FIGURES xi

LIST OF ABBREVIATIONS xiv

ABSTRACT xix

CHAPTER I: INTRODUCTION 1

Epidemiology and sequela of obesity 1

Adipocytes and their physiological roles 2

Development of obesity and associated complications 3

Obesity, inflammation and impaired adipogenesis 4

Adipogenesis - An overview 6

Preadipocytes 6

Process of adipocyte differentiation 7

Initial growth arrest 8

Induction of adipocyte differentiation with hormonal stimuli 8

Mitotic clonal expansion 10

Terminal differentiation 12

Transcriptional regulation of adipogenesis 12

Early transcription factors - C/EBPβ and C/EBPδ 13

Early transcription factor - CREB 16

Proadipogenic transcription factor - C/EBPα 16

Proadipogenic transcription factor - PPARγ 18

Regulation of the PPARγ transcriptional activity 19

Role of PPARγ in adipogenesis 20

Extracellular factors regulating adipocyte differentiation 21

Prostaglandins 22

PGF2α - A potent inhibitor of adipogenesis 24

PGF2α synthesis, signaling and functions 24

Anti-adipogenic effects of PGF2α in cultured preadipocytes 25

Mechanisms of PGF2α-mediated inhibition of adipocyte differentiation 26
PGF2α inhibits adipogenesis through calcineurin-dependent signaling
Role of PGF2α in physiological inhibition of adipose tissue development in vivo

Summary

CHAPTER II: MATERIALS AND METHODS
Cell culture and adipocyte differentiation
Plasmid constructs
Dominant-negative gp130
EpoR/gp130-WT, 1F and 4F
STAT1, IL-11 and CRTC2 specific shRNAs
MSCV-DsRed2-PPARγ2
Nurr1-WT, Nurr1-AA and Nurr1-KLL
Dominant-negative Nurr1
ER-caCRTC2
YFP-CRTC2
K-CREB and CREB R314A
Retroviral generation and infection of 3T3-L1 cells
Immunoblot analysis
Quantitative real-time PCR (qRT-PCR) analyses
Enzyme-linked immunosorbent assay (ELISA)
Immunofluorescence
Luciferase assay
Statistical analysis

CHAPTER III: THE PGF2α/CALCINEURIN-SIGNALING PATHWAY
INHIBITS ADIPOGENESIS VIA AN AUTOCRINE/PARACRINE-
MEDIATED IL-11/GP130/STAT1-DEPENDENT SIGNALING CASCADE
Introduction
Interleukin-11 (IL-11) cytokine
IL-11 signaling
Role of IL-11 in inhibiting adipogenesis
STAT transcription factors
Expression of STATs during adipogenesis
Role STAT3 in adipogenesis
Role of STAT1 in adipogenesis
Role of ERK in adipogenesis
Results
Activation of the PGF2α/calcineurin-signaling pathway induces the expression of IL-11 in differentiating 3T3-L1 preadipocytes
IL-11 and gp130 cytokine co-receptor-dependent signaling play a role in mediating the inhibitory effects of PGF2α on adipocyte differentiation
gp130 cytokine co-receptor-dependent signaling is sufficient to inhibit adipocyte differentiation by blocking the expression of PPARγ
C-terminal tyrosine residues involved in the activation of STAT transcription factors play a critical role in mediating the inhibitory effects of gp130 signaling on adipocyte differentiation

A critical role for STAT1 in mediating the inhibitory effects of gp130, IL-11 and PGF2α on adipocyte differentiation

STAT1 depletion enhances the efficiency of adipocyte differentiation: Evidence for an intrinsic negative regulatory role

Discussion

CHAPTER IV: THE PGF2α/CALCINEURIN-SIGNALING PATHWAY UPREGULATES THE EXPRESSION OF THE ORPHAN NUCLEAR HORMONE RECEPTOR NURR1 THROUGH A CRTC/CREB-DEPENDENT MECHANISM: A POTENTIAL ROLE IN INHIBITING ADIPOCYTE DIFFERENTIATION

Introduction

Nurr1 and its family members (NR4A Family)

Expression and functions of NR4A family members

Role of Nurr1 and its family members in adipogenesis

Structure and regulation of Nurr1 activity

Regulation of gene expression by Nurr1 and its family members

Regulation of Nurr1 expression by the calcineurin phosphatase and the CRTC2/CREB transcriptional complex

CREB transcription factor

CRTC family of transcriptional co-activators

Results

The PGF2α/calcineurin-signaling pathway induces Nurr1 expression in differentiating 3T3-L1 preadipocytes

Nurr1 plays a potential role in mediating the inhibitory effects of PGF2α on adipocyte differentiation

Nurr1-mediated inhibition of adipogenesis requires its direct DNA binding

Nurr1-mediated inhibition of adipogenesis does not require its heterodimerization with RXR

CREB and its association with CRTC2 are required for PGF2α-mediated Nurr1 expression during adipocyte differentiation

The PGF2α/calcineurin-signaling pathway induces the activation and nuclear translocation of CRTC2 co-activator during adipogenesis

PGF2α requires the CRTC proteins to induce Nurr1 expression during adipogenesis

CRTC2 activity is sufficient to induce Nurr1 expression and to inhibit adipocyte differentiation

CRTC co-activator proteins play a critical role in the regulation of normal adipocyte differentiation

Discussion
LIST OF TABLES

Table | Page
-----|-----
1. Nucleotide sequence of primers used for qRT-PCR | 43
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Schematic model showing the adipocyte differentiation and the sequential expression of adipogenic transcription factors</td>
<td>9</td>
</tr>
<tr>
<td>2.</td>
<td>Schematic model showing the PGF2α-mediated inhibition of adipocyte differentiation via activation of the calcineurin phosphatase</td>
<td>27</td>
</tr>
<tr>
<td>3.</td>
<td>Schematic model showing the IL-11/gp130-dependent signaling pathways</td>
<td>47</td>
</tr>
<tr>
<td>4.</td>
<td>PGF2α treatment of differentiating 3T3-L1 preadipocytes induces the calcineurin-dependent expression and secretion of IL-11.</td>
<td>53</td>
</tr>
<tr>
<td>5.</td>
<td>gp130-dependent signaling play a role in mediating the inhibitory effects of PGF2α on adipocyte differentiation.</td>
<td>57</td>
</tr>
<tr>
<td>6.</td>
<td>IL-11 play a role in mediating the inhibitory effects of PGF2α on adipocyte differentiation.</td>
<td>60</td>
</tr>
<tr>
<td>7.</td>
<td>Schematic model illustrating the chimeric EpoR/gp30 receptors used in this study.</td>
<td>63</td>
</tr>
<tr>
<td>8.</td>
<td>gp130-dependent signaling is sufficient to inhibit adipocyte differentiation.</td>
<td>64</td>
</tr>
<tr>
<td>9.</td>
<td>Tyrosine residues involved in STAT transcription factor activation play a role in mediating the inhibitory effects of gp130-dependent signaling on adipocyte differentiation.</td>
<td>66</td>
</tr>
<tr>
<td>10.</td>
<td>gp130-dependent selective activation of STAT1, but not STAT3 inhibits adipocyte differentiation.</td>
<td>70</td>
</tr>
</tbody>
</table>
11. STAT1-depletion rescues adipocyte differentiation from the EpoR/gp130-chimeric receptor signaling. 73
12. STAT1-depletion rescues adipocyte differentiation from the IL-11 cytokine 75
13. STAT1-depletion rescues adipocyte differentiation from PGF2α. 77
14. STAT1-depletion enhances the efficiency of adipocyte differentiation in response to suboptimal adipogenic stimulation. 80
15. Schematic model illustrating the proposed mechanism by which PGF2α inhibits adipogenesis. 89
16. Schematic model showing the structure of Nurr1 and the modes of regulation of gene expression by Nurr1 94
17. Schematic model showing the activation of the CRTC2 co-activator 99
18. The PGF2α/calcineurin-signaling pathway induces Nurr1 expression, but not other family members during adipocyte differentiation 102
19. Evidence that Nurr1 potentially plays a role in mediating the inhibitory effects of PGF2α on adipogenesis 106
20. Ectopic expression of Nurr1 inhibits adipogenesis via a DNA binding and RxR-independent mechanism 109
21. PGF2α induces Nurr1 expression through CREB-dependent mechanism during adipogenesis 112
22. The PGF2α/calcineurin-signaling pathway induces sustained activation of CRTC2 115
23. CRTC co-activators are required for the PGF2α to induce Nurr1 expression during adipocyte differentiation 118
24. Conditional and selective activation of the constitutively active CRTC2 is sufficient to induce Nurr1 expression and to inhibit adipogenesis 121
25. CRTC is required for normal adipocyte differentiation 125
26. Schematic model showing that the PGF2α/calcineurin-signaling pathway induces Nurr1 expression via CRTC2/CREB transcriptional xii
complex to inhibit adipogenesis

27. Schematic model showing the signaling pathways involved in mediating the inhibitory effects of PGF2α on adipogenesis
## LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGIF</td>
<td>Adipogenesis inhibitory factor</td>
</tr>
<tr>
<td>Akr</td>
<td>Aldo-keto reductase</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>aP2</td>
<td>Adipocyte protein 2</td>
</tr>
<tr>
<td>BrdU</td>
<td>Bromodeoxyuridine</td>
</tr>
<tr>
<td>bZIP</td>
<td>Basic Leucine Zipper Domain</td>
</tr>
<tr>
<td>C/EBP</td>
<td>Ccaat-enhance-binding protein</td>
</tr>
<tr>
<td>CaMK</td>
<td>Calmodulin kinase</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CBP</td>
<td>CREB binding protein</td>
</tr>
<tr>
<td>CDC</td>
<td>Center for Disease Control and Prevention</td>
</tr>
<tr>
<td>Cdc25c</td>
<td>Cell division cycle 25 homolog c</td>
</tr>
<tr>
<td>Cdc45I</td>
<td>Cell division cycle 45 homolog</td>
</tr>
<tr>
<td>CDK2</td>
<td>Cyclin-dependent kinase 2</td>
</tr>
<tr>
<td>ChIP</td>
<td>Chromatin immunoprecipitation</td>
</tr>
<tr>
<td>CLC</td>
<td>Cardiotrophin-like cytokine</td>
</tr>
<tr>
<td>CNTF</td>
<td>Ciliary neurotrophic factor</td>
</tr>
<tr>
<td>CoREAST</td>
<td>Co-repressor for element-1-silencing transcription factor</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
</tr>
<tr>
<td>---------</td>
<td>-----------</td>
</tr>
<tr>
<td>CRE</td>
<td>cAMP response element</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP response element-binding protein</td>
</tr>
<tr>
<td>CRTC</td>
<td>CREB-regulated transcription co-activator</td>
</tr>
<tr>
<td>CsA</td>
<td>Cyclosporin A</td>
</tr>
<tr>
<td>CsA</td>
<td>Cyclosporine</td>
</tr>
<tr>
<td>CT-1</td>
<td>Cardiotrophin-1</td>
</tr>
<tr>
<td>DEC1</td>
<td>Deleted in esophageal cancer 1</td>
</tr>
<tr>
<td>DN</td>
<td>Dominant-negative</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>Epo</td>
<td>Erythropoietin</td>
</tr>
<tr>
<td>EpoR</td>
<td>Erythropoietin receptor</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen receptor</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>GAS</td>
<td>Gamma activated sequence</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescence protein</td>
</tr>
<tr>
<td>Gins1</td>
<td>GINS complex subunit 1</td>
</tr>
<tr>
<td>GLUT4</td>
<td>Glucose transporter type 4</td>
</tr>
<tr>
<td>gp130</td>
<td>Glycoprotein 130</td>
</tr>
<tr>
<td>GPCR</td>
<td>G protein-coupled receptor</td>
</tr>
<tr>
<td>GSK-3β</td>
<td>Glycogen synthase kinase-3 beta</td>
</tr>
<tr>
<td>HDAC</td>
<td>Histone deacetylase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>HIF-1</td>
<td>Hypoxia-inducible factor-1</td>
</tr>
<tr>
<td>HPRT</td>
<td>Hypoxanthine phosphoribosyltransferase</td>
</tr>
<tr>
<td>IGF-1</td>
<td>Insulin-like growth factor 1</td>
</tr>
<tr>
<td>IL-11</td>
<td>Interleukin 11</td>
</tr>
<tr>
<td>IL-11Rα</td>
<td>IL-11 receptor alpha</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin 6</td>
</tr>
<tr>
<td>INFγ</td>
<td>Interferon gamma</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus kinase</td>
</tr>
<tr>
<td>kdm4b</td>
<td>Lysine (K)-specific demethylase 4b</td>
</tr>
<tr>
<td>KRAB</td>
<td>Kruppel associated box</td>
</tr>
<tr>
<td>LIF</td>
<td>Leukemia inhibitory factor</td>
</tr>
<tr>
<td>LPL</td>
<td>Lipoprotein lipase</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
</tr>
<tr>
<td>MCE</td>
<td>Mitotic clonal expansion</td>
</tr>
<tr>
<td>Mcm3</td>
<td>Mini-chromosome maintenance complex component 3</td>
</tr>
<tr>
<td>MDI</td>
<td>Methyl isobutyl xanthine, Dexamethasone, Insulin</td>
</tr>
<tr>
<td>MEF</td>
<td>Mouse embryonic fibroblast</td>
</tr>
<tr>
<td>NBRE</td>
<td>Nerve growth factor I-B response element</td>
</tr>
<tr>
<td>NCoR</td>
<td>Nuclear receptor co-repressor</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor κB</td>
</tr>
<tr>
<td>NFAT</td>
<td>Nuclear factor of activated T cells</td>
</tr>
<tr>
<td>Nor1</td>
<td>Neuron-derived orphan receptor 1</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>Nurr1</td>
<td>Nuclear receptor related 1</td>
</tr>
<tr>
<td>ORO</td>
<td>Oil Red O</td>
</tr>
<tr>
<td>OSM</td>
<td>Oncostatin M</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PG</td>
<td>Prostaglandin</td>
</tr>
<tr>
<td>PGF2α</td>
<td>Prostaglandin F2 alpha</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PPARγ</td>
<td>Peroxisome proliferator-activated receptor gamma</td>
</tr>
<tr>
<td>PREF-1</td>
<td>Preadipocyte factor-1</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative real-time PCR</td>
</tr>
<tr>
<td>Rb</td>
<td>Retinoblastoma</td>
</tr>
<tr>
<td>RIP 140</td>
<td>Receptor interacting protein 140</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RXR</td>
<td>Retinoid X receptor</td>
</tr>
<tr>
<td>SAMP6</td>
<td>P6 strain of senescence-accelerated mice</td>
</tr>
<tr>
<td>SCD1</td>
<td>Stearoyl-CoA desaturase 1</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SIK</td>
<td>Salt inducible kinase</td>
</tr>
<tr>
<td>siRNA</td>
<td>small interfering RNA</td>
</tr>
<tr>
<td>SMRT</td>
<td>Silencing mediator for retinoid and thyroid receptor</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------------------------</td>
</tr>
<tr>
<td>TCF 4</td>
<td>Transcription factor 4</td>
</tr>
<tr>
<td>TFIID</td>
<td>Transcription factor II D</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumor necrosis factor alpha</td>
</tr>
<tr>
<td>WAT</td>
<td>White adipose tissue</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-type</td>
</tr>
<tr>
<td>YFP</td>
<td>Yellow fluorescence protein</td>
</tr>
</tbody>
</table>
ABSTRACT

Prostaglandin F2α (PGF2α) is a potent physiological inhibitor of adipocyte differentiation, that has recently been shown to play an important role in the regulation of adipose tissue development in vivo, where it acts to oppose the development of obesity and its associated co-morbidities. Hence, it is of considerable interest to understand the underlying molecular mechanisms by which PGF2α inhibits adipogenesis. In this respect, previous studies from our laboratory have demonstrated that PGF2α inhibits adipogenesis via activation of the calcium/calmodulin-regulated, serine/threonine phosphatase, calcineurin. However, the precise molecular mechanism by which the PGF2α/calcineurin-signaling pathway inhibits adipogenesis is not known. Accordingly, the goal of this dissertation project was to identify the underlying molecular mechanisms by which this pathway inhibits adipocyte differentiation. Since the best known function of calcineurin is the regulation of gene expression, our central hypothesis was that calcineurin activation induces the expression of a gene(s) directly responsible for inhibiting adipogenesis. To test this hypothesis, we initially performed a microarray analysis to identify candidate calcineurin-regulated genes likely to be responsible. As a result of this analysis, we identified two excellent candidate genes that were strongly induced by PGF2α in a calcineurin-dependent fashion: the IL-11 cytokine and the Nurr1 orphan nuclear hormone receptor. This dissertation describes our investigations into the
potential roles of IL-11 and Nurr1 in mediating the inhibitory effects of the PGF2α/calcineurin-signaling pathway on adipogenesis.

Initially, we demonstrate that activation of the PGF2α/calcineurin signaling pathway in differentiating 3T3-L1 preadipocytes induces the expression and secretion of IL-11. First, using a combined shRNA and dominant-negative mutant approach, we provide evidence that IL-11 acts in an autocrine fashion via the gp130 cytokine co-receptor signaling subunit to inhibit adipogenesis. Second, by taking advantage of a well-characterized panel of chimeric gp130 receptor mutants, we demonstrate that the activation of gp130-dependent signaling is sufficient to inhibit adipogenesis, and more specifically, that the gp130-mediated activation of the STAT1 transcription factor is necessary for this effect. Conversely, we find that the depletion of endogenous STAT1 rescues adipogenesis from the anti-adipogenic effects of both IL-11 and PGF2α. Collectively, our findings support a model in which PGF2α inhibits adipocyte differentiation by establishing a calcineurin-dependent, IL-11-mediated autocrine negative feedback loop, that acts via gp130 to block adipogenesis through the essential actions of the STAT1 transcription factor.

In addition to IL-11, we find that Nurr1, an orphan nuclear hormone receptor, is also highly upregulated by the PGF2α/calcineurin-signaling pathway during the early stages of adipogenesis. Using a panel of CREB transcription factor mutants and a shRNA knockdown approach, we show that the CREB transcription factor and more specifically, its association with members of the CREB-regulated transcriptional co-activator (CRTC) family of proteins is required for PGF2α to induce Nurr1 expression.
Furthermore, using a conditionally active CRTC2 mutant, we demonstrate that activation of CRTC proteins is sufficient to both induce Nurr1 expression and inhibit adipocyte differentiation. Finally, using a dominant-negative approach, we provide evidence that Nurr1 is involved in mediating the inhibitory effects of PGF2α on adipogenesis. Further, using Nurr1 mutants that are either deficient in their ability to bind DNA or heterodimerize with RxR, we demonstrate that Nurr1 requires its direct DNA binding activity, but not its interaction with RxR to inhibit adipogenesis. Taken together, these results demonstrate that, in addition to the IL-11 cytokine, the PGF2α/calcineurin-signaling pathway also activates a CRTC/CREB/Nurr1-dependent signaling cascade to inhibit adipocyte differentiation.

In summary, my studies have helped to identify and delineate two independent signaling pathways that function downstream of calcineurin to mediate the inhibitory effects of PGF2α on adipogenesis: the IL-11/gp130/STAT1- and the CRTC/CREB/Nurr1-signaling pathways. Thus, the current findings of this dissertation project provide significant new insights into the molecular mechanisms by which the PGF2α/calcineurin-signaling pathway acts to inhibit adipocyte differentiation.
CHAPTER I

INTRODUCTION

1. Epidemiology and sequela of obesity: Obesity, an excessive accumulation of adipose tissue, is a major public health problem and is emerging as a worldwide epidemic in both developed and developing countries. The Centers for Disease Control and Prevention (CDC) report that about one-third of USA adults (34.9%) are already obese in 2012, while childhood obesity is increasing at an alarming rate with 18% children in USA aged between 6 and 11 years being classified as obese (Ogden, 2013). Further, the World Health Organization (WHO) reports that obesity has doubled worldwide since 1980 and about 500 million adults worldwide are currently obese (WHO, 2013). Obesity is a major risk factor for the development of several co-morbidities such as insulin resistance, type 2 diabetes, coronary artery disease, hypertension, hyperlipidemia and several cancers (De Pergola and Silvestris, 2013; Kahn and Flier, 2000; Kahn et al., 2006; Nakamura et al., 2013; Van Gaal et al., 2006). These obesity-associated diseases are linked to reduced life expectancy and premature death (Olshansky et al., 2005). Hence, it is important to understand the development of adipose tissue and obesity at the molecular level in order to identify better therapeutic targets to treat obesity and its associated complications.
2. Adipocytes and their physiological roles: Adipose tissue is primarily comprised of specialized, lipid-storing cells called adipocytes. These specialized cells are evolutionarily developed to store excess energy in the form of triglycerides during abundant availability of food and to release energy in response to starvation and flight or fight (Rosen and Spiegelman, 2006; Spiegelman and Flier, 2001). In addition to this important role in energy homeostasis, adipocytes also perform an important endocrine function and secrete a variety of biologically active molecules such as leptin, adiponectin, resistin, TNFα, IL-6, angiotensinogen, fatty acids and others (Fruhbeck et al., 2001; Kershaw and Flier, 2004; Vazquez-Vela et al., 2008; Waki and Tontonoz, 2007). Collectively, these molecules are called adipokines and are known to regulate a variety of functions including food intake, insulin responsiveness, cardiovascular function, vascular remodeling and the immune response (Trujillo and Scherer, 2006; Vazquez-Vela et al., 2008; Waki and Tontonoz, 2007). Importantly, leptin secreted predominantly by adipocytes acts on the feeding center in the hypothalamus and inhibits appetite and food intake (Friedman and Halaas, 1998). In fact, the ob/ob mouse that is deficient in leptin and the db/db mouse that has a defective leptin receptor develop obesity at a very early age and are used as models to study obesity and type 2 diabetes (Chen et al., 1996; Coleman, 1978; Drel et al., 2006). In addition, adiponectin secreted by adipocytes increase the insulin sensitivity, whereas resistin, IL-6 and TNFα are shown to induce insulin resistance (Trujillo and Scherer, 2006). Thus, adipokines regulate whole body metabolism by regulating food intake and insulin sensitivity. Collectively, adipocytes
regulate energy homeostasis through storage and release of excess energy, and whole body metabolism by secreting a variety of adipokines.

3. **Development of obesity and associated complications:** Adipocytes contribute to the development of adipose tissue mass by two mechanisms: 1) an increase in the size of adipocytes (hypertrophy) due to storage of energy as triglycerides, and 2) an increase in the number of adipocytes (hyperplasia) due to *de novo* generation of adipocytes known as adipogenesis (Hirsch and Batchelor, 1976; Spalding et al., 2008). Both adipocyte hyperplasia and hypertrophy play a crucial role in the development of obesity. Adipocyte hyperplasia plays a critical role in setting the number of adipocytes during early stages of childhood development and the number then stays constant into adulthood. This initial hyperplasia determines the degree of adipose tissue cellularity and subsequent propensity to develop obesity in adulthood (Brook et al., 1972; Hirsch and Batchelor, 1976; Knittle et al., 1979; Prins and O'Rahilly, 1997). In the adult, in the settings of positive energy balance, adipocyte hypertrophy through excessive accumulation of triglycerides contributes to the excessive accumulation of adipose tissue mass resulting in the formation of large, lipid-filled, dysfunctional, hypertrophic adipocytes (Hoffstedt et al., 2010; Krotkiewski et al., 1983; Weyer et al., 2000). These large dysfunctional hypertrophic adipocytes eventually become unable to store any additional energy. As long as new, small insulin-sensitive adipocytes are generated from the precursor cells through adipogenesis, the excess energy can be stored by these new cells (Gray and Vidal-Puig, 2007; Tan and Vidal-Puig, 2008; Virtue and Vidal-Puig, 2010). However,
this essential adipogenic process is inhibited in extreme obesity by inflammatory mediators as discussed later in this chapter. In the absence of new adipocytes, the excess energy can be “spilled-over” and deposited as fat in non-adipose tissues such as liver, skeletal muscle, heart muscles, pancreatic islets and the perivascular tissues. Since these tissues are not specialized to store lipids, this can result in lipotoxicity in these tissues leading to tissue dysfunction such as insulin resistance and inability to secrete insulin (Gray and Vidal-Puig, 2007; Tan and Vidal-Puig, 2008; Virtue and Vidal-Puig, 2010). In fact, transplantation of intact functional adipose tissue into both a lipodystrophy mouse model (Gavrilova et al., 2000; Moitra et al., 1998) and a leptin deficient obese mouse model with severe metabolic abnormalities (Sennello et al., 2006) resulted in improvement in overall metabolic profile and insulin sensitivity. This shows that the lack of functional adipose tissue capable of storing excess energy contributes to the development of metabolic abnormalities in obesity. In addition, the large dysfunctional adipocytes begin to secrete a more pathological array of adipokines such as resistin, TNFα and IL-6 that are known to induce insulin resistance (Qatanani and Lazar, 2007). Collectively, impaired adipogenesis leading to lipotoxicity to non-adipose tissues and pathological array of adipokines secreted by dysfunctional adipocytes contribute to the development of obesity associated complications such as insulin resistance and type 2 diabetes.

4. Obesity, inflammation and impaired adipogenesis: Though the total number of adipocytes remains constant in adulthood, about 10% of the adipocytes are renewed
annually throughout life (Spalding et al., 2008). The newly generated small insulin-sensitive adipocytes are essential as they store excess energy and prevent lipid spill-over to non-adipose tissues (Tan and Vidal-Puig, 2008; Virtue and Vidal-Puig, 2010). Thus, adipogenesis increases the overall lipid buffering capacity of adipose tissue and prevents lipotoxicity to non-adipose tissues. However, obesity is considered as an inflammatory condition and a variety of inflammatory cells such as macrophages, T lymphocytes, B lymphocytes, natural killer cells and neutrophils are recruited into adipose tissue (Cildir et al., 2013; Nishimura et al., 2009; Talukdar et al., 2012; Weisberg et al., 2003; Winer et al., 2011; Xu et al., 2003). These inflammatory cells produce a variety of inflammatory mediators such as IL-6 and TNFα, which create a low-grade chronic inflammatory condition in adipose tissue (Chawla et al., 2011; Gregor and Hotamisligil, 2011). In addition, the large dysfunctional adipocytes in obesity also secrete various inflammatory mediators (Qatanani and Lazar, 2007). These inflammatory mediators are known to potently inhibit the differentiation of preadipocytes into mature adipocytes (Gustafson and Smith, 2006; Xu et al., 1999). In fact, several line of evidence show that adipogenesis is impaired in obese patients. The number of preadipocytes that undergo differentiation is decreased in obese patients (Isakson et al., 2009) and these patients show a decreased number of committed preadipocytes (Tchoukalova et al., 2007). Further, the expression of genes involved in adipogenesis is decreased in type 2 diabetic patients (Dubois et al., 2006). Collectively, this evidence shows that preadipocytes from obese individuals have impaired potential to differentiate into adipocytes. In the absence of new insulin-sensitive adipocytes, the lipid buffering capacity of adipose tissue is lost, resulting in lipid spill
over, lipotoxicity and subsequent development of type 2 diabetes (Tan and Vidal-Puig, 2008; Virtue and Vidal-Puig, 2010). The direct link between obesity, inflammation and diabetes is evident from the finding that the obese mouse model that lacks the expression of TNFα shows enhanced insulin sensitivity and glucose homeostasis (Uysal et al., 1997). Given the potential role of the inflammatory mediators in inhibiting adipogenesis, it is important to understand the molecular mechanisms by which they inhibit adipogenesis to identify better therapeutic targets to block this inhibitory effect on adipogenesis and consequently, to treat the obesity-associated complications such as type 2 diabetes.

5. Adipogenesis - An overview: Adipogenesis is a highly complex, multistep process that involves the initial commitment of multipotent stem cells into the adipogenic lineage and subsequent terminal differentiation into mature adipocytes. Cell culture systems are widely used to understand the adipocyte differentiation and they faithfully recapitulate the \textit{in vivo} adipogenic process (Armani et al., 2010).

5.1 Preadipocytes: Much of our current understanding about the molecular mechanisms of adipocyte differentiation comes from studies using preadipocyte cell culture systems (Rosen and MacDougald, 2006). Preadipocytes are cells committed to the adipocyte lineage and have lost their potential to differentiate into other cell types. Both primary preadipocytes and established preadipocyte cell lines are used to study adipocyte differentiation (Green and Meuth, 1974; Gregoire et al., 1990; Nougues et al., 1988).
Primary preadipocytes are derived from the vascular stromal fraction of adipose tissue. These primary preadipocytes can be induced to differentiate into mature adipocytes with a combination of adipogenic factors such as dexamethasone, insulin and 3-isobutyl-1-methylxanthine (Armani et al., 2010). However, these primary cells cannot be cultured for prolonged periods and do not maintain the differentiation state for extended periods of time (Otto and Lane, 2005). In contrast, immortalized preadipocyte cell lines such as 3T3-L1 and 3T3-F442A were developed from non-clonal Swiss 3T3-L1 cells by Green and associates in the 1970s and can be induced to differentiate into mature adipocytes (Green and Meuth, 1974; Green and Kehinde, 1975; Green and Kehinde, 1976). These two cell lines recapitulate much of the key features of adipogenesis in vivo such as morphological changes, expression of lipogenic enzymes, extensive lipid accumulation and sensitivity to key hormones that have effects on this cell type. In fact, subcutaneous implantation of 3T3-F442A cells into mice can form functional adipose tissue (Green and Kehinde, 1979). Therefore, the 3T3-L1 and 3T3-F422A cell lines provide a perfect model to study adipocyte differentiation in vitro.

5.2 Process of adipocyte differentiation: The differentiation of preadipocytes into mature, lipid-filled adipocytes is a multistep process which includes initial growth arrest, mitotic clonal expansion and terminal differentiation into mature adipocytes (Gregoire et al., 1998). The differentiation process is regulated by a sequential expression of transcription factors including C/EBP family members and PPARγ. In this transcriptional cascade, C/EBPβ and C/EBPδ are expressed within few hours of induction of
adipogenesis, whereas C/EBPα and PPARγ are expressed later and then, they coordinately induce the expression of adipocyte specific genes (Figure 1) (Rosen and MacDougald, 2006).

5.3 Initial growth arrest: In the preadipocyte cell culture system, growth arrest is a prerequisite for the differentiation of preadipocytes into mature adipocytes. The initial growth arrest is achieved in cultured cell lines after contact inhibition and the cells arrest at the G₀/G₁ cell cycle boundary (Patel and Lane, 2000). However, absolute cell-cell contact is not required as confluent 3T3-F442A cells shifted to methylcellulose-stabilized suspension culture still differentiate into adipocytes (Pairault and Green, 1979). Hence, growth arrest, but not cell-cell contact, is required for preadipocytes to differentiate into adipocytes.

5.4 Induction of adipocyte differentiation with hormonal stimuli: Growth-arrested, post-confluent preadipocytes can be induced to terminally differentiate into mature, lipid-filled, morphologically distinct adipocytes with a hormonal cocktail that consists of 3-isobutyl-1-Methylxanthine, Dexamethasone and pharmacological concentrations of Insulin or insulin-like growth factor 1 (IGF-1) in the presence of fetal bovine serum (Rosen and Spiegelman, 2000; Schmidt et al., 1990). This hormonal cocktail is referred as MDI. Mechanistically, 3-isobutyl-1-methylxanthine inhibits cAMP phosphodiesterase and transiently increases intracellular cAMP concentration, which results in the activation of protein kinase A (PKA) and subsequent activation of the downstream signaling
FIGURE 1. Schematic model showing the adipocyte differentiation and the sequential expression of adipogenic transcription factors.

Upon stimulation with the adipogenic cocktail MDI, two-day post confluent 3T3-L1 preadipocytes differentiate into mature adipocytes in 6 to 8 days after stimulation. During the differentiation process, C/EBPβ and C/EBPδ are expressed within a few hours after the induction of adipogenesis. Once expressed, C/EBPβ and C/EBPδ bind to the C/EBPα and PPARγ promoters and induce their expression. Then, C/EBPα and PPARγ reinforce each other’s expression and induce adipocyte specific genes to confer the adipocyte phenotype.
pathways such as the cAMP response element-binding protein (CREB) transcription factor (Rosen and Spiegelman, 2000). CREB has been shown to regulate adipogenesis at multiple stages (Fox et al., 2008; Reusch et al., 2000; Zhang et al., 2004a) and is discussed later in this chapter. Dexamethasone is a glucocorticoid and is known to activate cytoplasmic, nuclear hormone, glucocorticoid receptors. It has been show to induce the expression of the early transcription factor, C/EBPδ (Cao et al., 1991). Insulin increases the percentage of cells that differentiate into mature adipocytes (Girard et al., 1994). Although preadipocytes express few insulin receptors (Reed and Lane, 1980), the pharmacological concentration of insulin has been shown to cross-activate IGF-1 receptor signaling during adipogeneic induction (Smith et al., 1988). The activation of the insulin and IGF-1 receptors results in the activation of multiple signaling pathways that regulate adipogenic process. Thus, the hormonal cocktail MDI along with fetal bovine serum potently induces the preadipocytes to differentiate into mature adipocytes.

5.5 Mitotic clonal expansion: When the post-confluent, growth-arrested preadipocytes in G0/G1 phase are stimulated with MDI, they synchronously re-enter the cell cycle and undergo two or three rounds of cell division, known as mitotic clonal expansion (MCE) (Cornelius et al., 1994; Tang et al., 2003b). The synchronous entry of growth-arrested preadipocytes into S phase is evidenced by changes in the cell cycle regulatory proteins such as expression and activation of cdk2-cyclin-E/A, down regulation of p27/kip1, hyperphosphorylation of Rb, translocation of cyclin D1 from the nuclei to cytoplasm and GSK-3β from cytoplasm to nuclei and incorporation of [³H] thymidine into DNA (Tang
et al., 2003b). The MCE is an important step for the differentiation of cultured preadipocytes into mature adipocytes. In fact, inhibiting cell cycle progression with rapamycin (Yeh et al., 1995a) or aphidicolin (Reichert and Eick, 1999) inhibits adipocyte differentiation. Collectively, these findings indicate that mitotic clonal expansion plays a critical role in adipocyte differentiation.

During the MCE process, DNA replication and epigenetic modifications in the chromatin structure at regulatory sequences increase the accessibility of critical cis-elements to transactivating factors, which activate or de-repress the expression of genes required for terminal differentiation into mature adipocytes (Musri et al., 2010). For example, initial studies showed that the SWI/SNF chromatin remodeling enzyme complex promotes transcription of PPARγ gene by C/EBPβ (Salma et al., 2004). In the case of the C/EBPα promoter, HDAC1 blocks its expression, but once PPARγ protein accumulates, it is able to target HDAC1 to proteasome degradation and allows activation of C/EBPα expression (Zuo et al., 2006). A recent study using DNase I Hypersensitive site analysis to identify open chromatin regions during adipogenesis showed that major chromatin remodeling events occur within the first 4 h of adipogenesis and one third of PPARγ target hotspots are primed for subsequent PPARγ binding by 4 h of adipogenic stimulation (Siersbaek et al., 2011). Further, the histone hyperacetylation associated with gene expression is selectively increased at the promoter regions of adipogenic genes, whereas the expression of several histone deacetylases and the deacetylase enzyme activity associated with gene repression are decreased during adipocyte differentiation (Yoo et al., 2006). Collectively, chromatin remodeling that occurs during MCE leads to
adipocyte specific gene transcription and subsequent terminal differentiation into mature adipocytes.

### 5.6 Terminal differentiation:

After 2 or 3 rounds of MCE, cells withdraw from the cell cycle, enter into permanent growth-arrest and terminally differentiate into mature adipocytes over 6 to 8 days after adipogenic stimulation. During this stage, the cells express adipocyte specific genes like aP2, GLUT4, leptin, adiponectin and others. Subsequently, the preadipocytes acquire biochemical and morphological characteristics of mature adipocytes through de novo lipogenesis and accumulation of fat droplets, thereby acquiring a spherical, lipid filled morphology. In addition, the differentiated cells also acquire insulin sensitivity and respond to hormones that are known to act on adipocytes in vivo (Gregoire et al., 1998).

### 6. Transcriptional regulation of adipogenesis:

Adipocyte differentiation is regulated by a sequential and orchestrated expression of transcription factors. Several transcription factors involved in this process have been identified. However, C/EBPs and PPARγ are considered as critical transcription factors that determine the terminal differentiation of adipocytes (Farmer, 2006). In this transcriptional cascade, C/EBPβ and C/EBPδ are expressed within a few hours of induction of adipogenesis (Cao et al., 1991; Yeh et al., 1995b). Once expressed, C/EBPβ and C/EBPδ coordinately bind to C/EBPα and PPARγ gene promoters and induce their expression. Once expressed, C/EBPα and PPARγ are able to bind to each other’s promoter and serve to reinforce each other’s expression via
positive feedback loop, thus ensuring their persistent expression in mature adipocytes (Rosen et al., 2002; Wu et al., 1996). More importantly, C/EBPα and PPARγ coordinately bind to the promoter of adipocyte specific genes and induce their expression to confer adipocyte phenotype (Tontonoz et al., 1994).

6.1 Early transcription factors - C/EBPβ and C/EBPδ: C/EBPβ and C/EBPδ are basic-leucine zipper family of transcription factors that are rapidly expressed within 4 h of induction of adipocyte differentiation and are referred to as the early transcription factors of adipogenesis (Cao et al., 1991; Yeh et al., 1995b). The role of C/EBPβ, but not C/EBPδ, in adipogenesis has been extensively studied.

Upon exposure to MDI, C/EBPβ expression is induced by methylisobutylxanthine which acts via CREB, whereas C/EBPδ expression is induced by dexamethasone (Cao et al., 1991; MacDougald et al., 1994; Zhang et al., 2004a). Further, CREB and its family members have been shown to bind to the proximal promoter of C/EBPβ and induce its expression within 4 h of adipogenic stimulation. Once expressed, C/EBPβ is phosphorylated by MAPK on Thr-188 between 2 to 4 h after induction and the phosphorylated C/EBPβ translocates into the nucleus (Tang et al., 2005), where this phosphorylation is sustained by cdk2 throughout the MCE phase (Li et al., 2007). Within the nucleus, C/EBPβ is further phosphorylated by GSK3β on Thr-179 or Ser-184 (Tang et al., 2005). These sequential phosphorylation events cause a conformational change that allows homodimerization and acquisition of DNA binding ability by C/EBPβ between 12
and 16 h after the induction of differentiation (Tang and Lane, 1999). In fact, C/EBPβ has been shown to bind to centromeric satellite DNA during this time period and the DNA binding activity coincides with the entry of cells into S phase of the cell cycle (Tang and Lane, 1999). After gaining DNA binding ability, C/EBPβ induces expression of cell cycle specific genes such as Cdc45I (cell division cycle 45 homolog), Mcm3 (minichromosome maintenance complex component 3), GIns1 (GINS complex subunit 1), Cdc25c (cell division cycle 25 homolog c) and Mcm3 histone demethylase during MCE (Guo et al., 2012). C/EBPβ also induces the expression of epigenetic modifiers kdm4b, a histone demethylase (Guo et al., 2012), and G9a, a histone dimethylase during MCE (Li et al., 2013). Once induced, kdm4b acts as a co-activator of C/EBPβ to induce the expression of cell cycle specific genes, whereas G9a dimethylates the proximal promoters of PPARγ and C/EBPα, and inhibits their expression until the cells exit the cell cycle (Guo et al., 2012; Li et al., 2013). These findings clearly demonstrate that C/EBPβ plays a crucial role in the regulation of MCE and progression of the adipogenic process.

The important role of C/EBPβ in adipocyte differentiation is evident from the finding that the mouse embryonic fibroblasts (MEFs) from C/EBPβ knockout mice fail to differentiate into adipocytes due to their inability to undergo MCE. In contrast, overexpression of C/EBPβ in these cells restores their capacity to differentiate into adipocytes (Tang et al., 2003a). The importance of C/EBPβ is further supported by the finding that a dominant-negative form of C/EBPβ, which prevents the nuclear translocation and DNA binding activity of endogenous C/EBPβ, inhibits differentiation
by blocking the MCE process (Zhang et al., 2004b). Interestingly, ectopic expression of C/EBPβ in the 3T3-L1 cells induces them to undergo adipogenesis without exogenous adipogenic stimuli (Yeh et al., 1995b), and overexpression of C/EBPβ induces the multipotent NIH-3T3 cell line to differentiate into adipocytes (Wu et al., 1995), suggesting that C/EBPβ is sufficient to induce adipogenesis in certain settings. In the in vivo setting, C/EBPβ knockout mice show a slight reduction in adipose tissue mass compare to their wild type counter parts, whereas C/EBPδ knockout mice develop with normal amounts of adipose tissue. Conversely, in C/EBPβ and C/EBPδ double knockout mice, 85% of the animals die on day 1 after birth and the remaining mice survive, but show a dramatic reduction in adipose tissue mass and the expression of adipocyte specific genes (Tanaka et al., 1997). This shows that C/EBPβ and C/EBPδ transcription factors complement each other and play a critical role in the development of adipose tissue.

In addition to the critical role of C/EBPβ and C/EBPδ proteins in the MCE, these proteins also regulate terminal differentiation by inducing C/EBPα and PPARγ expression. C/EBPα and PPARγ possess functional C/EBP binding sites in their promoters and are induced by C/EBP proteins (Christy et al., 1991; Clarke et al., 1997; Wu et al., 1996). Specifically, ectopic expression of C/EBPβ alone, or in combination with C/EBPδ in NIH 3T3-L1 cell line induces PPARγ expression and converts these cells into adipocytes (Wu et al., 1996).
6.2 Early transcription factor - cAMP responsive element binding protein (CREB): CREB is constitutively expressed in preadipocytes and is phosphorylated upon addition of adipogenic cocktail by signaling pathways initiated by methylisobutylxanthine and insulin through PKA and ERK signaling, respectively (Klemm et al., 1998; Martini et al., 2009; Reusch et al., 2000). The important role of CREB in adipogenesis is evident from the findings that the activation of a constitutively active CREB is sufficient to induce adipogenesis in the absence of adipogenic cocktail, whereas either dominant-negative CREB or siRNA-mediated knockdown of CREB inhibits adipogenesis (Fox et al., 2006; Reusch et al., 2000). Indeed, CREB binds to the C/EBPβ promoter along with its family members and induces its expression (Fox et al., 2006; Niehof et al., 1997; Zhang et al., 2004a). It also induces expression of cyclin D1 during the early stages of adipogenesis, which is required for the MCE process (Fox et al., 2008). Further, CREB directly binds to the PPARγ2 promoter and induces its expression (Fox et al., 2006). In addition, CREB also binds to the promoters of several other adipocyte-specific genes (Reusch et al., 2000). Collectively, CREB plays an important role in adipogenesis by regulating the expression of multiple adipogenic genes.

6.3 Proadipogenic transcription factor - C/EBPα: When preadipocytes are induced to differentiate with the adipogenic cocktail, C/EBPα is expressed after 2 days of induction (Cao et al., 1991; Yeh et al., 1995b). The early transcription factors C/EBPβ and C/EBPδ directly bind to the consensus binding sites in the proximal promoter of C/EBPα and induce its expression (Cao et al., 1991). Once expressed, C/EBPα binds to the promoter
of the adipocyte specific genes such as aP2, GLUT4 and SCD1, and transactivates their expression (Christy et al., 1989; Kaestner et al., 1990). Collectively, this results in terminal differentiation into mature adipocytes. In addition, C/EBPα is also an anti-mitotic factor and stimulates the cells to exit MCE by inducing the expression of cell cycle inhibitor, p21 and by increasing its stability (Harris et al., 2001; Timchenko et al., 1996). Further, C/EBPα inhibits cdk2 activity by directly interacting with p21 and cdk2, thereby preventing cell cycle progression (Harris et al., 2001). Thus, C/EBPα regulates adipogenesis by inhibiting MCE and inducing the expression of adipocyte specific genes.

The important role of C/EBPα in adipocyte differentiation is evident from the loss and gain of function experiments in preadipocyte cell lines. The expression of antisense C/EBPα RNA in 3T3-L1 cells inhibits adipogenesis and prevents the expression of several adipocyte specific genes (Lin and Lane, 1992), whereas expression of C/EBPα in preadipocytes and mouse fibroblasts induces these cells to differentiate into adipocytes without the addition of adipogenic stimuli (Freytag et al., 1994; Lin and Lane, 1994). These results suggest that C/EBPα is both necessary and sufficient to induce adipogenesis. The crucial in vivo role of C/EBPα in adipose tissue development is evident from the analysis of C/EBPα knockout mice, as these mice die within a few hours after birth due to impairment of hepatic gluconeogenesis (Wang et al., 1995). Interestingly, these mice survived with selective expression of C/EBPα in liver, but failed to develop white adipose tissue, except in the mammary fat pat (Linhart et al., 2001). Further, replacement of C/EBPα with C/EBPβ in a mouse model results in decreased
development of white adipose tissue compared to its wild type litter mates (Chen et al., 2000). Collectively, these findings show that C/EBPα plays a critical role in the adipocyte differentiation and the development of adipose tissue in vivo.

In addition to its role in adipocyte differentiation and the expression of adipocyte specific genes, C/EBPα plays a crucial role in the insulin sensitivity of adipocytes. In fact, fibroblasts from C/EBPα null mice express low levels of PPARγ and fail to differentiate into mature adipocytes. While these cells differentiate into adipocytes with ectopic expression of PPARγ, these adipocytes show a complete absence of insulin-stimulated glucose transport due to decreased expression and tyrosine phosphorylation of the insulin receptor and its downstream signaling protein, IRS-1 (Wu et al., 1999). This shows that C/EBPα specifically induces genes involved in the insulin sensitivity of mature adipocytes.

6.4 Proadipogenic transcription factor - PPARγ: PPARγ belongs to the nuclear-receptor superfamily of ligand-activated transcription factors (Feige et al., 2006) and is expressed as two isoforms, PPARγ1 and PPARγ2 from the same gene through alternative splicing and promoter usage (Zhu et al., 1995). Though PPARγ1 and PPARγ2 are expressed in adipocytes, PPARγ1 is expressed ubiquitously, whereas PPARγ2 expression is exclusively restricted in adipocytes (Vidal-Puig et al., 1996). PPARγ induces gene expression by binding to the promoter of its target genes at consensus nuclear hormone
binding sites as a heterodimer with its nuclear binding partner, retinoid X receptor (RXR) (Palmer et al., 1995).

### 6.4.1 Regulation of the PPAR\(\gamma\) transcriptional activity:

PPAR\(\gamma\) is a ligand-activated transcription factor and while several lipid metabolites including polyunsaturated fatty acids, prostanoids, prostaglandin 15 deoxy -\(\Delta 12, 14\) prostgandin J2 are considered as putative ligands for PPAR\(\gamma\), an endogenous ligand that potently activates PPAR\(\gamma\) has not yet been identified (Tontonoz and Spiegelman, 2008). Interestingly, PPAR\(\gamma\) is the target for antidiabetic thiazolidinedione drugs (Lehmann et al., 1995) and these drugs are shown to increase adipocyte differentiation in vitro (Kletzien et al., 1992; Sandouk et al., 1993) and increase adipose tissue mass in vivo (Hallakou et al., 1997).

In addition to its ligands, PPAR\(\gamma\) activity is also regulated by several co-activators and co-repressors. In the absence of a ligand, PPAR\(\gamma\) binds to NCoR (nuclear receptor co-repressor) and SMRT (silencing mediator for retinoid and thyroid receptor) transcriptional co-repressors (Guan et al., 2005), resulting in the inhibition of its transcriptional activity, whereas blocking the expression of both NCoR and SMRT by siRNA in 3T3-L1 cells results in their more efficient differentiation into adipocytes (Yu et al., 2005). Another co-repressor called receptor interacting protein 140 (RIP 140) has also been shown to inhibit PPAR\(\gamma\) transcriptional activity (Debevec et al., 2007). Upon binding to a ligand, the co-repressor complex associated with PPAR\(\gamma\) is replaced by co-activators, resulting in the gene transcription (Perissi and Rosenfeld, 2005). Several transcriptional co-activators including CBP/p300, steroid receptor coactivators and...
TRAP200/DRIP205 are known to directly interact with PPARγ and induce its transcriptional activity (Powell et al., 2007). In addition, several post translational modifications are known to regulate the transcriptional activity of PPARγ. The phosphorylation of PPARγ at S112 induced by ERK activation inhibits the transcriptional activity of PPARγ and blocks adipogenesis (Camp and Tafuri, 1997; Hu et al., 1996). Further, sumoylation of PPARγ at K107 inhibits its transcriptional activity (Ohshima et al., 2004; Yamashita et al., 2004). In addition, PPARγ is poly ubiquitinated and degraded by the proteasome (Floyd and Stephens, 2002; Hauser et al., 2000). Thus, it is clear that the PPARγ activity is regulated by multiple mechanisms, including multiple co-activators, co-repressors and post translational modifications.

6.4.2 Role of PPARγ in adipogenesis: During the adipogenic process PPARγ is expressed after 2 days of induction and its expression is induced by the early transcription factors C/EBPβ and C/EBPδ (Christy et al., 1991; Clarke et al., 1997; Wu et al., 1995). The delayed expression of PPARγ is important due to its anti-mitotic activity, which induces the cells to exit the cell cycle and undergo permanent growth-arrest (Altiok et al., 1997). Once expressed, the more important function of PPARγ is to induce the expression of genes involved in lipogenesis and insulin sensitivity to confer the adipocyte phenotype (Rosen et al., 2000). Most adipocyte specific genes contain PPARγ binding sites and are shown to be regulated by PPARγ. A recent study with genome-wide ChIP-seq profiling of PPARγ:RxR-binding sites in differentiating 3T3-L1 cells demonstrated that the PPARγ:RxR heterodimer binds to more than 5000 gene promoters, with the majority of
the genes involved in glucose and lipid metabolism (Nielsen et al., 2008). In addition, PPARγ also induces the expression of C/EBPα. Thus, a positive feedback loop exists between PPARγ and C/EBPα to reinforce each other expression and to maintain their expression in the mature adipocytes (Otto and Lane, 2005; Rosen et al., 2002).

Multiple evidence show that PPARγ is the master regulator of adipogenesis and the failure of expression of PPARγ prevents adipogenesis. In fact, the ectopic expression of PPARγ induces fibroblasts to express adipocyte specific genes without adipogenic stimuli and subsequently, these cells accumulate triglycerides (Tontonoz et al., 1994). In contrast, embryonic stem cells that lack PPARγ fail to differentiate into adipocytes (Rosen et al., 1999). Interestingly, PPARγ can induce adipogenesis in C/EBPα deficient MEF, whereas C/EBPα cannot induce adipogenesis in the absence of PPARγ (Rosen et al., 2002). This shows that PPARγ alone is sufficient to induce adipogenesis. Though germline PPARγ deficiency is embryonic lethal (Barak et al., 1999), selective depletion of PPARγ2 in a mouse model resulted in decreased white adipose tissue mass and decreased insulin sensitivity (Rosen et al., 1999). Collectively, both in vitro and in vivo studies show that PPARγ is both sufficient and necessary for adipogenesis and is the master regulator of adipocyte differentiation.

7. Extracellular factors regulating adipocyte differentiation: The efficiency of adipocyte differentiation both in vitro and in vivo is strongly influenced by the environmental milieu, with a fine balance of extracellular factors that positively and
negatively regulate the adipogenic process (MacDougald and Mandrup, 2002). The extracellular factors regulate adipogenesis through activation of multiple signaling pathways that ultimately induce or inhibit the expression of the above mentioned key adipogenic transcription factors. Various factors including several hormones, cytokines, growth factors and some pharmacological compounds are known to positively regulate adipogenesis (MacDougald and Mandrup, 2002). For example, insulin and IGF-1 are potent inducers of adipogenesis and are required for complete differentiation of preadipocytes into mature adipocytes (Girard et al., 1994; Smith et al., 1988). In contrast, several factors including Wnt, epidermal growth factor, resistin, PREF-1 and pro-inflammatory cytokines TNFα, IL-6 and IL-11 are known to inhibit adipogenesis by blocking the expression of key adipogenic transcription factors (MacDougald and Mandrup, 2002). For example, Wnts, a family of secreted proteins, are considered as potent inhibitors of adipocyte differentiation. Interestingly, inhibition of Wnt signaling using a dominant-negative form of its downstream signaling protein TCF4 in 3T3-L1 cells induces these cells to spontaneously differentiate into adipocytes in the absence of adipogenic stimuli (Bennett et al., 2002; Ross et al., 2000). Another important class of agents that are known to influence adipocyte differentiation both positively and negatively is the prostaglandins which are discussed below.

7.1 Prostaglandins: Prostaglandins (PGs) are a group of lipid mediators and are synthesized through a series of enzymatic process from membrane phospholipids. First, arachidonic acid is liberated from membrane phospholipids by phospholipase A2. Then,
it is converted to PGH2, a common precursor of all PGs, by cyclooxygenase enzymes. Finally, PGH2 is metabolized to various PGs such as PGD2, PGE2, PGF2α, PGI2 and thromboxane A2 by the action of specific PG synthases (Breyer et al., 2001; Ricciotti and FitzGerald, 2011). After biosynthesis, prostaglandins are rapidly transported out of the cell and act in an autocrine and paracrine manner on their cognate PG receptors that belong to the membrane spanning G-protein couple receptors (Breyer et al., 2001; Ricciotti and FitzGerald, 2011). Though PGs are known to play an important role in mediating inflammation, they also regulate several cellular processes including adipocyte differentiation. Specific PGs have been shown to either promote or inhibit adipocyte differentiation. PGI and PGD2 metabolite PGJ2 have been shown to have a positive effect on adipocyte differentiation (Forman et al., 1995; Reginato et al., 1998). PGI promotes adipogenesis by inducing the expression of early transcription factors C/EBPβ and C/EBPδ through a cAMP-dependent mechanism (Aubert et al., 2000), while PGD2 metabolite 15-deoxy-Δ12, 14-prostaglandine J2 has been shown to potently induce adipocyte differentiation, possibly through its role as a putative endogenous ligand of PPARγ (Forman et al., 1995). In contrast, PGE2 and PGF2α are known as inhibitors of adipocyte differentiation (Inazumi et al., 2011; Serrero et al., 1992; Tsuboi et al., 2004). PGE2 and a selective agonist of EP receptor inhibit adipocyte differentiation by blocking the expression of PPARγ, whereas an EP receptor antagonist promotes adipocyte differentiation (Tsuboi et al., 2004). More importantly, PGF2α is a potent physiological inhibitor adipogenesis (Volat et al., 2012) and is discussed below.
7.2 PGF2α - A potent inhibitor of adipogenesis:

7.2.1 PGF2α synthesis, signaling and functions: PGF2α is synthesized, as described earlier, from arachidonic acid by a series of enzymatic reactions. The terminal enzymes that catalyze the conversion of PGH2 to PGF2α are aldo-keto reductases (Akrs). Though several tissue specific isoforms of Akrs are expressed, Akr1b7 and Akr1b3 are highly expressed in preadipocytes and act as PGF2α synthases in these cells (Fujimori et al., 2010b; Tirard et al., 2007). When Akr1b7 expression was depleted in a mouse model, these mice showed a significantly decreased level of PGF2α in their adipose tissue, which shows that Akr1b7 plays a critical role in the PGF2α synthesis in vivo (Volat et al., 2012). Once synthesized and released, PGF2α binds and activates a specific Gαq coupled GPCR called the FP prostanoid receptor (Breyer et al., 2001; Narumiya et al., 1999). Activation of the FP receptor results in the stimulation of phospholipase C, an increase in intracellular calcium and the subsequent activation of several downstream signaling pathways including phosphotidylinositol-3 kinase, protein kinase C, mitogen-activated protein kinases, tyrosine kinases and stimulation of the Rho small G-protein and the activation of the β-catenin/TCF-signaling pathway (Breyer et al., 2001; Fujino and Regan, 2001; Pierce et al., 1999; Watanabe et al., 1994; Watanabe et al., 1995). The PGF2α-mediated signaling pathways have been shown to regulate various biological processes including bone remodeling, reproduction, inflammation, and vascular and endometrial remodeling (Agas et al., 2013; Ricciotti and FitzGerald, 2011). Importantly, PGF2α has been shown to potently inhibit adipocyte differentiation both in vitro and in
vivo (Casimir et al., 1996; Lepak and Serrero, 1993; Serrero et al., 1992; Volat et al., 2012).

7.2.2 Anti-adipogenic effect of PGF2α in cultured preadipocytes: Initial studies showed that arachidonic acid, the precursor of prostaglandins, inhibits adipocyte differentiation and the pretreatment with cyclooxygenase inhibitors block this inhibitory effect (Casimir et al., 1996). This showed that the prostaglandins synthesized from arachidonic acid inhibit adipogenesis. Further, prostaglandins F2α, E2 and D2 were able to mimic the inhibitory effect of arachidonic acid when added to the adipogenic cocktail. Among these prostaglandins, PGF2α is the most effective and highly potent in inhibiting adipocyte differentiation (Casimir et al., 1996). More importantly, PGF2α is produced by preadipocytes themselves and its production is highest in dividing nonconfluent cells. But its level dropped by approximately 70% after stimulation and the level remained at low throughout the differentiation process (Miller et al., 1996). This shows that PGF2α seems to act as a negative regulator of adipocyte differentiation. Further, the activation of the cognate PGF2α receptor (FP receptor) with a selective agonist, fluprostenol inhibits preadipocytes differentiation, whereas FP receptor antagonists prevents the inhibitory effects of fluprostenol on adipogenesis (Casimir et al., 1996; Miller et al., 1996). This confirms that the activation of FP receptor inhibits adipogenesis. FP receptors are present throughout differentiation, as the membranes prepared from either 3T3-L1 preadipocytes or adipocytes exhibits specific binding for PGF2α (Miller et al., 1996). Given the fact that endogenous PGF2α production decreases during the differentiation and the FP
receptors present throughout the differentiation process, the FP-receptor mediated inhibition of adipogenesis appears to be regulated by the alteration in the PGF2α concentration rather than the receptor expression (Miller et al., 1996). In addition, when several prostaglandins were examined, structurally similar prostaglandin 9 alpha, 11 beta-PGF2α potently inhibited adipogenesis, suggesting that the structural characteristics of PGF2α is required for its inhibitory effect on adipogenesis (Lepak and Serrero, 1993).

7.2.3 Mechanisms of PGF2α-mediated inhibition of adipocyte differentiation:
Several mechanisms have been proposed for the inhibitory effects of PGF2α on adipogenesis. Initial studies showed that the activation of FP receptor with fluprostenol transiently increases the intracellular calcium and a calcium/calmodulin-dependent protein kinase inhibitor rescues the inhibitory effect (Miller et al., 1996). This study suggested that the PGF2α-induced increase in intracellular calcium inhibits adipogenesis by activation of the calcium-dependent downstream signaling pathways. Similarly, a number of studies have shown that increases in intracellular calcium concentration during the early phase of human and 3T3-L1 preadipocyte differentiation act to potently inhibit adipogenesis (Neal and Clipstone, 2002; Ntambi and Takova, 1996; Shi et al., 2000). Further, PGF2α-mediated activation of ERK has been linked to the inhibition of adipogenesis by causing an inhibitory phosphorylation of PPARγ (Reginato et al., 1998). In addition, PGF2α has been shown to inhibit adipogenesis through upregulation of HIF-1 transcription factor under normoxic condition (Liu and Clipstone, 2008). HIF-1 induces
FIGURE 2. Schematic model showing the PGF2α-mediated inhibition of adipocyte differentiation via activation of the calcineurin phosphatase.

PGF2α activates Gαq coupled G-protein coupled receptor and increases the intracellular concentration of Ca^{2+}. The increased intracellular Ca^{2+} activates calcium-dependent phosphatase, calcineurin. The PGF2α/calcineurin-signaling pathway inhibits adipocyte differentiation by blocking the expression of late transcription factors (C/EBPα and PPARγ), but the early transcription factors (C/EBPβ and C/EBPδ) are not affected.
the expression of a HDAC associated transcriptional repressor DEC1, which has been shown to directly repress the PPARγ promoter. Interestingly, trichostatin A, an HDAC inhibitor rescues differentiation from PGF2α, suggesting the role of a HDAC regulated gene, possibly DEC1 in mediating the inhibitory effects of PGF2α on adipogenesis (Liu and Clipstone, 2008). More importantly, studies from our laboratory have shown that PGF2α inhibits adipogenesis through a calcium-dependent phosphatase calcineurin (Liu and Clipstone, 2007), which is discussed below.

7.2.4 PGF2α inhibits adipogenesis through calcineurin-dependent signaling: Initial studies from our laboratory showed that calcineurin activity is required for the inhibitory effects of calcium ionophore, as the presence of the calcineurin inhibitors either CsA or FK506, rescued differentiation from the inhibitory effect of calcium inophore on adipogenesis (Neal and Clipstone, 2002). In addition, ectopic expression of a calcium-independent, constitutively active calcineurin inhibited differentiation of 3T3-L1 cells into adipocytes (Neal and Clipstone, 2002). Collectively, these findings show that calcineurin activity inhibits adipocyte differentiation. Later, studies from our laboratory showed that the calcineurin phosphatase acts as a potent downstream effector in mediating the inhibitory effects of PGF2α (Liu and Clipstone, 2007). In fact, PGF2α requires Gαq, but not Gβγ subunits of G-protein to inhibit adipocyte differentiation (Liu and Clipstone, 2007). The activation of Gαq is well known to activate phospholipase C, which results in a subsequent increase in intracellular calcium (Katritch et al., 2013).
When the 3T3-L1 cells are induced to differentiate in the presence of PGF2α along with an inhibitor of receptor-mediated plasma membrane Ca\(^{2+}\)-influx or a membrane permeable Ca\(^{2+}\)-chelator, both inhibitors rescue adipocyte differentiation, suggesting that PGF2α-induced increase in intracellular calcium is responsible for the inhibition of adipogenesis (Liu and Clipstone, 2007). It is also well known that the activity of calcineurin phosphatase is regulated by intracellular calcium and calcineurin acts as a negative regulator of adipocyte differentiation (Neal and Clipstone, 2002). Interestingly, pretreatment with either a calcineurin inhibitor, cyclosporin A or FK506 or expression of a calcineurin inhibitory VIVIT peptide completely rescues adipocyte differentiation from the PGF2α-mediated inhibitory effect, suggesting that PGF2α acts through calcineurin phosphatase to inhibit adipogenesis. Interestingly, PGF2α does not prevent the mitotic clonal expansion during adipogenesis as well as expression of early transcription factors C/EBPβ and C/EBPδ, whereas the expression of key adipogenic factors C/EBPα and PPARγ is inhibited by PGF2α (Figure 2) (Liu and Clipstone, 2007). However, the precise molecular mechanism by which the PGF2α/calcineurin-signaling pathway inhibits adipogenesis by blocking the C/EBPα and PPARγ expression is not known.

### 7.2.5 Role of PGF2α in physiological inhibition of adipose tissue development in vivo:

A recent study using Akr1b7 knockout mice shows that PGF2α plays a critical negative regulatory role in the development of adipose tissue in vivo (Volat et al., 2012). As mentioned earlier, Akr1b7 is a terminal enzyme involved in the biosynthesis of
PGF2α from its precursor PGH2 and is highly expressed in adipocyte precursor cells. Further, its expression is down regulated during adipocyte differentiation, whereas overexpression of Akr1b7 inhibits differentiation process of 3T3-L1 preadipocytes. Interestingly, Akr1b7 knockout mice showed a significantly decreased level of PGF2α in adipose tissue compared to wild type littermates and an increase in basal white adipose tissue mass under normal feeding condition. Under a high fat diet, these animals rapidly gained weight with a five-fold increase in plasma leptin level compared to their wild type counterparts and developed insulin resistance. In contrast, daily administration of a synthetic PGF2α, cloprostenol for 3 months to Akr1b7-/- mice resulted in a significant decrease in plasma leptin level, which indicates a reduced white adipose tissue mass. Further, BrdU treatment to assess adipose tissue expansion in high fat diet fed Akr1b7-/- mice showed a 3.5 fold increase in BrdU-positive adipocyte-associated nuclei in gonadal WAT from Akr1b7-/- compared with wild type, whereas cloprostanol treatment reduced the production of new adipocytes under this condition to the level of WT mice fed with HF-diet (Volat et al., 2012). This suggests that PGF2α inhibits adipocyte differentiation and the generation of new adipocytes. Collectively, it is evident that PGF2α acts as a physiological inhibitor of adipose tissue development in vivo. Hence, it will be interesting to decipher the underlying molecular mechanisms involved in mediating the inhibitory effects of PGF2α on adipocyte differentiation.

8. Summary: Adipocyte differentiation is a highly complex, well regulated cellular process that is influenced by several positive and negative exogenous factors. One such
factor that is known to potently inhibit adipogenesis both *in vitro* and *in vivo* is PGF2α. Previous studies from our laboratory have shown that PGF2α acts through calcium-dependent phosphatase, calcineurin to inhibit adipocyte differentiation. However, the precise molecular mechanism by which the PGF2α/calcineurin-signaling pathway inhibits adipogenesis is not known. In this dissertation project, we have explored the underlying molecular mechanisms involved in mediating the inhibitory effects of PGF2α on adipogenesis and found that IL-11, an IL-6 cytokine family member and Nurr1, an orphan nuclear receptor are strongly induced by the PGF2α/calcineurin-signaling pathway and play a role in mediating its inhibitory effects on adipocyte differentiation. We have further explored the underlying mechanisms by which IL-11 and Nurr1 act to inhibit adipogenesis and have delineated the pathways and mechanisms involved. Collectively, our studies have afforded significant new insights into the mechanism by which the physiologically relevant anti-adipogenic factor PGF2α acts to inhibit the adipogenic process.
CHAPTER II
MATERIALS AND METHODS

1. Cell culture and adipocyte differentiation: The 3T3-L1 preadipocyte cells were plated at the concentration of 8x10^4 cells per well of a 6-well plate and were grown to confluence in DMEM/high glucose media (HyClone) supplemented with 10% fetal bovine serum (Atlanta Biologicals). After 2 days, the confluent cells were treated for 48 h with growth media plus the adipogenic cocktail, MDI (0.5 mM Methylisobutylxanthine, 1 µM Dexamethasone, and 10 µg/ml Insulin; all from Sigma). Cells were re-fed with growth media containing 10 µg/ml insulin at day 2 and every 2 days thereafter with growth media alone. Where indicated, cells were also treated for the first 48 h of the induction period with the indicated concentrations of PGF2α (Calbiochem), 1 µg/ml cyclosporin A (Calbiochem), recombinant murine IL-11 (50 ng/ml; Peprotech) and recombinant mouse erythropoietin (40 ng/ml; BD Pharmingen). In some experiments, conditioned media collected from 3T3-L1 cells ectopically expressing cDNAs encoding either murine IL-11 or murine erythropoietin were used instead of recombinant cytokines with identical results. To assess the extent of adipocyte differentiation, cells were fixed on day 8 post-stimulation with 10% formalin for 60 min at 4°C, then after two washes with phosphate buffered saline (PBS), were stained with filtered Oil Red O solution (Sigma) for 2 hrs at room temperature. After staining, the cells were washed with PBS to remove unbound dye and the plates were scanned. Cells were then counter stained with
hematoxylin (Sigma) for 4 min and, after washing with PBS, were visualized by light microscopy and photographed (40X).

2. Plasmid constructs:

2.1 Dominant-negative gp130: The dominant-negative version of gp130 (DN-gp130) was generated by using a murine gp130 cDNA (Open Biosystems; clone I.D. 6834623) as a template in a polymerase chain reaction (PCR) using PfuTurbo (Stratagene) together with gene-specific primers designed to delete the C-terminal 213 amino acids containing conserved amino acid residues known to be essential for gp130-mediated signaling events (Taga and Kishimoto, 1997) and replacing them with a FLAG epitope tag followed by a stop codon. The resulting DN-gp130 construct was then subsequently introduced into the MSCV-GFP retroviral expression vector.

2.2 EpoR/gp130-WT, 1F and 4F: The wild-type EpoR/gp130 chimera and corresponding mutant chimeras containing tyrosine-to-phenylalanine substitutions in conserved residues of the gp130 cytoplasmic domain were kind gifts of Dr. Fred Schaper, RWTH Aachen, Germany (Schaper et al., 1998; Schmitz et al., 2000). The wild type EpoR/gp130 chimera was PCR-amplified using gene-specific primers and the resulting PCR product was digested with SalI and MfeI, and introduced into the XhoI/EcoRI site of the MSCV-GFP retroviral vector, giving rise to MSCV-EG-WT. To generate MSCV-EG-1F and MSCV-EG-4F, Nsi I/Bam HI fragments corresponding to the C-terminal region of murine gp130 containing either a Y759F single substitution (1F) or
Y767F/Y814F/Y905F/Y915F quadruple substitutions (4F) were introduced into Nsi I/Bam HI-digested MSCV-EG-WT backbone. The EpoR/gp130-4F “add back” mutants were generated using PCR cloning techniques to fuse peptide sequences previously reported to selectively activate either STAT1 (PTPSFGYDKPHV), or STAT3 (PTPSFGYFKQHV) (Gerhartz et al., 1996), in-frame with the extreme C-terminus of EG-4F. Briefly, EG-4F was used as a template in a PCR reaction with the forward primer 5’-CTTCA TGCAT GTCAT CTTCT AGGC-3’ together with either 5’-AGGAT CCTAC
ACGTG TGGCT TGTCG TATCC GAAGG ATGTT GGCAGT CTGTGA TAAG
AAACT TTTAG GC-3’ or 5’-GAGGACTCTACACGTGCTGC TTGAA GTATC
CGAAG GATGTT GGGCGT CTGTG GTAAGAAACT TTTAG GC-3’ as reverse
primers. The resulting PCR products were digested with Nsi I/Bam HI and introduced into the corresponding sites of MSCV-EG-4F giving rise to EG-4F-STAT1 and EG-4F-STAT3, respectively.

2.3 STAT1, IL-11 and CRTC2 specific shRNAs: To generate retroviral constructs capable of expressing shRNAs specific for STAT1, plasmids encoding STAT1-specific shRNAs in the lentiviral vector pLKO.1 were purchased from Open Biosystems (Clone I.D. TRCN0000054924 and TRCN0000054927) and used as templates in a PCR reaction together with the forward primer 5’-CTCTC AATTG TATCG ATCAC GAGAC
TAGCC TC-3’; and the reverse primer 5’-CCTCA AGCTT GGATG AATAC TGCCA
TTTGT CTCTG-3’. The resulting PCR fragments were digested with Mfe I and Hind III and cloned into the pSR-GFP/Neo vector (OligoEngine) previously digested with Eco RI
and Hind III to generate pSR-STAT1-924 and pSR-STAT1-927, respectively. The shRNA retroviral constructs specific for murine IL-11 and murine CRTC2 were generated by the identical approach using plasmids encoding IL-11 (Open Biosystems; Clone I.D. TRCN0000066459) and CRTC2 (Open Biosystems; Clone I.D. TRCN00000176098)-specific shRNAs in the lentiviral vector pLKO.1. The control shRNA retroviral vector specific for firefly luciferase has been described previously (Liu and Clipstone, 2008).

2.4 MSCV-DsRed2-PPARγ2: The MSCV-DsRed2-PPARγ2 construct was generated by isolating a blunted Afl III/Xba I fragment of the full-length murine PPARγ2 cDNA from pSVSport-PPARγ2 (Addgene) and introducing it into MSCV-DsRed2 at the blunted EcoRI site.

2.5 Nurr1-WT, Nurr1-AA and Nurr1-KLL: Plasmids encoding the murine wild type Nurr1 (Nurr1-WT), the DNA binding defective Nurr1-C280A/E281A (Nurr1-AA), and the RxR binding-defective Nurr1-K554A/L555A/L556A (Nurr1-KLL) in the pCMX expression vector were kindly provided by Dr. Thomas Perlmann (Ludwig Institute for Cancer Research, Karolinska Institute, Stockholm, Sweden). To generate a FLAG-tagged version of WT-Nurr1, the pCMX-wtNurr1 plasmid was used in a polymerase chain reaction using PfuTurbo (Stratagene) and 5’- CTCAGA TCTGAG ACCATG GACTAC AAGGAC GACGAT GACAAG ATGCCT TGTGTT CAGGCG CAGTAT GG - 3’ as a forward primer together with 5’- CTCGAT CATATG CGTAGT GTAGTT
CTG - 3’ as a reverse primer. The resulting PCR product encoding the FLAG peptide fused in-frame with the 5’ end of the wtNurr1 sequence was digested with Bgl II and Nde I, then ligated into the Bgl II/Xho I-digested pMSCV-GFP retroviral expression vector together with an Nde I/Xho I fragment encompassing the 3’ end of the wtNurr1 cDNA isolated from pCMX-wtNurr1. FLAG-tagged versions of Nurr1-KLL and Nurr1-AA were constructed in a similar manner using Nde I/Xho I fragments isolated from pCMX-Nurr1-KLL and pCMX-Nurr1-AA, respectively.

2.6 Dominant-negative Nurr1: The dominant-negative Nurr1 (DN-Nurr1) construct is comprised of Nurr1 amino acids from 94 to 365, lacking both N- and C-terminal transactivation domains, fused in-frame with the N-terminal 100 amino acids of the ZPF-10 KRAB family member containing the KRAB transcriptional repressor domain. First, an Ndelta94-Nurr1 construct lacking the N-terminal 94 amino acids of Nurr1 was generated by using the pCMX-wtNurr1 plasmid in a PCR reaction with 5’- TCTGAG ACCATG TTCTAC CCATAC GACGTC CCAGAC TACGCT GTAGAA GACATT CAGATG CAC - 3’ as the forward primer and 5’ - CTCGAT CATATG CGTAGT GGCCAC GTAGTT CTG - 3’ as a reverse primer. The resulting PCR product was digested with Bgl II and Nde I and ligated together with an Nde I/Xho I fragment encompassing the 3’ end of the wtNurr1 cDNA isolated from pCMX-wtNurr1 into Bgl II/Xho I-digested MSCV-GFP to give the MSCV-Ndelta94Nurr1 construct. Second, the ZPF-10 plasmid (Open Biosystems) was used in a polymerase chain reaction together with 5’- ACAGGA TCCCTC TCCCCC CTCACC TCCGCT GAGTCT GATCAT
GGATGC TAAGTC ACTAAC TGCCTG GTC - 3’ as a forward and 5’ - GAGCTC GAGTTA AACTGA TGATTT GATTTC AAATGC AG - 3’ as a reverse primer. The resulting 400 bp fragment encompassing the amino acids 343-365 of Nurr1 fused in frame with the N-terminal 100 amino acids of ZPF-10 containing the KRAB transcriptional repressor domain was digested with Bam HI and Xho I and ligated together with a 1477 bp Spe I/Bam HI fragment from MSCV-Ndelta94Nurr1 comprising amino acids 94-365 of Nurr1 into the MSCV-GFP vector digested with Spe I and Xho I.

2.7 ER-caCRTC2: To insert wild type FLAG-tagged murine CRTC2 into the MSCV-GFP retroviral expression vector, the pcDNA3-FLAG-TORC2 construct (Addgene) was used in a PCR reaction together with 5’ - CTCAGA TCTACC ATGGAC TACAAG GACGAC GATGA C - 3’ as the forward primer and 5’ - GAGCTC GAGTCA TTGGAG CCGGTC ACTGCG GAATGA CTCC  - 3’ as the reverse primer. The resulting PCR fragment was digested with Bgl II and Xho I and ligated into Bgl II/Xho I-digested MSCV-GFP.

The constitutively active CRTC2 mutant containing serine to alanine substitution at amino acids 171 and 275 was generated using the mutagenic primers 5’ - CACTTA ACAGGA CAAGCG CTGACT CTGCTC TTCAC  - 3’ and 5’ - AACACA GGAGGC GCCCTA CCTGAC C - 3’ together with the QuickChange II site-directed mutagenesis kit from Stratagene. The tamoxifen-regulated conditionally active CRTC2 mutant was created by first performing a PCR reaction with a plasmid encoding the tamoxifen-regulated mutant version of murine estrogen receptor as template and 5’ - CTCGGA
TCCACC ATGGAC TACAAG GACGAC GATGAC AAGCTG CAGGAT CCATCT
GCTGGA GACATG AGGGCT GC - 3’ as forward primer and 5’ - GAGGAA TTCGAT
CGTGTT GGGAA G C CTC TGCT - 3’ as the reverse primer. The resulting 900 bp
fragment was digested with Bam HI and Eco RI and ligated into Spe I/Eco RI-digested
MSCV-caCRTC2 together with a 700 bp Spe I-Bgl II fragment from MSCV-GFP.

2.8 YFP-CRTC2: To generate an YFP-CRTC2 construct, pEYFP-C1 was used as a
template in a PCR reaction with 5’ - CTCAGA TCTGCC ACCATG GTGAGC
AAGGGC GAGGAG CTG - 3’ as forward primer and 5’ - GAGGAA TTCGGA
TCCGAG TCCGGA CTTGTA CAGCTC GTCCATG - 3’ as reverse primer. The
resulting 729 bp fragment was digested with Bgl II and Eco RI and ligated together with
an Eco RI/Xho I-digested 2152 bp fragment of wtCRCT2 into Bgl II/Sal I-digested
MSCV-GFP.

2.9 K-CREB and CREB R314A: To generate MSCV-CREB, a plasmid encoding rat
CREB (kind gift from Dr. Basabi Rana, Loyola University Chicago) was used as a
template in a PCR reaction together with 5’ - CTCCTC GAGACC ATGACC ATGGAC
TCTGGA GCAGAC AACCAG - 3’ as a forward primer and 5’ - GAGGAA TTCCTA
ATCTGA CTTGTG GCAGTA AAGGTC - 3’ as reverse primer and the resulting 1034
bp PCR fragment was digested with Xho I and Eco RI and ligated into Xho I/Eco RI-
digested MSCV-GFP. The dominant-negative K-CREB mutant was generated by PCR
mutagenesis using the primers 5’- GCAAGA GAATGT CTTATA AAGAAG AAAG -
3’ and 5’ - CTTTCT TCTTTA TAAGAC ATTCTC TTGC - 3’ together with the primers listed above to introduce the R288L/R289I substitutions into the wtCREB sequence. CREB-R314A was generated by a similar strategy using the primers 5’ - GAAAGA ATATGT GAAATG TTTAGA GAAC GC AGTGGC AGTGCT TG - 3’ and 5’ - CAAGCA CTGCCA CTGCCT TCTCTA AACATT TCACAT ATTCTT TC - 3’.

The integrity of all constructs was confirmed by DNA sequence analysis.

3. Retroviral generation and infection of 3T3-L1 cells: Retroviral expression vectors were co-transfected with pVSV-G (Clontech) into the GP293 pantropic packaging cell line (Clontech) using Lipofectamine Plus (Invitrogen). Media was replaced after 24 h and viral supernatants were collected after 24 h and 48 h post-transfection and stored at -80°C. For retroviral infections, 5x10⁴ 3T3-L1 cells were plated per well of a 6-well plate, the next day media was replaced with retroviral particle-containing growth media supplemented with 8 µg/ml polybrene (Sigma) and the plates were centrifuged in a swinging bucket rotor at 1,000 rpm for 90 min at room temperature, then returned to the incubator overnight. After 24 h, cells were subjected to a second round of infection, essentially as described above, then expanded in regular growth medium prior to seeding for subsequent experiments.

4. Immunoblot analysis: Whole cell lysates were prepared by adding boiling SDS sample buffer (120 mM Tris-HCl pH 6.8, 4% sodium dodecyl sulfate, 20% glycerol) directly to cells on the plate. The cell lysates were collected by scraping and boiled for 10
min, then sonicated for 10s using a Digital sonifier (Branson) at an amplitude of 11% and stored at -80°C. An equal amount of protein from each treatment was resolved by SDS-PAGE gel electrophoresis, transferred to nitrocellulose membrane, and subjected to immunoblot analysis with the following primary antibodies: anti-STAT1 (#9172), anti-phospho-STAT1 (Tyr701; #9171), anti-STAT3 (#9132), anti-phospho-STAT3 (Tyr705; #9131), anti-C/EBPβ (LAP) (#3087), anti-C/EBPδ (#2318) and anti-C/EBPα (#8178) from Cell Signaling Technologies; anti-PPARγ (sc-7196), anti-phospho ERK and anti-ERK (sc-93) from Santa Cruz Biotechnology; anti-aP2 (#10004944) from Cayman Chemical Company; anti-mouse EpoR (#AF1390) and anti-Nurr1 (AF2156) from R&D systems. Appropriate horseradish peroxidase-conjugated secondary antibodies (anti-rabbit, anti-mouse, anti-goat) were purchased from Vector laboratories, and detected by enhanced chemiluminescence using ECL reagent (Thermo Scientific).

5. Quantitative Real-time PCR (qRT-PCR) analyses: Total RNA was isolated from 3T3-L1 cells at 48 h post-differentiation using an RNeasy kit (Qiagen). cDNA was synthesized from 1 µg of mRNA using random primer and reverse transcriptase (Promega). The expression of the relevant genes was determined by qRT-PCR using a SYBR Green-based detection system (SA Biosciences) and hypoxanthine phosphoribosyltransferase (HPRT) gene as an internal control for normalization. The primers used are shown in Table.1. All qRT-PCR measurements were performed using an iCycler real-time PCR detection system (Bio-Rad) and the fold change in mRNA expression was determined by the ΔΔCT method.
6. **Enzyme-linked immunosorbent assay (ELISA):** Cell culture supernatants were collected at the indicated time post-induction of adipocyte differentiation and assayed by ELISA for the presence of IL-11 (Cat. No. DY418, R&D Systems) according to the manufacturer’s instructions.

7. **Immunofluorescence:** 3T3-L1 preadipocytes were infected with YFP-CRTC2 encoding retroviruses as described earlier and induced to undergo adipogenesis using standard MDI-induced differentiation protocol in the presence of MDI alone, PGF2α (25 nM) or PGF2α (25 nM) plus CsA (1 µg/ml). At the indicated time points, cells were fixed with 3.7% formalin for 15 min at room temperature and then, washed 3 times with PBS. The cells were permeabilized with 0.05% saponin in PBS and then, washed 3 times with PBS. Then, the cells were incubated in dark with DAPI (0.5 µg/ml) in PBS for 10 min at room temperature and then, washed 3 times with PBS. To keep the cells wet, 2 ml of PBS was added to each well. The cells were examined and counted using fluorescence microscopy (Olympus Inc, Center Valley, PA). At least 100 cells expressing YFP-CRTC2 were counted per well and classified as cells with YFP-CRTC2 either completely nuclear, completely cytoplasmic, more nuclear than cytoplasmic and more cytoplasmic than nuclear. Then, the cells with YFP-CRTC2 completely nucleus and more nuclear than cytoplasm were grouped as nuclear, whereas with completely cytoplasmic and more cytoplasmic than nuclear were grouped as cytoplasmic.
8. Luciferase assay: HEK293T cells were plated at the concentration of 5x10^4 cells per well in 24 well plates. Next day, the cells were transfected with plasmids encoding either GFP, Nurr1-WT (100 ng/well) or DN-Nurr1 (0, 100, 200 and 400 ng/well) as indicated along with appropriate amount of GFP control plasmid to make the final concentration of plasmids to 500 ng/well. Simultaneously, the cells were also transfected with the Nurr1 reporter, (NBRE)3-TK-LUC (Firefly; 50 ng/well) and pRG-TK (Renilla; 25 ng/well) reporter plasmids. The transfection was performed using Attractene transfection reagent (Qiagen, Cat. No. 301005) following manufacturer’s instructions. At 24 h post-transfection, Luciferase bioluminescence for each well was determined using Dual-luciferase reporter assay system (Promega, Cat. No. E1910) following the manufacturer’s instructions and TD-20/20 Luminometer (Turner Designs, Sunnyvale, CA). Firefly luciferase activity of each well was corrected using the corresponding Renilla luciferase activity as a transfection efficiency control. The fold effect of (NBRE)3 activation by DN-Nurr1 was determined with comparison to that of Nurr1-WT expression alone.

To determine the activation of ER-caCRTC2, HEK293T cells were plated as described above and next day, the cells were transfected with either GFP or ER-caCRTC2 plasmids at the concentration of 400 ng/well along with the CREB reporter, CRE-Luc (Firefly; 50 ng/well) and pRG-TK (Renilla; 25 ng/well) reporter plasmids as described above. At 16 h post-transfection, cells were treated with either vehicle or tamoxifen (500 nM). Cells were lysed at 4 h post-treatment and luciferase activity was measured as described above.
9. **Statistical analysis:** The data are expressed as the mean ± S.E.M. For comparison between groups, either one-way ANOVA followed by Bonferroni *post hoc* test or Student’s *t* test was employed. Differences were considered to be statistically significant at *p* < 0.05.

### TABLE 1. Nucleotide sequence of primers used for qRT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Il-11</em></td>
<td>5’-CTGTGGGGACATGAA CTGTG-3’</td>
<td>5’-CGTCAGCTGGGAATT TGTCT-3’</td>
</tr>
<tr>
<td><em>Il-6</em></td>
<td>5’-AGTTGGCTTTCTTGGA CTGA-3’</td>
<td>5’-TCTGCAAGTGCATCAT CGTT-3’</td>
</tr>
<tr>
<td><em>Hprt</em></td>
<td>5’-GTTGGATAACAGGGCA GACTT TGTTG-3’</td>
<td>5’-GAGGGTAGGCTGGCC TATAGGCT-3’</td>
</tr>
<tr>
<td><em>Nurr1</em></td>
<td>5’-CGGTTTCAGAAGTGCC TAGC-3’</td>
<td>5’-TTGCCCTGGAACCTGGA ATAG-3’</td>
</tr>
<tr>
<td><em>Nur77</em></td>
<td>5’-ATGCCTCCCCCTACCA TCTT-3’</td>
<td>5’-TCTGCCCACCTTTCCGAT AAC-3’</td>
</tr>
<tr>
<td><em>Nor1</em></td>
<td>5’-CCAAAACCAAGAGCCC ACTA-3’</td>
<td>5’-GGCCGTCAGAAGGTTG TAGA-3’</td>
</tr>
<tr>
<td></td>
<td>5’-CTGCCCTCACAACAA AGAG-3’</td>
<td>5’-GGCCGTCAGAAGGTTG TAGA-3’</td>
</tr>
<tr>
<td></td>
<td>5’-TCAGCCTTTTTGGAG CTGTT-3’</td>
<td>5’-TAAACCATGTCGCTCT GTGA-3’</td>
</tr>
<tr>
<td></td>
<td>5’-AGAAGGTCAGAAGAA ATGC-3’</td>
<td>5’-TAGTGGGCTCCCTTGG TTGG-3’</td>
</tr>
<tr>
<td><em>Crtc1</em></td>
<td>5’-CACCAGAGCAACATGA CACC-3’</td>
<td>5’-GCCTTCTTTGAGTCCT CCC ATGA-3’</td>
</tr>
<tr>
<td><em>Crtc2</em></td>
<td>5’-TGGATGCTAAAAGTCCC TGCT-3’</td>
<td>5’-TAGCAGGCTGTCAGG AGAT-3’</td>
</tr>
<tr>
<td><em>Crtc3</em></td>
<td>5’-TTCCAGCCATCACTTC ATCA-3’</td>
<td>5’-AGCTCTCATCCAGGTG CTGT-3’</td>
</tr>
</tbody>
</table>
CHAPTER III

THE PGF2α/CALCINEURIN-SIGNALING PATHWAY INHIBITS ADIPOGENESIS VIA AN AUTOCRINE/PARACRINE-MEDIATED IL-11/GP130/STAT1-DEPENDENT SIGNALING CASCADE

1. Introduction: Adipocytes are specialized cells that form adipose tissue and are known to regulate energy homeostasis and whole body metabolism (Rosen and Spiegelman, 2006). The excessive accumulation of adipose tissue due to hypertrophy and hyperplasia of adipocytes contributes to the development of obesity which is associated with co-morbidities such as insulin resistance, type 2 diabetes, cardiovascular diseases and some cancers (De Pergola and Silvestris, 2013; Kahn and Flier, 2000; Kahn et al., 2006; Nakamura et al., 2013; Nishimura et al., 2009; Van Gaal et al., 2006). Recent findings support that the generation of new insulin-sensitive adipocytes through adipogenesis plays a protective role in the development of type 2 diabetes in obesity (Tan and Vidal-Puig, 2008; Virtue and Vidal-Puig, 2010). However, obesity is considered as an inflammatory condition (Gregor and Hotamisligil, 2011) and several inflammatory mediators are known to inhibit adipogenesis (Gustafson and Smith, 2006; Xu et al., 1999). Given the protective role of adipogenesis in the development of obesity associated complications, it is important to understand the molecular mechanisms by which these inflammatory mediators inhibit adipogenesis to identify better targets to treat obesity and the associated complications. An inflammatory mediator that is known to inhibit
adipogenesis both in vitro and in vivo is PGF2α (Casimir et al., 1996; Liu and Clipstone, 2007; Miller et al., 1996; Volat et al., 2012). In our attempts to understand the underlying molecular mechanisms, our previous studies showed that PGF2α inhibits adipocyte differentiation through activation of the calcium-dependent phosphatase, calcineurin (Liu and Clipstone, 2007). In this chapter, we further extended our studies and demonstrate that the PGF2α/calcineurin-signaling pathway inhibits adipogenesis through activation of an autocrine/paracrine-mediated IL-11/gp130/STAT1-dependent signaling cascade.

2. Interleukin-11 (IL-11) cytokine: IL-11 is a multifunctional cytokine belonging to IL-6 family of cytokines which also consists of IL-6, LIF (leukemia inhibitory factor), OSM (oncostatin M), CNTF (ciliary neurotrophic factor), CT-1 (cardiotrophin-1) and CLC (cardiotrophin-like cytokine) (Heinrich et al., 2003; Taga and Kishimoto, 1997). All these family members bind to specific transmembrane, non-signaling α-subunit and signal through recruitment of a common β-subunit called glycoprotein 130 (gp130) (Heinrich et al., 2003; Taga and Kishimoto, 1997). Though gp130 is ubiquitously expressed, expression of specific α-chain determines the tissue specificity of each IL-6 family members. IL-11 is a peliotrophic cytokine with multiple biological effects including hematopoiesis, lymphopoiesis, immune response and cell differentiation (Bozza et al., 2001; Du et al., 1993; Suga et al., 2001; Weich et al., 1997). In addition, IL-11 is over expressed in certain tumors and has been shown to play a role in the development and metastasis of certain tumors (Grivennikov, 2013; Onnis et al., 2013).
2.1 **IL-11 signaling:** IL-11 signals through a hexameric receptor complex consisting of two molecules each of ligand-binding IL-11 α-subunit (IL-11Rα) and the gp130 co-receptor β-chain signaling receptor (Barton et al., 2000). The IL-11Rα subunit lacks intrinsic signaling capacity, however, binding of IL-11 to IL-11Rα promotes the formation of a complex with gp130 and subsequent gp130 heterodimerization, thereby allowing the activation of the gp130-associated JAK kinases and phosphorylation of critical conserved tyrosine residues within the gp130 cytoplasmic domain that are responsible for the recruitment and activation of specific downstream signaling molecules (Heinrich et al., 2003; Taga and Kishimoto, 1997). Specifically, phosphorylation of Y759 within the gp130 cytoplasmic domain forms a docking site for the SHP2 adaptor protein that is responsible for triggering the activation of the Ras-Raf-mitogen-activated protein kinase (MAPK) signaling pathway, whereas phosphorylation of Y767, Y814, Y905 and Y915 results in the recruitment of STAT1 and STAT3 to gp130. Once recruited to the receptor complex, STAT1 and STAT3 are phosphorylated by JAKs and then, they homo and/or heterodimerize, translocate to the nucleus and regulate gene expression (Figure 3) (Heinrich et al., 2003).

2.2 **Role of IL-11 in inhibiting adipogenesis:** IL-11 was initially cloned as an adipogenesis inhibitory factor (AGIF) from human bone-marrow derived stromal cell line as the supernatant collected from the COS-1 cells that overexpress AGIF cDNA inhibited differentiation of 3T3-L1 cells into adipocytes and thus, provided initial evidence that IL-11 is a potent inhibitor of adipocyte differentiation (Kawashima et al., 1991).
FIGURE 3. Schematic model showing the IL-11/gp130-dependent signaling pathways.

IL-11 binds to non-signaling α chain and recruits signaling subunit, gp130 co-receptor. Then, the JAKs associated with gp130 phosphorylate specific tyrosine residues in the cytoplasmic domain of gp130, resulting in the recruitment of STATs, which are then phosphorylated by JAKs, homo/heterodimerize, translocate to nucleus and induce gene expression.
In human long-term marrow culture that has a potential to differentiate into myeloid cells and adipocytes, IL-11 inhibited these cells to differentiate into adipocytes, whereas promoted them to differentiate into hematopoietic cells (Keller et al., 1993). This further provided evidence that IL-11 is a potent inhibitor of adipogenesis in hematopoietic microenvironment. Further, IL-11 secreted by breast tumor cells inhibited the differentiation of fibroblast into adipocytes (Meng et al., 2001). Thus, several studies using cell culture system show that IL-11 inhibits adipogenesis. The physiological role of IL-11 in regulating adipogenesis is evident from the analysis of the P6 strain of senescence-accelerated mice (SAMP6). These mice showed a decrease in osteoclastogenesis and osteoblastogenesis, whereas they showed increased adipose tissue in bone marrow compared to control mice (Kajkenova et al., 1997). Further analysis of the bone marrow stromal cells from these mice showed a decreased expression of IL-11 and an increased potential to differentiate into adipocytes (Kodama et al., 1998). Further, transgenic mice with IL-11 overexpression showed a significantly increased bone mass and cortical thickness, suggesting that IL-11 promotes osteoblastogenesis (Takeuchi et al., 2002). These findings show that IL-11 physiologically inhibits adipocyte differentiation, whereas promotes osteoblast differentiation. Collectively, therefore, multiple evidence support that IL-11 is a potent inhibitor of adipocyte differentiation. However, the precise molecular mechanisms and downstream signaling pathways involved in mediating the inhibitory effects of IL-11 on adipogenesis are not known.
3. **STAT transcription factors:** The STAT proteins are a family of latent cytoplasmic transcription factors that consist of STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6. These proteins are activated by interferons, IL-6 family of cytokines, growth hormone, erythropoietin, leptin and others (Brierley and Fish, 2005). Binding of these factors to their cognate receptors activate JAKs, which in turn phosphorylate specific tyrosine residues in the cytoplasmic tail of the receptor to generate docking sites for STAT proteins and subsequently, STATs are recruited to the receptor complex (Fu et al., 1992; Platanias et al., 1994; Schindler et al., 1992). Once recruited to the receptor complex, STAT proteins are phosphorylated by JAKs and then, they homo or heterodimerize, translocate to nucleus and induce gene expression (Darnell, 1997). The genes induced by these STAT transcription factors are known to regulate various biological and pathological process including cell growth and differentiation, metabolism, hematopoiesis, host defense, immunoregulation and cancer development (Bromberg and Darnell, 2000; O'Shea et al., 2013; Xu et al., 2013).

3.1 **Expression of STATs during adipogenesis:** Studies with 3T3-L1 cells and human subcutaneous preadipocytes showed that several STAT proteins are expressed and regulated during adipogenesis (Harp et al., 2001; Stephens et al., 1996). STAT1, STAT3 and STAT5 proteins are minimally expressed in undifferentiated 3T3-L1 cells, whereas their expression significantly increases after adipogenic stimulation. While STAT3 expression remains at the same level, STAT1 and STAT5 expression decreases after 24 h of induction and again increases after 96 h. Further, STAT6 is expressed at high level in
preadipocytes and the level of expression remains constant during adipogenic process. In contrast, STAT2 and STAT4 are not expressed in preadipocytes (Stephens et al., 1996). Although several STAT family members are expressed during adipogenesis, STAT5 has extensively been studied and has been shown to positively regulate adipogenesis (White and Stephens, 2010). Recent evidences show that STAT3 positively regulates adipogenesis (Wang et al., 2009; Zhang et al., 2011), whereas STAT1 inhibits adipogenesis (McGillicuddy et al., 2009) which is discussed below.

3.2 Role of STAT3 in adipogenesis: While STAT3 level remains constant throughout adipogenesis, it is highly phosphorylated and translocated into nucleus within 2 h of adipogenic stimulation. This phosphorylation seems to be regulated by JAK2 as both the inhibitor of JAK2/STAT3 activation, AG490 and depleting JAK2 expression using siRNA decrease the STAT3 phosphorylation during adipogenesis (Wang et al., 2009). Initial studies linked the early phosphorylation and activation of STAT3 to cell proliferation and mitotic clonal expansion phase of adipogenesis (Deng et al., 2000). Further, STAT3 has been shown to bind to DNA during this phase. Later studies showed that STAT3 directly bind to C/EBPβ promoter during the early stages of adipogenesis and regulates its expression (Zhang et al., 2011). This is a highly possible mechanism as multiple evidence shows that C/EBPβ plays an important role in the regulation of critical mitotic clonal expansion phase of adipogenesis (Tang et al., 2003a). Further evidence for the role of STAT3 in adipogenesis is evident from the findings that the expression of a dominant-negative STAT3 and knockdown of STAT3 expression using siRNA blocks
adipogenesis (Wang et al., 2009). Collectively, STAT3 plays a positive role in adipogenesis by regulating C/EBPβ expression and mitotic clonal expansion.

3.3 **Role of STAT1 in adipogenesis:** The activation of STAT1 appears to inhibit adipocyte differentiation as the stimulation of adipogenesis in the presence of INFγ, a potent activator of STAT1, inhibits adipogenesis (McGillicuddy et al., 2009). In mature adipocytes, INFγ treatment resulted in strong activation and nuclear translocation of STAT1 which correlated with a substantial decrease in PPARγ expression (Waite et al., 2001). Further studies identified a potential consensus sequence of interferon-γ-activated site (GAS) elements in the PPARγ2 promoter and indeed, STAT1 homodimers are shown to bind an IFNγ-responsive site within the PPARγ2 promoter in 3T3-L1 cells (Hogan and Stephens, 2001). Collectively, the available evidence suggests that activation of the STAT1 transcription factor inhibits adipogenesis through possibly inhibiting the expression of PPARγ.

4. **Role of ERK in adipogenesis:** ERK has been shown to both positively and negatively regulate adipocyte differentiation. While ERK has been shown to positively regulate adipogenesis by phosphorylating C/EBPβ during the early stage of adipogenesis (Tang et al., 2005), angiotensin-mediated activation of ERK has been shown to inhibit adipocyte differentiation (Fuentes et al., 2010). Further, mechanical stretch induced activation of ERK has been shown to inhibit adipogenesis through down regulation of PPARγ expression (Tanabe et al., 2004). Thus activation of ERK during early stage of
differentiation promotes adipogenesis, whereas its activation in the later stage inhibits adipogenesis by blocking PPARγ expression.

In this chapter, we investigated the role IL-11 in mediating the inhibitory effects of PGF2α on adipogenesis. First, we show that PGF2α induces IL-11 expression during adipogenesis in a calcineurin-dependent mechanism. Next, using IL-11 specific shRNA and a dominant-negative gp130, we demonstrate that IL-11/gp130-mediated signaling is required for the inhibitory effects of PGF2α on adipogenesis. Further, using a panel of chimeric, mutant gp130 receptors, we identify that the gp130-mediated activation of STAT1 transcription factor inhibits adipogenesis. In addition, using STAT1 specific shRNAs, we demonstrate that STAT1 is required for the inhibitory effects of IL-11 and PGF2α on adipogenesis. Collectively, our results provide evidence that the PGF2α/calcineurin-signaling pathway inhibits adipocyte differentiation through activation of an autocrine/paracrine IL-11/gp130/STAT1-dependent signaling.

5. RESULTS

5.1 Activation of the PGF2α/calcineurin-signaling pathway induces the expression of IL-11 in differentiating 3T3-L1 preadipocytes. In previous studies we had demonstrated that PGF2α inhibits the early stages of adipocyte differentiation via the essential actions of the calcineurin phosphatase (Liu and Clipstone, 2007). Since calcineurin is a well-known positive regulator of a number of transcription factor (Crabtree, 2001), we hypothesized that the PGF2α/calcineurin-signaling pathway was likely to inhibit adipogenesis via the increased expression of a gene(s) capable of
FIGURE 4. **PGF2α treatment of differentiating 3T3-L1 preadipocytes induces the calcineurin-dependent expression and secretion of IL-11.**

Two-day post-confluent 3T3-L1 preadipocytes were induced to undergo adipocyte differentiation by treatment with MDI in the presence of either vehicle, PGF2α (25 nM) alone or PGF2α (25 nM) plus Cyclosporin A (CsA) (1 µg/ml). At 48 h post-stimulation,
total RNA and cell culture supernatants were collected and the expression of IL-11 was determined by qRT-PCR (A) and ELISA (B), respectively. The expression of IL-6 was determined by qRT-PCR (C). The data is shown as mean ± SEM (either one-way ANOVA followed Bonferroni post hoc test or Student’s t test; p<0.05; n=3). ND; not detectable. Results are representative of at least three independent experiments.
inhibiting the differentiation process. In order to test this hypothesis, we initially performed a microarray gene expression profiling experiment to identify candidate anti-adipogenic genes induced by PGF2α in a calcineurin-dependent fashion during the early stages of 3T3-L1 preadipocyte differentiation. Amongst the most highly induced genes identified by this analysis was the multifunctional cytokine IL-11. This observation was of significant interest, as IL-11 had previously been shown to be a potent inhibitor of adipocyte differentiation (Kawashima et al., 1991; Keller et al., 1993).

To confirm our initial microarray result, we performed quantitative RT-PCR and ELISA experiments to analyze the expression of IL-11 in 3T3-L1 cells induced to undergo adipocyte differentiation in the presence or absence of PGF2α. As shown in Figure 4, while 3T3-L1 preadipocytes induced to undergo adipocyte differentiation by the standard MDI-induced protocol expressed essentially undetectable levels of IL-11 at 48 h-post stimulation, significant levels of both IL-11 mRNA (Fig. 4A) and protein (Fig. 4B) were readily observed in cells induced to differentiate in the presence of PGF2α, an effect that was attenuated in the presence of the calcineurin-specific inhibitor cyclosporin A (CsA). In fact, we observed that treatment of non-differentiated 3T3-L1 cells with PGF2α alone was sufficient to induce IL-11 expression, although in this case the level of induction (~3-fold; data not shown) was significantly less than that observed in the presence of the MDI adipogenic cocktail (23-fold; see Fig. 4A). As IL-11 is a member of the IL-6 family of cytokines (Taga and Kishimoto, 1997), we also investigated whether PGF2α affected the expression of other family members during the differentiation process. In this respect, while our initial microarray data and subsequent
qRT-PCR analysis revealed that the expression of IL-6 itself was induced by PGF2α treatment, the level of induction observed (3.5-fold) was considerably lower compared to the effect on IL-11 (Fig. 4C), whereas no substantial effect of PGF2α on the expression of other members of this family was observed. Taken together, therefore, our results indicate that the activation of the PGF2α/calcineurin-signaling pathway synergizes with MDI-induced signals during the early stages of 3T3-L1 preadipocyte differentiation to specifically induce the robust expression and subsequent secretion of the IL-11 cytokine, which given the known anti-adipogenic effects of IL-11 (Kawashima et al., 1991; Keller et al., 1993), raised the possibility that PGF2α might act to inhibit adipogenesis by means of an IL-11 mediated autocrine mechanism.

5.2 IL-11 and gp130 cytokine co-receptor-dependent signaling play a role in mediating the inhibitory effects of PGF2α on adipocyte differentiation. Since IL-11 signaling is known to be critically dependent upon the actions of the gp130 cytokine co-receptor signaling subunit (Barton et al., 2000), we initially took advantage of a well-characterized dominant-negative form of gp130 (DN-gp130) (Kumanogoh et al., 1997) to examine the potential role of IL-11 in mediating the inhibitory effects of the PGF2α/calcineurin-signaling pathway on adipocyte differentiation. This dominant-negative gp130 mutant comprises the extracellular domain of gp130 known to be responsible for interacting with IL-11/IL-11Rα complexes, but lacks the cytoplasmic domain containing the conserved residues required for signal transduction. Consequently, ectopic expression of DN-gp130 should act to attenuate gp130-dependent signaling
FIGURE 5. gp130-dependent signaling play a role in mediating the inhibitory effects of PGF2α on adipocyte differentiation.

3T3-L1 preadipocytes transduced with either MSCV-GFP or MSCV-DN-gp130 retroviruses were induced to undergo differentiation with MDI in the presence of the indicated concentrations of PGF2α for the initial 48 h and the extent of adipocyte differentiation was determined at day 8 by either ORO staining (A) or immunoblotting...
for the expression of PPARγ and aP2 (B). Equal protein loading was confirmed by probing with a control antibody directed against ERK. Results are representative of at least three independent experiments.
events by sequestering IL-11 in non-functional complexes incapable of signaling. Thus, to initially establish the potential role of gp130 signaling in mediating the inhibitory effects of PGF2α on adipocyte differentiation, 3T3-L1 preadipocytes were transduced with either a control retrovirus or a retrovirus encoding DN-gp130, and then induced to undergo differentiation by the standard MDI-induced adipogenic protocol in the presence of various concentrations of PGF2α. As expected, the graded doses of PGF2α served to produce a potent dose-dependent inhibition of adipocyte differentiation in control cells, as measured by a decrease in cells staining positive for Oil Red O (ORO) (Fig. 5A) and the decreased expression of the adipocyte-specific genes PPARγ and aP2 (Fig. 5B). In contrast, we found that ectopic expression of DN-gp130 was able to greatly attenuate the inhibitory activity of PGF2α, allowing 3T3-L1 preadipocyte cells to differentiate in the presence of concentrations of PGF2α that were able to potently inhibit the differentiation of control cells (Figs 5A, B). This result demonstrates that inhibiting gp130-dependent signaling in 3T3-L1 preadipocytes is able to partially rescue adipocyte differentiation in cultures containing PGF2α, thereby suggesting a critical role for gp130 signaling in mediating the inhibitory effects of PGF2α on adipocyte differentiation.

While the gp130 cytokine co-receptor signaling subunit is an essential component of the IL-11 receptor (Barton et al., 2000), it is also known to be involved in mediating the signaling of other members of the IL-6 family of cytokines (Heinrich et al., 2003; Taga and Kishimoto, 1997). Consequently, to more directly assess the role of IL-11 in
FIGURE 6. **IL-11 play a role in mediating the inhibitory effects of PGF2α on adipocyte differentiation.**

(A, B) 3T3-L1 preadipocytes transduced with retroviruses encoding either control or IL-11-specific shRNA were induced to undergo differentiation with MDI in the absence or presence of PGF2α (12.5 nM) for the initial 48 h. After 8 days, the extent of adipocyte differentiation was assessed by ORO staining (A) or immunoblotting for the expression of PPARγ and aP2 (B). Equal protein loading was confirmed by probing with a control antibody directed against ERK. (C) The efficiency of knockdown of IL-11 mRNA was
determined by qRT-PCR using RNA isolated from PGF2α-treated cells at the 48 h time point. The data is shown as mean ± SEM (One-way ANOVA followed by Bonferroni post hoc test; p<0.05; n=3). Results are representative of at least three independent experiments.
mediating the inhibitory effects of PGF2α on adipocyte differentiation, we adopted a shRNA-mediated knockdown strategy in which 3T3-L1 preadipocytes were transduced with retroviruses encoding either a control shRNA or an shRNA-specific for IL-11, then subsequently examined for their ability to undergo adipocyte differentiation in the presence of an inhibitory concentration of PGF2α (12.5 nM). As expected, the ability of control shRNA-transduced 3T3-L1 preadipocytes to undergo adipocyte differentiation under these conditions was potently inhibited, as determined by both loss of ORO-staining cells and decreased expression of PPARγ and aP2 (Fig. 6A, B). Conversely, in IL-11 specific shRNA-transduced cultures induced to undergo differentiation in the presence of this same inhibitory concentration of PGF2α, we consistently observed the rescue of adipocyte differentiation, as indicated by the presence of significant patches of ORO-staining cells and the expression of both PPARγ and aP2 (Fig. 6A, B). In these experiments we found that the IL-11 specific shRNA was typically able to reduce the level of IL-11 induced in PGF2α-treated 3T3-L1 preadipocytes by approximately 70% compared to control shRNA-expressing cells (see Fig. 6C). These results therefore provide evidence that IL-11 likely plays an important role in mediating the inhibitory effects of the PGF2α/calcineurin-signaling pathway on adipocyte differentiation and are consistent with a model in which PGF2α-induces the initial expression and subsequent secretion of IL-11, which then acts in an autocrine/paracrine fashion to inhibit adipocyte differentiation by means of a gp130 cytokine co-receptor-dependent pathway.
FIGURE 7. Schematic model illustrating the chimeric EpoR/gp30 receptors used in this study.

The array of chimeric EpoR/gp130 receptors used in this study comprising of the extracellular ligand-binding domain of the EpoR (grey ovals) fused to various amino acid-substituted versions of the intracellular domain of gp130 (open rectangles) are shown. The position of the conserved tyrosine residues in the gp130 cytoplasmic domain and corresponding tyrosine to phenylalanine substitutions are indicated, as are the synthetic peptide sequences used for the selective activation of STAT1 and STAT3 in the EG-4F-STAT1 and EG-4F-STAT3 “add-back” mutants.
FIGURE 8. **gp130-dependent signaling is sufficient to inhibit adipocyte differentiation.**

3T3-L1 preadipocyte cells were initially infected with either MSCV-GFP or MSCV-EG-WT retroviruses then super-infected with either MSCV-DsRed or MSCV-DsRed-PPARγ retroviruses, as indicated. (A) The expression of EG-WT and PPARγ was confirmed by immunoblotting with antibodies specific for EpoR and PPARα, while equal protein loading was confirmed by immunoblotting with a control antibody directed against ERK. (B, C) Two-day post-confluent cells were then induced to differentiate with MDI in the presence or absence of Epo for the initial 48 h and the degree of adipocyte differentiation was assessed at day 8 post-stimulation by ORO staining (B) and immunoblotting for the expression of PPARγ and aP2 (C). Equal protein loading was confirmed by probing with a control antibody directed against ERK. Results are representative of more than three independent experiments.
5.3 gp130 cytokine co-receptor-dependent signaling is sufficient to inhibit adipocyte differentiation by blocking the expression of PPARγ. Next, we determined whether gp130 signaling per se was sufficient to inhibit adipocyte differentiation. For this experiment, we took advantage of a previously characterized chimeric form of gp130 in which the cytoplasmic signaling domain of gp130 is fused to the extracellular ligand-binding domain of the erythropoietin receptor (EpoR/gp130; EG-WT; see Fig. 7), which allows gp130-specific signaling events to be induced in response to stimulation with erythropoietin (Epo) (Schaper et al., 1998; Schmitz et al., 2000). As shown in Fig. 8, the presence of Epo in the culture medium had no effect on the ability of control GFP-expressing cells to undergo adipocyte differentiation, but conversely was able to potently inhibit the differentiation of cells ectopically expressing the EG-WT chimeric receptor, as measured by a decrease in both ORO staining and the expression of PPARγ and aP2 (Fig. 8B, C). Furthermore, we found that ectopic expression of PPARγ in cells expressing the EG-WT chimeric receptor was able to bypass the inhibitory effects of Epo on adipocyte differentiation in these cells (Fig. 8B, C). Taken together, these data indicate that gp130 signaling per se is sufficient to inhibit adipocyte differentiation, most likely by acting to block the expression of the pro-adipogenic master regulatory transcription factor, PPARγ.

5.4 C-terminal tyrosine residues involved in the activation of STAT transcription factors play a critical role in mediating the inhibitory effects of gp130 signaling on adipocyte differentiation. Signaling via gp130 is critically dependent upon conserved tyrosine residues located within the cytoplasmic domain, which are phosphorylated in
FIGURE 9. Tyrosine residues involved in STAT transcription factor activation play a role in mediating the inhibitory effects of gp130-dependent signaling on adipocyte differentiation.

(A) 3T3-L1 preadipocyte cells were infected with either MSCV-GFP, EG-WT, EG-1F, or EG-4F encoding retroviruses and the expression of each chimeric receptor was determined by immunoblotting with an antibody specific for EpoR, while equal protein loading was confirmed by immunoblotting with a control antibody directed against ERK.
(B) To confirm the ability of the chimeric receptors to activate STAT1 and STAT3, whole cell extracts from cells induced to differentiate with MDI in the presence of Epo were analyzed by immunoblotting at the indicated times with antibodies specific for pSTAT1-Y701, STAT1, pSTAT3-Y705, and STAT3. (C) To determine the activation of ERK by the chimeric receptors, whole cell extracts from 90% confluent cells treated with Epo in serum free medium were collected at the indicated time and analyzed by immunoblotting with antibodies specific for pERK and ERK. (D, E) Two-day post-confluent cells were induced to differentiate with MDI in the presence of Epo for the initial 48 h. After 8 days, adipocyte differentiation was assessed by ORO staining (C) and immunoblotting for the expression of PPARγ and aP2 (D). Results are representative of more than three independent experiments.
response to receptor activation and are responsible for recruiting cytoplasmic signaling molecules involved in the propagation of specific downstream signaling pathways (Heinrich et al., 2003; Taga and Kishimoto, 1997). IL-11 signaling mediated via the gp130 co-receptor signaling subunit is known to primarily activate two independent downstream effectors pathways: the ERK and JAK/STAT signaling pathways (Heinrich et al., 2003; Taga and Kishimoto, 1997). In order to ascertain which of these gp130-dependent signaling events were responsible for mediating the inhibition of adipocyte differentiation, we took advantage of previously well-characterized EpoR/gp130 chimeric mutants known to be selectively deficient in the activation of one or another of these signaling pathways (Schaper et al., 1998; Schmitz et al., 2000). Specifically, we utilized chimeric mutant receptors with either a Y759F substitution (EG-1F) that is known to be selectively deficient in the activation of the downstream ERK pathway, or a quadruple chimeric mutant receptor containing Y767F, Y814F, Y905F and Y915F substitutions (EG-4F) that is selectively deficient in the activation of the STAT1 and STAT3 latent transcription factors (see Fig. 7 for details). Each of these mutant receptors, along with EG-WT, was independently introduced into 3T3-L1 preadipocytes by retroviral transduction, and immunoblot analysis was performed to confirm that each receptor was expressed at comparable levels (Fig. 9A). Subsequently, cells were then induced to undergo adipocyte differentiation by the standard MDI-induced protocol in either the presence or absence of Epo, and the effects on differentiation were assessed. As shown in Figure 9D and E, the presence of Epo was able to inhibit the differentiation of EG-1F-expressing cells to essentially the same degree as that observed for cells expressing EG-
WT. By contrast, the presence of Epo had no effect on the ability of EG-4F expressing cells to differentiate. As shown in Figure 9B, immunoblot analysis confirmed that unlike EG-WT and EG-1F, the EG-4F receptor was deficient in its ability to efficiently activate STAT1 and STAT3 in response to Epo stimulation. Further, as shown in Figure 9C, unlike EG-4F, the EG-1F receptor was deficient in its ability to activate ERK. These data therefore demonstrate that the four C-terminal tyrosine residues present in the cytoplasmic domain of gp130 are required for the gp130-mediated inhibition of adipocyte differentiation. Moreover, since these latter tyrosine residues are required for the gp130-mediated activation of STAT1 and STAT3, the data further suggests a potentially important role for the activation of STAT transcription factors in mediating the inhibitory effects of gp130 co-receptor signaling on adipocyte differentiation.

5.5 A critical role for STAT1 in mediating the inhibitory effects of gp130, IL-11 and PGF2α on adipocyte differentiation. To further examine the potential role of STAT1 and STAT3, we next generated chimeric gp130 “add-back” receptors in which consensus peptide sequences previously reported to selectively activate either STAT1 or STAT3 (Gerhartz et al., 1996) were fused in-frame to the C-terminus of STAT signaling-deficient EG-4F mutant receptor, thereby generating EG-4F-STAT1 and EG-4F-STAT3, respectively (see Fig. 7). Each of these mutant “add-back” receptors, along with the parental EG-4F receptor, was independently expressed in 3T3-L1 preadipocytes (Fig. 10A) and their ability to activate STATs (Fig. 10B) and inhibit adipocyte differentiation (Fig. 10C, D) was assessed. As shown in Figure 10B, Epo-treatment of cells expressing
FIGURE 10. **gp130-dependent selective activation of STAT1, but not STAT3 inhibits adipocyte differentiation.**

(A) 3T3-L1 preadipocyte cells were infected with either EG-4F, EG-4F-STAT1 or EG-4F-STAT3 encoding retroviruses and their expression was assessed by immunoblotting the whole cell extracts with an antibody specific for EpoR, and ERK as a control for equal protein loading. (B) To determine the activation selectivity of the chimeric add-back receptors, the above cells were induced to differentiate with MDI in the presence of Epo and whole cell extracts prepared at the indicated times were analyzed by immunoblotting
with antibodies specific for pSTAT1-Y701, STAT1, pSTAT3-Y705 and STAT3. (C, D)
Two-day post-confluent cells from (A) were induced to differentiate with MDI in the
presence of Epo and after 8 days, the extent of adipocyte differentiation was assessed by
ORO staining (C) and immunoblotting for the expression of PPARγ and aP2 (D). Results
are representative of more than three independent experiments.
the EG-4F-STAT1 mutant receptor resulted in the preferential activation of STAT1, whereas Epo-stimulation of EG-4F-STAT3-expressing cells led to the preferential activation of STAT3. We note that although the EG-4F receptor is completely deficient in its ability to activate STAT1, it is still able to induce residual levels of STAT3 activation, albeit considerably reduced compared to EG-WT (see Fig 10B). Based upon analysis of mutant receptors lacking all cytoplasmic tyrosine residues, we believe that this effect is likely due to the ability of gp130-associated JAK2 to very inefficiently activate parallel gp130-independent pathways leading to STAT3 activation (data not shown). Nonetheless, it is clear from our data that EG-4F-STAT1 and EG-4F-STAT3 preferentially activate STAT1 and STAT3, respectively. When 3T3-L1 preadipocytes expressing these mutant receptors are induced to differentiate in the presence of Epo, we find that only those cells expressing EG-4F-STAT1 are blocked from undergoing adipogenesis, as measured by a decrease in both ORO staining (Fig. 10C) and the expression of PPARγ and aP2 (Fig. 10D). These data therefore suggest that it is the activation of the STAT1 transcription factor, rather than STAT3, that is associated with the ability of gp130 to inhibit adipocyte differentiation.

In order to further define the role of STAT1 as a critical anti-adipogenic effector of the gp130-signaling pathway, we next chose to determine whether STAT1 function was necessary for the ability of gp130-induced signaling to inhibit adipocyte differentiation. To accomplish this goal, we adopted a shRNA knockdown strategy using STAT1-specific shRNAs to stably deplete endogenous STAT1 levels in 3T3-L1 preadipocytes. By initially screening a panel of STAT1-specific shRNAs we were able to
FIGURE 11. STAT1-depletion rescues adipocyte differentiation from the EpoR/gp130-chimeric receptor signaling.

(A) 3T3-L1 preadipocyte cells transduced with retroviruses encoding either a control shRNA or one of two independent STAT1-specific shRNAs (924 & 927), were superinfected with a MSCV-EG-WT encoding retrovirus and whole cell extracts from these cells were analyzed by immunoblotting with antibodies specific for STAT1, EpoR and
STAT3 as a protein loading control and to show specificity of the shRNA knockdown. (B, C) These independent cell populations were then induced to differentiate with MDI in the presence of Epo for the initial 48 h and after 8 days, the extent of adipocyte differentiation was assessed by ORO staining (B) and immunoblotting for the expression of PPARγ and aP2 (C). Results are representative of two independent experiments.
FIGURE 12. **STAT1-depletion rescues adipocyte differentiation from the IL-11 cytokine.**

3T3-L1 preadipocyte cells transduced with either control shRNA or STAT1-specific shRNA (924 & 927) encoding retroviruses were induced to differentiate with MDI in the presence of IL-11 for the initial 48 h and the extent of adipocyte differentiation was assessed at day 8 by either ORO staining (A) or immunoblotting for the expression of PPARγ and aP2 (B). Results are representative of two independent experiments.
identify two independent shRNAs capable of efficiently and specifically depleting endogenous levels of STAT1 (Fig. 11A). 3T3-L1 preadipocytes expressing either of these two independent STAT1-specific shRNAs or a control shRNA were then each superinfected with a retrovirus encoding EG-WT and induced to undergo adipocyte differentiation in the presence of Epo. As expected, when control shRNA expressing cells were treated with Epo their differentiation was potently inhibited (Fig. 11B, C). In contrast, we found that the inhibitory effect of Epo was greatly attenuated in cells expressing either of the two STAT1-specific shRNAs (Fig. 11B, C). Similarly, we found that these two STAT1-specific shRNAs also greatly attenuated the ability of exogenous IL-11 to inhibit adipocyte differentiation (Fig. 12 A, B). It is noteworthy that in each case the degree of rescue caused by each of the STAT1-specific shRNAs was proportional to their degree of knockdown of endogenous STAT1 levels.

Next, we wished to examine the requirement for STAT1 as a downstream anti-adipogenic effector of the PGF2α/calcineurin-signaling pathway. As shown in Fig. 13A, B, while PGF2α effectively inhibited the differentiation of control shRNA-expressing cells as expected, we found that depletion of endogenous STAT1 levels with either of our STAT1-specific shRNAs allowed for significant rescue of adipogenesis in the presence of inhibitory concentrations of PGF2α capable of potently inhibiting control cells. Taken together, these data identify an important role for STAT1 as a critical downstream effector in mediating the inhibitory effects of PGF2α and IL-11/gp130-signaling on adipocyte differentiation.
FIGURE 13. STAT1-depletion rescues adipocyte differentiation from PGF2α.

3T3-L1 preadipocyte cells transduced with either control shRNA or STAT1-specific shRNA (924 & 927) encoding retroviruses were induced to differentiate with MDI in the
presence of increasing concentrations of PGF2α for the initial 48 h and the extent of adipocyte differentiation was assessed after 8 days by ORO staining (A) and immunoblotting for the expression of PPARγ and aP2 (B). Results are representative of at least five independent experiments.
5.6 STAT1 depletion enhances the efficiency of adipocyte differentiation: Evidence for an intrinsic negative regulatory role: In addition to the well-known anti-adipogenic effects of exogenous PGF2α, prior studies have revealed that PGF2α also serves as an important endogenous negative regulator of adipogenesis that is naturally synthesized transiently by preadipocytes early during the normal differentiation process, and acts in an autocrine-mediated, negative feedback loop to restrict the overall adipogenic capacity of these cells (Fujimori et al., 2010a; Fujimori et al., 2010b; Silvestri et al., 2013). This led us to hypothesize that this endogenous PGF2α-mediated negative feedback pathway is also likely to involve the calcineurin/IL-11/gp130/STAT1 signaling axis that we have described above. Consistent with this notion we find that 3T3-L1 preadipocyte cells induced to differentiate by the standard MDI-induced adipogenic protocol transiently secrete low levels of IL-11 early during the differentiation process, peaking at 24 hrs post-induction and falling to background levels by 48 hrs (Fig. 14A). Moreover, we find that this increase in IL-11 secretion is inhibited in the presence of CsA, indicating that it is dependent upon the activation of calcineurin, which is presumably induced in response to endogenously produced PGF2α. It is noteworthy, however, that the level of IL-11 secretion detected here during normal adipocyte differentiation is considerably less than that induced in response to exogenous PGF2α (0.278 pg/ml versus 11.6 ng/ml).

Having demonstrated that IL-11 is naturally produced during the normal process of adipocyte differentiation, we next sought to determine whether the IL-11/gp130/STAT1 signaling axis served as an intrinsic negative regulator of the adipocyte differentiation pathway designed to restrict the adipogenic potential of preadipocyte cells.
FIGURE 14. STAT1-depletion enhances the efficiency of adipocyte differentiation in response to suboptimal adipogenic stimulation.
(A) 3T3-L1 cells were induced to differentiate by the standard MDI-induced protocol in the presence and absence of CsA and culture supernatants were assayed after 24 h for the presence of IL-11 by ELISA, (B, C) 3T3-L1 preadipocyte cells transduced with either control shRNA or STAT1-specific shRNA (924) encoding retroviruses were exposed to a suboptimal adipogenic cocktail comprising of decreasing serial dilutions of MD alone in the absence of exogenously added insulin. After 8 days, the extent of adipocyte differentiation was assessed by ORO staining (B) and immunoblotting for the expression of PPARγ and aP2 (C). The extent of STAT1 depletion was confirmed by immunoblotting for STAT1, while immunoblot analysis of STAT3 served as a control for knockdown specificity and equal protein loading. The data is shown as mean ± SEM (Student’s $t$ test; $p<0.05$; $n=3$). ND; not detectable. Results are representative of three independent experiments.
We reasoned that if this were the case, then inhibition of this pathway should enhance the
efficiency of the differentiation process. For these experiments we took advantage of the
observation that treatment of 3T3-L1 preadipocytes with suboptimal adipogenic stimuli
(reduced concentrations of Mix and Dex in the absence of insulin) results in barely
detectable levels of adipocyte differentiation (Fig. 14B, C). However, in contrast, we find
that when the integrity of the IL-11/gp130/STAT1 signaling pathway is abrogated by
depletion of endogenous STAT1 proteins, a significant degree of adipocyte
differentiation is observed in response to these suboptimal doses of adipogenic stimuli
(Fig 14B, C). In fact, we find that even in the presence of optimal doses of adipogenic
stimuli, we consistently observe enhanced levels of adipocyte differentiation (as indicated
by increased ORO staining and expression of the adipogenic marker proteins, PPARγ and
aP2), in cells in which the IL-11/gp130/STAT1 signaling axis has been inhibited, either
as a result of STAT1 knockdown or via ectopic expression of DN-gp130 (see Fig. 5 and
13). Hence, these results strongly suggest that the IL-11/gp130/STAT1 signaling pathway
does indeed represent an intrinsic negative regulatory pathway that serves to restrict the
adipogenic potential of preadipocyte cells.

6. DISCUSSION
Recent studies identifying an emerging in vivo role for PGF2α as an important
endogenous negative regulator of adipocyte differentiation involved in opposing the
development of obesity and its contingent pathological metabolic sequelae (Volat et al.,
2012), have raised considerable interest in elucidating the molecular pathways and
mechanisms by which this important prostaglandin mediates its inhibitory effects on adipogenesis. Building upon our previous identification of calcineurin as a critical downstream anti-adipogenic effector of PGF2α (Liu and Clipstone, 2007), we have now further delineated the molecular mechanism by which this signaling pathway inhibits adipocyte differentiation. Collectively, our findings support a model in which exposure of differentiating 3T3-L1 preadipocytes to PGF2α results in the initial calcineurin-dependent expression and secretion of the IL-11 cytokine, which then acts in an autocrine/paracrine fashion to inhibit adipocyte differentiation via the essential actions of the gp130 cytokine co-receptor signaling subunit and the subsequent activation of the STAT1 transcription factor.

Our results clearly identify IL-11 as a downstream transcriptional target of the PGF2α/calcineurin-signaling pathway in 3T3-L1 preadipocytes. Interestingly, this same pathway has also recently been shown to induce IL-11 expression in endometrial adenocarcinoma cells via the calcineurin-dependent activation of the nuclear factor of activated T cells (NFAT) transcription factor family (Sales et al., 2010). By analogy with this finding, together with the knowledge that NFAT proteins are expressed in 3T3-L1 preadipocytes (Ho et al., 1998), it is tempting to speculate that NFAT proteins are also likely to play a role in coupling the PGF2α/calcineurin-signaling pathway to the expression of IL-11 in these cells. However, the recent demonstration that IL-11 can be transcriptionally regulated by the hypoxia-inducible factor 1 transcription factor (Onnis et al., 2013), which we have previously shown is activated in 3T3-L1 cells in response to
PGF2α stimulation (Liu and Clipstone, 2008), raises the possibility that this pathway may also play a role.

IL-11 has long been known to be a potent inhibitor of adipocyte differentiation (Kawashima et al., 1991; Keller et al., 1993). Moreover, 3T3-L1 preadipocytes are known to express functional IL-11 receptors (Tenney et al., 2005). Hence, our observation that activation of the PGF2α/calcineurin-signaling pathway in 3T3-L1 preadipocytes induces the expression and subsequent secretion of IL-11, strongly suggested a model in which PGF2α inhibits adipocyte differentiation by means of an autocrine IL-11-mediated negative regulatory pathway. Evidence in support of this model is provided by our dual observation that both the shRNA-mediated knockdown of IL-11 and the dominant-negative inhibition of the gp130 cytokine co-receptor signaling subunit, an essential signaling component of the IL-11 receptor, are both able to rescue 3T3-L1 preadipocyte differentiation in the presence of inhibitory concentrations of PGF2α. Together, these results demonstrate that the ability of PGF2α to inhibit adipocyte differentiation is dependent upon both IL-11 and gp130 cytokine co-receptor signaling. In this regard, although the DN-gp130 mutant used in our experiments is capable of inhibiting IL-11 signaling, it can also inhibit signaling via all other members of the IL-6 cytokine family, whose receptors all share the same gp130 cytokine co-receptor signaling subunit (Heinrich et al., 2003; Taga and Kishimoto, 1997). However, of this family of cytokines, we find that only IL-11 and, to a somewhat lesser extent, IL-6 (Fig.4), is significantly induced by the PGF2α/calcineurin-signaling pathway in differentiating 3T3-L1 preadipocytes. Hence, based upon our collective data, we conclude that IL-11, acting
in an autocrine fashion via a critical gp130 cytokine co-receptor-dependent mechanism, most likely plays the prominent role in mediating the inhibitory effects of the PGF2α/calcineurin-signaling pathway on adipocyte differentiation, although we do not rule out the possibility that IL-6, and potentially other related family members, may also additionally contribute.

Having identified a clear role for the gp130-signaling axis in mediating the inhibitory effects of the PGF2α/calcineurin-signaling pathway on adipocyte differentiation, and having further showed that the activation of gp130-dependent signaling per se is sufficient to inhibit the adipogenic process, our attention naturally focused towards investigating the underlying gp130-dependent mechanisms involved. In this respect, signaling via the gp130 cytokine co-receptor signaling subunit has been extensively characterized and is known to primarily involve the activation of both the STAT1 and STAT3 latent transcription factors and the MAPK signaling pathway (Heinrich et al., 2003). By turning to previously characterized chimeric mutants of gp130 known to be selectively deficient in the activation of either one of these latter pathways (Schaper et al., 1998; Schmitz et al., 2000), we were able to interrogate their respective roles in mediating the inhibitory effects of gp130 signaling on adipogenesis. Surprisingly, although the MAPK signaling pathway has previously been shown to inhibit adipocyte differentiation in certain contexts (Font de Mora et al., 1997; Tanabe et al., 2004), we found that tyrosine 759, responsible for coupling gp130 to the activation of MAPK, was completely dispensable for the inhibitory effects of gp130 signaling. Conversely, we found that the ability of gp130 to inhibit adipocyte differentiation was critically
dependent upon the conserved tyrosine residues known to be involved in the activation of STAT1 and STAT3, suggesting a potentially important role for STAT transcription factors. By adopting the use of additional gp130 mutant receptors that are preferentially able to activate either STAT1 or STAT3 (Gerhartz et al., 1996), we were able to demonstrate that the gp130-mediated inhibition of adipogenesis correlated most closely with the activation of STAT1, but not STAT3. More significantly, we found that the shRNA-mediated depletion of endogenous STAT1 protein served to largely attenuate the inhibitory effects of either IL-11 or chimeric EpoR/gp130 receptor signaling on 3T3-L1 preadipocyte differentiation, as well as significantly rescue adipogenesis in the presence of PGF2α. Taken together, therefore, our data provides evidence that STAT1 plays an important role in mediating the inhibitory effects of the PGF2α/calcineurin-signaling pathway on adipogenesis, where it appears to serve as a critical negative regulatory effector downstream of gp130 receptor complexes to couple PGF2α/calcineurin-induced autocrine IL-11 cytokine signaling-events to the inhibition of adipocyte differentiation (see Fig. 15).

Our identification of STAT1 as a negative regulator of adipocyte differentiation is consistent with prior studies that have suggested a potential role for STAT1 in mediating the anti-adipogenic effects of IFNγ (McGillicuddy et al., 2009; Waite et al., 2001). Interestingly, in contrast to the anti-adipogenic role of STAT1, other members of the STAT transcription factor family, namely STAT3 and STAT5 (Richard and Stephens, 2011), have conversely been reported to play pro-adipogenic roles. In this regard, STAT3 has been proposed to play a role in promoting the expression of the early transcription
factor C/EBPβ and is believed to play a critical role in the mitotic clonal expansion phase of adipogenesis (Deng et al., 2000; Zhang et al., 2011), whereas STAT5 has been reported to make a more direct contribution towards adipocyte differentiation by directly helping promote the expression of PPARγ (Kawai et al., 2007; Nanbu-Wakao et al., 2002). STAT proteins therefore play complex, diverse and opposing roles in the regulation of adipocyte differentiation. By virtue of their established regulatory roles and specific activation in response to diverse ligand-activated plasma membrane receptors (Levy and Darnell, 2002; Levy and Darnell, 2002), STAT proteins offer the opportunity to modulate and fine-tune the efficiency of the adipogenic process in response to changing extracellular cues. However, exactly how the relative activities of these related transcription factors are integrated at the level of the transcriptome to influence the adipogenic cell fate decision remains to be determined.

Mechanistically, it is currently unclear how STAT1 activation results in the inhibition of adipocyte differentiation. However, we believe that STAT1 likely acts at a point proximal to the expression of the PPARγ master adipogenic transcription factor, as our data shows that gp130 signaling is sufficient to attenuate the expression of PPARγ in a STAT1-dependent manner, whereas ectopic expression of PPARγ2 is able to readily bypass the inhibitory effects of gp130-mediated signaling (see Fig. 8). While STAT1 is commonly thought to primarily play a role as a positively acting transcription factor, it is evident that it can also act to inhibit transcriptional events in certain biological contexts (Furukawa et al., 2009; Liu et al., 2008; Ramana et al., 2000). Hence, we envision that STAT1 may contribute towards the inhibition of adipocyte differentiation via either of
two non-mutually exclusive mechanisms. On the one hand, the most direct mechanism by which STAT1 may inhibit adipogenesis is via the direct repression of a critical pro-adipogenic gene(s) that is required for adipogenesis. In this regard, it is perhaps noteworthy that a nucleotide region capable of specifically binding STAT1 in vitro has previously been identified in the immediate upstream regulatory region of the murine PPARγ2 promoter and has been proposed to play a negative regulatory role in the expression of this critical pro-adipogenic transcription factor (Hogan and Stephens, 2001), thereby raising the possibility that STAT1 may inhibit adipogenesis by directly blocking the expression of PPARγ itself. Alternatively, STAT1 may contribute to the inhibition of adipocyte differentiation via a more indirect mechanism in which STAT1 is responsible for inducing the expression of a distinct anti-adipogenic gene(s) that is in turn itself responsible for inhibiting the adipogenic process. In this respect, the increased expression of many genes and microRNAs are known to be capable of inhibiting adipogenesis by preventing expression of PPARγ (McGregor and Choi, 2011; Rosen and MacDougald, 2006), although whether any of these known inhibitors act as functional downstream anti-adipogenic effectors of STAT1 remains to be determined. Consequently, the respective roles of either of these two non-mutually exclusive pathways in mediating the STAT1-dependent inhibition of adipocyte differentiation and the precise molecular mechanism(s) involved await further investigation.

Finally, while PGF2α has long been known to be a potent exogenous inhibitor of adipocyte differentiation in vitro (Casimir et al., 1996; Lepak and Serrero, 1993; Liu and Clipstone, 2007; Miller et al., 1996; Serrero et al., 1992), more recent data has
FIGURE 15. Schematic model illustrating the proposed mechanism by which PGF2α inhibits adipogenesis.

The PGF2α/calcineurin-signaling pathway induces IL-11 expression, which in turn binds to the non-signaling IL-11Rα receptor subunit, recruits the gp130 co-receptor signaling subunit and then, activates STAT1 transcription factor. It is the activation of STAT1 transcription factor that mediates the inhibitory effects of PGF2α on adipogenesis.
highlighted an emerging *in vivo* role for PGF2α as an important endogenous negative regulator of adipogenesis that is naturally synthesized by preadipocytes early during the normal differentiation process, and appears to participate in an autocrine-mediated, negative feedback loop that acts to restrict the overall adipogenic capacity of these cells (Fujimori et al., 2010a; Fujimori et al., 2010b; Silvestri et al., 2013). We believe that this latter negative feedback loop likely proceeds, at least in part, via the calcineurin/IL-11/gp130/STAT1-mediated pathway that we have described here. In support of this notion, we find that low levels of IL-11 are secreted in a calcineurin-dependent fashion during the normal MDI-induced differentiation of 3T3-L1 preadipocytes, presumably in response to the autocrine action of elevated levels of endogenously produced PGF2α. Furthermore, we find that inhibition of the endogenous calcineurin/IL-11/gp130/STAT1 signaling pathway results in enhanced differentiation of 3T3-L1 preadipocytes, especially under conditions of suboptimal adipogenic stimulation (Fig. 5 and Fig. 14). These results therefore argue that this pathway represents an intrinsic negative regulatory feedback mechanism that serves to restrict the differentiation of preadipocyte cells and thereby plays a major role in determining the efficiency of the adipogenic process. Given the fact that adipocyte-derived PGF2α production has been implicated as an important negative regulator of adipose tissue expansion *in vivo*, involved in opposing the development of obesity and its attendant diseases (Volat et al., 2012), it is tempting to speculate that these effects, at least in part, are also mediated by this same calcineurin/IL-11/gp130/STAT1-signaling module.
CHAPTER IV

THE PGF2α/CALCINEURIN-SIGNALING PATHWAY UPREGULATES THE EXPRESSION OF THE ORPHAN NUCLEAR HORMONE RECEPTOR NURR1 THROUGH A CRTC/CREB-DEPENDENT MECHANISM: A POTENTIAL ROLE IN INHIBITING ADIPOCYTE DIFFERENTIATION

1. Introduction: In addition to the increased expression of the IL-11 cytokine documented in the previous chapter, our microarray analysis revealed that the PGF2α/calcineurin-signaling pathway also highly induced the expression of an orphan nuclear hormone receptor, Nurr1 which is a member of the NR4A family of nuclear hormone receptors which also includes Nur77 and Nor1. This was of interest as the members of this family had previously been implicated in the negative regulation of adipocyte differentiation. However, the role of Nurr1 in the PGF2α-mediated inhibition of adipogenesis had not previously been investigated.

2. Nurr1 and its family members (NR4A Family):

2.1 Expression and functions of NR4A family members: Nurr1 and its related family members are considered to be immediate early genes and are rapidly induced by a wide variety of physiological stimuli, including cAMP, phorbol esters, prostaglandins, calcium, fatty acids, stress and growth factors (Maxwell and Muscat, 2006; Pearen and Muscat, 2010). They are expressed in multiple tissues including adipose tissue, skeletal muscle, kidney, heart, T-cells, macrophages, liver and brain.
(Maxwell and Muscat, 2006; Pearen and Muscat, 2010). They are known to play a diverse role in the regulation of cellular and physiological functions including a critical role in metabolism, inflammation and vascular remodeling (McMorrow and Murphy, 2011; Zhao and Bruemmer, 2010). Importantly, Nurr1 is critical for the development of dopaminergic neurons (Zetterstrom et al., 1997) and mutations in Nurr1 have been linked to the development of Parkinson’s disease (Le et al., 2003; Sirin et al., 2010). Nurr1 has also been shown to inhibit the proliferation of haematopoietic stem cells (Sirin et al., 2010).

2.2 Role of Nurr1 and its family members in adipogenesis: While these receptors are expressed at very low to undetectable levels in undifferentiated 3T3-L1 preadipocytes, they are rapidly and transiently induced by MDI stimulation, reaching a peak level within the first 4 h of the differentiation process, which then decreases to the basal level within 16 to 24 h (Fu et al., 2005; Soukas et al., 2001). Despite their rapid expression after adipogenic stimulation, their exact role in normal adipogenic process is not known. However, the over expression of Nurr1 and its family members are shown to inhibit adipocyte differentiation (Chao et al., 2008). Conversely, Au et al (2008) suggested that NR4A receptors are not required for adipogenesis, as the expression of a well characterized-dominant-negative Nur77 that is known to block the function of all three members Nur77, Nurr1 and Nor1 did not block adipogenesis (Au et al., 2008). While we find that Nurr1 is highly induced by PGF2α during adipogenesis, its exact role in mediating the inhibitory effects of PGF2α on adipogenesis is not known.
2.3 Structure and regulation of Nurr1 activity: Like other nuclear hormone receptors, NR4A family members contain a highly conserved central DNA binding domain, an N-terminal ligand-independent, AF-1 transactivation domain and a C-terminal ligand-binding, AF-2 transactivation domain (Maruyama et al., 1998). In contrast to other nuclear hormone receptors, the crystal structure of Nurr1 shows that its ligand-binding pocket is filled with bulky hydrophobic side chains and thus, it cannot be regulated by a ligand (Flaig et al., 2005; Wang et al., 2003). In fact, the crystal structures show that Nurr1 assumes the conformation of an active receptor even in the absence of a ligand (Wang et al., 2003). Considering this observation, it seems that Nurr1 functions as a constitutively active, ligand-independent receptor and its activity is primarily dependent on its expression and posttranslational modifications. In fact, the mitogen-activated protein kinase (ERK1, 2/ERK5) has been shown to positively regulate the transcriptional activity of Nurr1 via phosphorylation of specific amino acids in the N-terminal AF-1 region, whereas LIM kinase 1 has been shown to negatively regulate Nurr1 transcriptional activity (Sacchetti et al., 2006). In addition, Nurr1 protein level has been shown to be regulated by the ubiquitin-proteasome pathway via its N-terminal region. Thus, the transcriptional activity of Nurr1 is regulated by multiple mechanisms (Alvarez-Castelao et al., 2013).
FIGURE 16. Schematic model showing the structure of Nurr1 and the modes of regulation of gene expression by Nurr1.
(A) Nurr1 contains an N-terminal AF-1 domain, a central DNA binding domain and a C-terminal AF-2 domain. (B-D) Nurr1 directly binds to DNA and regulates gene expression as either monomer, homodimer or as a heterodimer with RxR. (E) Nurr1 indirectly inhibits gene expression through interacting with the DNA bound NF-κB and recruitment of co-repressor, CoREST.
2.4 Regulation of gene expression by Nurr1 and its family members: NR4A family members regulate gene expression as either monomers or homodimers by binding to canonical DNA binding site called the nerve growth factor-induced protein B-responsive element (NBRE) (Fig. 16B, C) (Maira et al., 1999; Paulsen et al., 1995; Philips et al., 1997). Further, Nurr1 and Nur77 also heterodimerize with the retinoid X receptor (RXR) and induce gene transcription by binding to DNA response elements composed of direct repeats of consensus nuclear hormone response elements spaced by five nucleotides called DR5 (Fig. 16D) (Perlmann and Jansson, 1995; Zetterstrom et al., 1997). While Nurr1 has previously been shown to act as a positive transcription factor by inducing gene expression via direct DNA binding to promoter regions (Davies et al., 2005; Lammi et al., 2004; Sakurada et al., 1999), it is also clear that Nurr1 is able to repress the expression of certain other genes (Kim et al., 2013; Kim et al., 2013; Mix et al., 2007; Wu et al., 2005). In addition, the effects of Nurr1 do not always require it to directly bind to DNA, as recent studies have indicated that Nurr1 can repress transcription of certain genes indirectly by forming a complex with DNA-bound NF-κB and recruitment of CoREST co-repressor complex (Fig. 16E) (Saijo et al., 2009).

2.5 Regulation of Nurr1 expression by the calcineurin phosphatase and the CRTC2/CREB transcriptional complex: The Nurr1 promoter contains a consensus cAMP response element (CRE) and has been shown to be regulated by the CREB transcription factor in multiple cell types (Ji et al., 2012; McEvoy et al., 2002; Zhao et al., 2011). Interestingly, CREB-induced Nurr1 expression is selectively regulated by the
CRTC2 co-activator (Conkright et al., 2003). More importantly, CRTC2 is activated by a cAMP and calcineurin-dependent mechanism (Screaton et al., 2004), as described later in this chapter and Nurr1 expression is widely used as a marker to determine the activation and the transcriptional regulation of CRTC2 (Conkright et al., 2003). In addition, a recent study using hippocampal neurons shows that Nurr1 expression is regulated by an increase in intracellular calcium through voltage-gated calcium channel and subsequent activation of the calcium-dependent phosphatase, calcineurin (Tokuoka et al., 2014). Thus, calcineurin appears to play a crucial role in the upregulation of Nurr1 expression in multiple cell types.

3. CREB transcription factor: CREB belongs to the basic leucine zipper (bZIP) family of transcription factors and is ubiquitously expressed in multiple tissues (Carlezon et al., 2005; Shaywitz and Greenberg, 1999). It is activated by a diverse array of extracellular stimuli including peptide hormones, growth factors, osmotic stress, ultraviolet irradiation and neuronal activity (Carlezon et al., 2005; Shaywitz and Greenberg, 1999). The transcriptional activity of CREB is regulated by the phosphorylation of serine 133 by different serine-threonine kinases including cAMP-dependent protein kinase A (PKA), protein kinase C (PKC), calmodulin kinases (CaMKs) and ribosomal S6 kinase (Gonzalez and Montminy, 1989; Gubina et al., 2001; Seamon et al., 1981; Sheng et al., 1991; Xing et al., 1996). Once CREB is phosphorylated on serine 133, it dimerizes, binds to its co-activators, CREB-binding protein (CBP) or p300 (Arias et al., 1994; Chrviva et al., 1993; Kwok et al., 1994) and, then induces the expression of its target genes through
binding to CREB-responsive element (CRE), which consist of either palindromic (5’-TGACGTCA-3’) or half-site (TGACG or CGTCA) sequences (Comb et al., 1986; Montminy et al., 1986).

CREB plays a diverse role in the regulation of various physiological and pathological process including cell differentiation, glucose and lipid metabolism in liver, immune function, cell survival in neurons and pancreatic β-cells, consolidation of memory, cancer progression and others (Carlezon et al., 2005; Herzig et al., 2001; Herzig et al., 2003; Hong et al., 2005; Pittenger et al., 2002; Wen et al., 2010). More importantly, CREB is constitutively expressed in preadipocytes (Reusch et al., 2000) and plays multiple roles during adipocyte differentiation by regulating the expression of several genes involved in adipogenesis, which is discussed in the chapter I of this dissertation.

4. CRTC family of transcriptional co-activators: The CRTC proteins are a family of recently identified co-activators of the CREB transcription factor and consist of three members: CRTC1, CRTC2 and CRTC3 (Conkright et al., 2003; Iourgenko et al., 2003). CRTC1 is highly expressed in brain, whereas CRTC2 and CRTC3 are abundantly expressed in multiple tissues (Conkright et al., 2003). Under basal conditions, CRTCs are highly phosphorylated and retained in the cytoplasm by interactions with 14-3-3 adaptor protein (Screaton et al., 2004). Specifically, CRTC2 is highly phosphorylated at serine 171 and 275 by salt inducible kinase (SIK1) and MARK1, respectively (Screaton et al., 2004). The phosphorylation of these residues form docking site for 14-3-3 adaptor proteins, thereby CRTC2 is retained in the cytoplasm in an inactive form. However, when
FIGURE 17. Schematic model showing the activation of the CRTC2 co-activator.

Under basal conditions, CRTC2 is highly phosphorylated and retained in the cytoplasm by the 14-3-3 adaptor protein. Upon stimulation with appropriate stimuli, CRTC2 is dephosphorylated by a cAMP and calcineurin-dependent mechanism and subsequently, translocates into the nucleus, where it interacts with CREB and enhances the transcriptional activity of the CREB.
the cells are stimulated with appropriate stimuli, SIK1 is inactivated by PKA-dependent inhibitory phosphorylation, whereas calcineurin dephosphorylates serine 275 (Screaton et al., 2004). The dephosphorylation of CRTC2 by these two mechanisms releases it from 14-3-3 adaptor proteins, resulting in the translocation of CRTC2 to nucleus where it binds to the bZIP domain of CREB and promote the transcriptional activity of CREB (Fig. 17) (Screaton et al., 2004). In fact, CRTCs do not modulate the DNA binding ability of CREB, but rather enhance the transcriptional activity of CREB by augmenting it’s interaction with the TAFII130 component of transcription factor II D (TFIID) (Conkright et al., 2003). More importantly, CRTCs regulate the transcriptional activity of CREB in a canonical serine 133 phosphorylation-independent mechanism. The activity of CRTC2 is terminated by the prolyl-isomerase Pin1, which interacts with serine 136 in the nuclear localization signal of CRTC2 and promotes its export from the nucleus to cytosol (Nakatsu et al., 2010).

CRTCs are known to regulate a variety of biological processes. CRTC1 regulates satiety, circadian clock, synaptic plasticity and memory in the brain (Sakamoto et al., 2013; Sekeres et al., 2012; Zhou et al., 2006), whereas CRTC2 and CRTC3 are primarily involved in the regulation of metabolism in the liver and adipose tissue (Dentin et al., 2007; Song et al., 2010; Wang et al., 2012). In the liver, CRTC2 is highly phosphorylated in response to insulin, resulting in its nuclear exclusion and degradation (Dentin et al., 2007), whereas glucagon causes dephosphorylation and nuclear translocation of CRTC2, resulting in the expression of gluconeogenic genes (Wang et al., 2012). CRTC3 has been shown to attenuate the β-adrenergic receptor signaling in adipose tissue by upregulating
the expression of regulator of G-protein signaling 2 (Rgs2) (Song et al., 2010). While the CRTC proteins are shown to play a diverse role in multiple tissues, their role in adipocyte differentiation is not known.

In this chapter, we investigated the molecular mechanism by which the PGF2α/calcineurin-signaling pathway induces Nurr1 expression during adipocyte differentiation and the functional significance of increased Nurr1 expression in mediating the inhibitory effect on adipogenesis. First, we show that PGF2α upregulates the expression Nurr1 in a calcineurin-dependent fashion. Next, we show that the expression of a dominant-negative Nurr1 partially rescues adipogenesis from the inhibitory effects of PGF2α. Then, using a panel of Nurr1 mutants, we demonstrate that the direct DNA binding of Nurr1, but not its heterodimerization with RxR is required to inhibit adipogenesis. In addition, using a panel of CREB mutants and the shRNA approach, we demonstrate that PGF2α-mediated activation of the CRTC/CREB transcriptional complex induces Nurr1 expression. Collectively, these results demonstrate that the PGF2α/calcineurin-signaling pathway inhibits adipogenesis by inducing the expression of Nurr1 through activation of the CRTC/CREB-transcriptional complex.

5. RESULTS

5.1 The PGF2α/calcineurin-signaling pathway induces Nurr1 expression in differentiating 3T3-L1 preadipocytes: Our previous studies showed that PGF2α inhibits adipocyte differentiation through a calcium-dependent phosphatase calcineurin (Liu and Clipstone, 2007), which is well-known to regulate gene expression through
Two-day post confluent 3T3-L1 preadipocytes were induced to undergo adipocyte differentiation by treatment with MDI in the presence of either vehicle, PGF2α (25 nM) alone or PGF2α (25 nM) plus cyclosporine A (CsA) (1 µg/ml). At 48 h post-stimulation, total RNA and total cell lysates were collected. (A) The expression of Nurr1 and Nur77 was determined by qRT-PCR and (B) Nurr1 expression was determined by immunoblotting. The data is shown as mean ± SEM (one-way ANOVA followed by Bonferroni post hoc test; p<0.05; n=3). Results are representative of at least three independent experiments.
activation of various transcriptional regulators. As previously discussed, we performed an initial microarray experiment to identify the potential downstream effectors of the PGF2α/calcineurin-signaling pathway during adipogenesis. In addition to the IL-11 cytokine discussed in the chapter III, our analysis also identified an orphan nuclear hormone receptor, Nurr1 as one of the most highly induced genes by the PGF2α/calcineurin-signaling pathway during adipogenesis. This was of interest, as Nurr1 and its family members are known to play a role in the regulation of cell differentiation and development, and their over expression has previously been shown to inhibit adipocyte differentiation (Chao et al., 2008).

To confirm the initial microarray data, 3T3-L1 cells were induced to differentiate with the MDI-induced differentiation protocol in the presence or absence of PGF2α or pretreatment with the calcineurin inhibitor, CsA. At 48 h post-induction, mRNA and protein were collected for qRT-PCR and immunoblotting, respectively. During adipogenesis, Nurr1 expression was undetectable with MDI alone, whereas PGF2α significantly induced Nurr1 expression at both the mRNA and protein level at 48 h post-stimulation (Fig. 18A, B). Importantly, the PGF2α-induced Nurr1 expression was attenuated to the basal level with CsA pretreatment, which indicates a role of calcineurin in mediating the PGF2α-induced Nurr1 expression during adipogenesis. Interestingly, another family member Nur77 was not significantly induced by PGF2α and Nor1 was undetectable with four different primer pairs. Collectively, these results demonstrate that PGF2α selectively upregulates Nurr1 expression through the activation of the calcineurin phosphatase.
5.2 Nurr1 plays a potential role in mediating the inhibitory effects of PGF2α on adipocyte differentiation: Having shown that PGF2α induces Nurr1 expression during adipogenesis, we next determined whether Nurr1 is necessary for PGF2α to inhibit adipocyte differentiation. To accomplish this, we initially tried to stably knockdown the expression of Nurr1 in 3T3-L1 cells using shRNA approach. However, our attempts to deplete the expression of Nurr1 by several shRNAs were unsuccessful. Hence, we took advantage of a dominant-negative mutant that is known to inhibit the activity of all three members of NR4A2 family of orphan nuclear receptors. This dominant-negative mutant contains the DNA binding domain of Nurr1 fused with the transcriptional repressor domain of the KRAB repressor (DN-Nurr1; Fig. 19A). First, we determined whether this DN-Nurr1 is functional by transient transfection luciferase reporter gene assay in HEK293T cells. As described in the materials and methods, we transfected these cells with equal concentrations of plasmid encoding wild-type Nurr1 (Nurr1-WT) and increasing concentrations of DN-Nurr1 plasmids. Simultaneously, the cells were also transfected with the Nurr1 reporter, (NBRE)3-TK-LUC and pRG-TK reporter plasmids. At 24 h post-transfection, cell lysates were prepared and the luciferase bioluminescence was determined. As shown in Figure 19B, Nurr1-WT expression showed a maximal luciferase activity, whereas there was a dose-dependent inhibition of Nurr1-WT activity by DN-Nurr1 as measured by the luciferase activity. This demonstrates that the DN-Nurr1 is functional and it inhibits the transcriptional activity of the wild-type Nurr1.
FIGURE 19. Evidence that Nurr1 potentially plays a role in mediating the inhibitory effects of PGF2α on adipogenesis.

(A) Schematic model illustrating the dominant-negative Nurr1 (DN-Nurr1). (B) HEK293T cells were transfected with Nurr1-WT (100 ng) and increasing concentrations of DN-Nurr1 (0, 100, 200 and 400 ng) along with (NBRE)3-TK-Luc (50 ng) and pRG-TK (25 ng) reporter plasmids. At 24 h post-transfection, luciferase assay was performed and the percent maximal luciferase activity was calculated. (C, D) 3T3-L1 preadipocytes transduced with either MSCV-GFP or DN-Nurr1 encoding retroviruses were induced to differentiate with MDI in the presence of the indicated concentrations of PGF2α for the initial 48 h and the extent of adipogenesis was determined at day 8 by either ORO staining (C) or immunoblotting for the expression of PPARγ and ERK as a control for equal loading (D). Results are representative of three independent experiments.
Next, to determine whether Nurr1 is involved in the PGF2α-mediated inhibition of adipocyte differentiation, we tested whether the ectopic expression of the DN-Nurr1 mutant was able to rescue cells from the inhibitory effects of PGF2α on adipogenesis. We expressed either MSCV-GFP or DN-Nurr1 in 3T3-L1 cells using retroviruses and induced these cells to differentiate by the MDI-induced differentiation protocol in the presence of increasing concentrations of PGF2α. As shown in Figure 19C, D, the differentiation of the control GFP-expressing cells was inhibited by PGF2α in a concentration-dependent fashion as evidenced by the decreased ORO staining and the decreased expression of PPARγ. Conversely, we observed a partial rescue of differentiation in DN-Nurr1 expressing cells at the concentration of PGF2α that clearly inhibited differentiation of control GFP expressing cells, which is evident from the increased ORO staining and the increased expression of PPARγ (Fig. 19C, D). These results demonstrate that Nurr1 is indeed required for mediating the inhibitory effects of PGF2α on adipogenesis.

5.3 Nurr1-mediated inhibition of adipogenesis requires its direct DNA binding: The overexpression of Nurr1 has previously been shown to inhibit adipocyte differentiation (Chao et al., 2008). Hence, we decided to use Nurr1-WT as a positive control in our experiments. Similar to the previous findings, the ectopic expression of Nurr1-WT inhibited adipogenesis, which is evidenced by the decreased ORO staining and the decreased expression of PPARγ (Fig. 20C, D). Furthermore, the ectopic expression of
FIGURE 20. **Ectopic expression of Nurr1 inhibits adipogenesis via a DNA binding and RXR-independent mechanism.**

(A) Schematic model showing the Nurr1 mutants and their ability to bind to either DNA or RxR. (B) 3T3-L1 preadipocytes were infected with either MSCV-GFP, Nurr1-WT, Nurr1-AA or Nurr1-KLL encoding retroviruses and their expression was determined by immunoblotting with an antibody specific for Nurr1, while equal protein loading was confirmed by immunoblotting with a control antibody directed against ERK. (C, D) Two-day post-confluent cells were induced to differentiate by the standard MDI-induced protocol. After 8 days, adipocyte differentiation was assessed by ORO staining (C) and immunoblotting for the expression of PPARγ, aP2 and ERK as a control for equal loading (D). (E) 3T3-L1 cells were initially infected with either MSCV-GFP or Nurr1-WT encoding retroviruses and then super-infected with either MSCV-Ds-Red or MSCV-Ds-Red-PPARγ encoding retroviruses, as indicated. Two-day post-confluent cells were induced to differentiate by standard MDI-induced differentiation protocol. After 8 days, the adipogenesis was determined by ORO staining. Results in (B, C & D) are representative of four independent experiments, whereas results in (E) are representative of two independent experiments.
PPAR\(\gamma\) in these cells was able to bypass the inhibitory effect of Nurr1-WT on adipogenesis (Fig. 20E). Taken together, these results demonstrate that Nurr1 is sufficient to inhibit adipocyte differentiation, most likely by a mechanism that is proximal to the expression of pro-adipogenic transcription factor, PPAR\(\gamma\).

Having shown that Nurr1 expression is sufficient to inhibit adipogenesis and plays a role in mediating the inhibitory effect of PGF2\(\alpha\) on adipocyte differentiation, we decided to gain insights into the mechanism by which Nurr1 mediates its inhibitory effect on adipogenesis. Being a transcription factor, Nurr1 most commonly regulates gene expression by directly binding to DNA (Lammi et al., 2004; Sakurada et al., 1999). However, Nurr1 has recently been shown to indirectly transrepress gene expression by interacting with DNA-bound NF\(\kappa\)B and promoting the recruitment of transcriptional co-repressor complex (Saijo et al., 2009). Hence, in order to determine whether Nurr1 interacts directly or indirectly with DNA to inhibit adipogenesis, we took advantage of the previously well-characterized mutant form of Nurr1 that is unable to bind the DNA, Nurr1-AA with C280A and E281A mutations in DNA binding domain (Fig. 20A) (Saijo et al., 2009). We expressed this mutant in 3T3-L1 cells by retroviral transduction system and initially determined that this mutant was expressed to a level comparable to that of Nurr1-WT expression (Fig. 20B). Then, we induced these cells to undergo adipogenesis with the standard MDI-induced differentiation protocol. As shown in Figure 20C, D, while Nurr1-WT expression inhibited adipogenesis as described earlier, Nurr1-AA expressing cells completely differentiated into mature adipocytes as evidenced by the increased ORO staining and the increased expression of adipocyte specific genes PPAR\(\gamma\).
and aP2. This shows that the ability of Nurr1 to inhibit adipocyte differentiation is strictly dependent upon its ability to directly bind to DNA.

**5.4 Nurr1-mediated inhibition of adipogenesis does not require its heterodimerization with RXR:** Nurr1 is known to regulate gene expression as monomer, homodimer or heterodimer with RXR (Maxwell and Muscat, 2006). Hence, to determine whether the heterodimerization of Nurr1 with RXR is required to inhibit adipogenesis, we took advantage of the previously well-characterized mutant form of Nurr1 that cannot heterodimerize with RXR, Nurr1-KLL with K555A, L556A and L557A mutation (Fig. 20A) (Aarnisalo et al., 2002). We expressed the Nurr1-KLL mutant in 3T3-L1 cells and initially determined that it was expressed to a comparable level to that of Nurr1-WT expression (Fig. 20B). When these cells were induced to undergo adipogenesis with MDI, similar to Nurr1-WT expressing cells, Nurr1-KLL expressing cells failed to differentiate into mature adipocytes, which is evident from the decreased ORO staining and the decreased expression of PPARγ and aP2 (Fig. 20C, D). This demonstrates that Nurr1 does not require its heterodimerization with RXR to inhibit adipocyte differentiation.

**5.5 CREB and its association with CRTC2 are required for PGF2α-mediated Nurr1 expression during adipocyte differentiation:** Having shown that Nurr1 is induced by the PGF2α/calcineurin-signaling pathway and plays a role in mediating the inhibitory effect of PGF2α on adipogenesis, we next sought to determine the signaling mechanism
FIGURE 21. **PGF2α induces Nurr1 expression through CREB-dependent mechanism during adipogenesis.**

3T3-L1 cells transduced with either MSCV-GFP, K-CREB or CREB R314A encoding retroviruses were induced to undergo differentiation with MDI in the presence or absence of PGF2α (25 nM) and mRNA was collected at 48 h post-induction. Nurr1 expression was determined by qRT-PCR. The data is shown as mean ± SEM (one-way ANOVA followed by Bonferroni post hoc test; p<0.05; n=3). Results are representative of two independent experiments.
by which Nurr1 expression is regulated by this pathway. In this regard, the Nurr1 promoter contains a CREB responsive element (CRE) and has been shown to be regulated by the CREB transcription factor in different cell types (Ji et al., 2012; McEvoy et al., 2002; Zhao et al., 2011). Interestingly, the CREB-induced Nurr1 expression is selectively regulated by a recently identified transcriptional co-activator, CRTC2 (Conkright et al., 2003). More importantly, CRTC2 is dephosphorylated and activated by the calcineurin phosphatase and then, it translocates into the nucleus and regulates the CREB transcriptional activity (Screaton et al., 2004). Hence, we sought to determine whether CREB and its association with CRTC2 are required for PGF2α to induce Nurr1 expression. To determine this, we took advantage of two well-characterized CREB mutants, K-CREB that blocks DNA binding of endogenous CREB and CREB R314A that blocks the direct interaction of CREB with CRTC2 (Walton et al., 1992; Xu et al., 2007). Thus, K-CREB prevents induction of CREB regulated genes and CREB R314A blocks the expression of CRTC2-regulated CREB-induced genes. We introduced either control MSCV-GFP or the CREB mutants independently by retroviral transduction into 3T3-L1 cells and induced these cells to differentiate with MDI in the presence or absence of PGF2α. At 48 h post-induction, mRNA was collected to determine the Nurr1 expression by qRT-PCR. As shown in Figure 21, while PGF2α significantly increased the expression of Nurr1 in control GFP expressing cells, ectopic expression of either K-CREB or CREB R314A significantly attenuated the PGF2α-induced Nurr1 expression during the early stages of adipogenesis. These results demonstrate that CREB, and more
specifically, its interaction with CRTC2 are required for the PGF2α-induced Nurr1 expression during adipocyte differentiation.

5.6 The PGF2α/calcineurin-signaling pathway induces the activation and nuclear translocation of CRTC2 co-activator during adipogenesis: Since calcineurin is known to activate CRTC2 and the CREB mutant that cannot interact with CRTC2 blocks the PGF2α-induced Nurr1 expression, we decided to determine whether the PGF2α/calcineurin-signaling pathway induces the activation and nuclear translocation of CRTC2 during adipogenesis. Initially, we attempted to determine the activation of the endogenous CRTC2 protein using a variety of commercially available antibodies. However, due to the poor quality of antibodies, we were unable to successfully determine the activation of the endogenous CRTC2. Hence, we used an YFP tagged CRTC2 to detect it’s activation by this pathway using immunofluorescence method. We expressed the YFP-CRTC2 in 3T3-L1 cells using retroviruses and induced these cells to undergo adipocyte differentiation with MDI in the presence or absence of PGF2α or pretreatment with the calcineurin inhibitor, CsA. As shown in Figure 22A and B, MDI alone induced a transient cytoplasmic to nuclear translocation of YFP-CRTC2 during the very early stage of adipocyte differentiation. In contrast, PGF2α induced a sustained nuclear translocation of YFP-CRTC2 for a prolonged period of time. This sustained translocation of CRTC2 into the nucleus by PGF2α was abolished by the pretreatment with a calcineurin inhibitor, CsA. This demonstrates that PGF2α induces a sustained cytoplasmic to nuclear
FIGURE 22. The PGF2α/calcineurin-signaling pathway induces sustained activation of CRTC2.
(A, B) 3T3-L1 preadipocytes transduced with YFP-CRTC2 encoding retrovirus was induced to differentiate with MDI in the presence of either vehicle, PGF2α (25 nM) alone or PGF2α (25 nM) plus CsA (1 µg/ml). The nuclear translocation of YFP-CRTC2 was determined by immunofluorescence at the indicated time points (A) and the percentage of cells with nuclear YFP-CRTC2 was calculated (B). The data is shown as mean ± SEM (One-way ANOVA followed by Bonferroni post hoc test; p<0.05; n=3). Results are representative of three independent experiments.
translocation of CRTC2 co-activator through a calcineurin-dependent pathway during adipogenesis.

5.7 PGF2α requires the CRTC proteins to induce Nurr1 expression during adipogenesis: Having shown that CRTC2 co-activator is activated by the PGF2α/calcineurin-signaling pathway, we next sought to determine whether CRTC2 is indeed required for PGF2α to induce Nurr1 expression during adipogenesis. Hence, we attempted to stably deplete the expression of CRTC2 by shRNA approach. Initially, we expressed either control shLUC or CRTC2 shRNA in 3T3-L1 cells using retroviruses and collected mRNA from the confluent cells to determine the knockdown efficiency of the shRNA by qRT-PCR. As shown in Figure 23A, the expression of CRTC2 was significantly inhibited by the CRTC2 shRNA. Unexpectedly, this shRNA also inhibited the expression of another family member, CRTC1 due to the fact that this shRNA partially recognizes the CRTC1 mRNA (Fig. 23C). Next, we determined the expression of Nurr1 in these cells in response to PGF2α during adipogenesis. As shown in Figure 23C, the control shLUC and CRTC2 shRNA expressing cells were induced to differentiate with MDI-induced differentiation protocol in the presence or absence of PGF2α and mRNA was collected at 48 h post-induction for qRT-PCR. As expected, while Nurr1 expression was significantly increased in response to PGF2α in control shLUC cells, the stable depletion of CRTC expression significantly attenuated Nurr1 expression in response to PGF2α. This demonstrates that CRTC proteins are required for the PGF2α-induced Nurr1 expression during adipocyte differentiation.
FIGURE 23. CRTC co-activators are required for the PGF2α to induce Nurr1 expression during adipocyte differentiation.

(A, B) 3T3-L1 cells infected with either control shLUC or CRTC2 shRNA encoding retroviruses were induced to differentiate with MDI in the presence or absence of PGF2α (25 nM) and the mRNA was collected at 0 and 48 h post-induction. The shRNA-specific
knockdown efficiency at 0 h (A) and Nurr1 expression at 48 h post-stimulation (B) were determined by qRT-PCR. The data is shown as mean ± SEM (either student’s t test or one-way ANOVA followed by Bonferroni post hoc test; p<0.05; n=3). Results are representative of two independent experiments. (C) Nucleotide alignment of CRTC2 shRNA with the coding sequences of CRTC2 and CRTC1.
5.8 CRTC2 activity is sufficient to induce Nurr1 expression and to inhibit adipocyte differentiation: Having shown that CRTC2 is activated by PGF2α and CRTC proteins are required for the PGF2α-induced Nurr1 expression, we decided to determine whether CRTC2 is sufficient to induce Nurr1 expression in differentiating 3T3-L1 cells. Hence, we generated a constitutively active CRTC2 (caCRTC2) by mutating the critical serine residues required for its cytoplasmic retention to alanine (S171A and S275A). Then, the caCRTC2 was fused to the hormone binding domain of estrogen receptor (ER-caCRTC2; Figure 24A), thereby CRTC2 activity can be conditionally activated in response to tamoxifen. First, as described in the materials and methods, we determined the conditional activation of ER-caCRTC2 by transient transfection luciferase reporter assay in HEK293T cells using a well characterized CREB reporter, CRE-Luc. As shown in Figure 24B, while luciferase activity was minimal in control GFP and untreated ER-caCRTC2 cells, tamoxifen treatment was able to significantly induce the luciferase activity in ER-caCRTC2 expressing cells. This shows that ER-caCRTC2 is functional and its activity can be selectively regulated by tamoxifen. Next, to determine whether the conditional activation of CRTC2 is sufficient to induce Nurr1 expression, we expressed either MSCV-GFP or ER-caCRTC2 in 3T3-L1 cells using retroviruses and induced these cells to differentiate using MDI in the presence or absence of tamoxifen. At 48 h post-induction, mRNA was collected to determine the Nurr1 expression by qRT-PCR. As shown in Figure 24C, Nurr1 was not induced by tamoxifen treatment in control GFP expressing cells. In contrast, Nurr1 expression was significantly increased in ER-caCRTC2 cells in the presence of tamoxifen compared to MDI alone. This shows that the
FIGURE 24. **Conditional and selective activation of the constitutively active CRTC2 is sufficient to induce Nurr1 expression and to inhibit adipogenesis.**

(A) Schematic model showing the tamoxifen inducible, constitutively active CRTC2 (ER-caCRTC2). (B) HEK293T cells were transfected with ER-caCRTC2 plasmid (400 ng) along with CRE-Luc (50 ng) and pRG-TK (25 ng) reporter plasmids. After 16 h post-transfection, cells were treated with tamoxifen and luciferase activity was determined at
4 h post-treatment. (C, D & E) 3T3-L1 cells transduced with either GFP or ER-caCRTC2 retroviruses were induced to differentiate with MDI in the presence or absence of tamoxifen (1 µM). At 48 h post induction, mRNA was collected and Nurr1 expression was determined by qRT-PCR (C). After 8 days, adipocyte differentiation was determined by ORO (D) and immunobloting for the expression of PPARγ, aP2 and ERK as a loading control for equal loading (E). The data is shown as mean ± SEM (One-way ANOVA followed by Bonferroni post hoc test; p<0.05; n=3). Results are representative of three independent experiments.
selective activation of CRTC2 is sufficient to induce Nurr1 expression during adipogenesis.

Next, having demonstrated that the activation of ER-caCRTC2 is sufficient to induce Nurr1 expression, we also determined whether the activation of ER-caCRTC2 is sufficient to inhibit adipogenesis. Hence, the control GFP or ER-caCRTC2 cells were stimulated to undergo adipogenesis with MDI-induced differentiation protocol in the presence or absence of tamoxifen for the initial 48 h. As shown in Figure 24D and E, control GFP expressing cells differentiated into adipocytes in the presence of tamoxifen. In contrast, while ER-caCRTC2 expressing cells differentiated into adipocytes with MDI alone, the selective activation of ER-caCRTC2 using tamoxifen inhibited adipogenesis, which is evident from the decreased ORO staining and the diminished expression of PPARγ and aP2 (Fig. 24D, E). This demonstrates that the selective activation of CRTC2 is sufficient to inhibit adipogenesis and thus, phenocopies the inhibitory effects of PGF2α on adipocyte differentiation. Collectively, our results demonstrate that the sustained activation of CRTC2, similar to PGF2α, is sufficient to induce Nurr1 expression and to inhibit adipocyte differentiation.

5.9 CRTC co-activator proteins play a critical role in the regulation of normal adipocyte differentiation: Having shown that CRTC co-activator proteins, more specifically CRTC2, are required for the PGF2α-induced Nurr1 expression and that CRTC2 activation is sufficient to inhibit adipogenesis, we next sought to directly determine whether CRTC protein is required for the PGF2α-mediated inhibition of
A.

<table>
<thead>
<tr>
<th></th>
<th>Ds-Red</th>
<th></th>
<th>PPARγ</th>
</tr>
</thead>
<tbody>
<tr>
<td>shLUC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>shRNA</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B.

<table>
<thead>
<tr>
<th></th>
<th>shLUC</th>
<th></th>
<th>CRTC2 shRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>C/EBPα</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PPARγ</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>aP2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

C.

<table>
<thead>
<tr>
<th></th>
<th>shLUC</th>
<th></th>
<th>CRTC2 shRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>C/EBPβ</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C/EBPδ</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
FIGURE 25. **CRTC is required for normal adipocyte differentiation.**

3T3-L1 cells were initially infected with either control shLUC or CRTC2 shRNA encoding retroviruses and then super-infected with either Ds-Red or Ds-Red PPARγ retroviruses, as indicated. Two-day post-confluent cells were induced to differentiate with MDI and adipogenesis was determined after 8 days of induction by ORO (A). (B, C) 3T3-L1 preadipocytes infected with either control shLUC or CRTC2 shRNA encoding retroviruses were induced to differentiate with MDI and the cell lysates were collected at the indicated times after adipogenic stimulation and, immunoblotting was performed for C/EBPβ, C/EBPδ, C/EBPα, PPARγ, aP2 and ERK as a loading control. The results in (A) are representative of three independent experiments, whereas the results in (B, C) are representative of two independent experiments.
adipogenesis. To accomplish this, we investigated the ability of CRTC depleted cells to differentiate in the presence of PGF2α. We induced either the control shLUC or CRTC2 shRNA expressing cells to differentiate using MDI-induced differentiation protocol in the presence or absence of PGF2α for the initial 48 h. Surprisingly, while control shLUC expressing cells completely differentiated into adipocytes, stable depletion of CRTC expression inhibited the ability of the cells to undergo adipocyte differentiation in the presence of MDI alone, as evidenced by the decreased ORO staining and the decreased expression of adipocyte specific genes PPARγ and aP2 (Fig. 25A, B). Hence, we were unable to dissect the role of CTRC in mediating the inhibitory effects of PGF2α on adipogenesis. However, this provided surprising evidence that the CRTC co-activators play a crucial positive regulatory role in the normal adipogenic process. Further, the ectopic expression of PPARγ in the CRTC2 shRNA expressing cells rescued their potential to differentiate into mature adipocytes (Fig. 25A), suggesting that CRTC regulates a critical step(s) proximal to the expression of PPARγ during normal adipocyte differentiation process. To further understand the underlying mechanisms, we determined the expression of key adipogenic transcription factors in the CRTC2 shRNA expressing cells during adipogenesis. As shown in Figure 25B and C, while the expression of the late transcription factors, C/EBPα and PPARγ and the early transcription factor, C/EBPβ were not affected by the depletion of CRTC protein expression, the expression of the early transcription factor C/EBPδ was decreased in these cells, suggesting that CRTC proteins might play a critical role in the regulation of C/EBPδ. Collectively, our results
demonstrate that CRTC proteins are required for normal adipocyte differentiation process and possibly, they regulate the expression of early transcription factor, C/EBPΔ.

6. DISCUSSION

In this dissertation chapter, we further investigated the mechanism by which PGF2α inhibits adipocyte differentiation and provide evidence that the increased expression of Nurr1 plays an important role in mediating the inhibitory effects of the PGF2α/calcineurin-signaling pathway on adipogenesis. Further, we have established that PGF2α induces Nurr1 expression via the calcineurin-dependent activation of CRTC proteins and the subsequent activation of the CREB transcription factor. Collectively, our results provide evidence that in addition to the IL-11/gp130/STAT1-signaling pathway described in chapter III, PGF2α can also inhibit adipogenesis by the means of a calcineurin/CRTC/CREB/Nurr1-dependent signaling mechanism.

While all the members of the NR4A family of transcription factors are known to be transiently expressed in 3T3-L1 cells during the initial few hours of adipogenesis and in response to various inflammatory mediators in different cell types (Chao et al., 2008; Pei et al., 2005), we found that PGF2α induces a sustained and prolonged expression of Nurr1. Interestingly, while PGF2α highly upregulates the expression of Nurr1, other family members Nur77 and Nor1 are not induced by PGF2α. More importantly, this increased PGF2α-induced Nurr1 expression was completely abrogated to the basal level with the inhibition of the calcineurin phosphatase activity, which demonstrates that PGF2α induces a sustained expression of Nurr1 via activation of the calcineurin
phosphatase. This is consistent with a recent finding in hippocampal neurons demonstrating that calcineurin plays an important role in Nurr1 expression in these cells (Tokuoka et al., 2014). Thus, our results demonstrate that Nurr1 is a selective transcriptional target of the PGF2α/calcineurin-signaling pathway during the early stages of adipogenesis.

While the ectopic expression of Nurr1 has previously been shown to inhibit adipocyte differentiation (Chao et al., 2008), we find that the PGF2α/calcineurin-signaling pathway induces a sustained expression of endogenous Nurr1. Further, the transient activation of its family member Nur77 has previously been shown to positively regulate adipogenesis, whereas the prolonged ectopic expression of Nur77 has been shown to inhibit adipogenesis (Fumoto et al., 2007). In our studies, while PGF2α inhibits adipogenesis, it also induces a sustained expression of endogenous Nurr1. Hence, our initial hypothesis was that the increased endogenous Nurr1, in addition to IL-11, mediates the inhibitory effects of PGF2α on adipogenesis. The direct evidence to support this hypothesis came from our observation that the expression of a dominant-negative Nurr1 was able to partially rescue adipogenesis from the inhibitory effects of PGF2α. This shows that the sustained expression of endogenous Nurr1 plays a critical role in mediating the inhibitory effects of PGF2α on adipogenesis. However, the mechanism by which Nurr1 inhibits adipogenesis and how the dominant-negative Nurr1 functions to rescue adipogenesis is unclear. It is possible that the dominant-negative Nurr1 binds to DNA and blocks the interaction of the endogenous Nurr1 to DNA. Alternatively, the dominant-negative Nurr1 may dimerize with the endogenous Nurr1, bind to DNA and
block gene expression through its KRAB repressor domain. Since, we observe a partial rescue from PGF2α by expressing the dominant-negative Nurr1 with KRAB repressor domain, we predict that most likely the dominant-negative Nurr1 acts to prevent the expression of a gene(s) that is induced by the sustained expression of endogenous Nurr1. In this respect, Nurr1 has been shown to induce the cell cycle inhibitor p18 and inhibit the proliferation of the haematopoietic stem cells (Sirin et al., 2010). However, while there is a possibility that Nurr1 could inhibit adipogenesis by blocking the mitotic clonal expansion by the similar mechanism, our previous studies demonstrate that PGF2α does not inhibit mitotic clonal expansion (Liu and Clipstone, 2007). Hence, we feel that Nurr1 might induce some other anti-adipogenic gene or microRNA to inhibit adipocyte differentiation. In contrast, since Nurr1 has also been shown to repress gene expression in multiple cell types (Jacobs et al., 2009; Kim et al., 2013), it is also possible that Nurr1 may also directly repress the expression of critical genes involved in adipogenesis.

Having provided evidence that Nurr1 is induced and plays a role in mediating the inhibitory effects of the PGF2α/calcineurin-signaling pathway on adipogenesis, we were then interested to understand the potential mechanisms by which Nurr1 inhibits adipogenesis. Nurr1 has been shown to regulate gene expression as either monomer, homodimer or as a heterodimer with its partner protein RXR (Maxwell and Muscat, 2006). Initially, the heterodimerization of Nurr1 with RXR has caught our attention, as the master regulator of adipogenesis PPARγ also requires RXR to induce gene expression (Palmer et al., 1995). Thus, it raised a possibility that the sequestration of RxR by Nurr1 might inhibit the availability of RXR to PPARγ, thereby adipogenesis is inhibited due to
the blockade of expression of the PPARγ-induced adipocyte specific genes. However, in our studies using a Nurr1 mutant that cannot heterodimerize with RXR, we found that this mutant is still able to inhibit adipogenesis. This suggests that Nurr1 does not require its interaction with RXR to inhibit adipocyte differentiation and RXR is not sequestrated. Further, Nurr1 is typically considered as a positive transcription factor and has been shown to regulate gene expression through its direct interaction with DNA (Davies et al., 2005; Lammi et al., 2004; Sakurada et al., 1999). However, the recent findings demonstrate that Nurr1 can also regulate gene expression without a direct interaction with DNA by binding to the DNA bound NF-κB and subsequent recruitment of repressor complex (Saijo et al., 2009). Hence, to delineate whether Nurr1 needs to bind to DNA or not to inhibit adipogenesis, we used a mutant Nurr1 that cannot directly bind to DNA. While the wild type Nurr1 inhibited adipogenesis, the Nurr1 mutant that cannot bind to DNA was unable to inhibit adipogenesis. This provided evidence that Nurr1 must directly bind to DNA in order to mediate its inhibitory effects on adipogenesis. However, the genes directly regulated by Nurr1 during adipocyte differentiation are not known. Collectively, our results provide evidence that while Nurr1 needs to directly bind to DNA to inhibit adipogenesis, its heterodimerization with RXR is dispensable to mediate its inhibitory effect on adipogenesis.

Having demonstrated that PGF2α induces Nurr1 expression through activation of the calcineurin phosphatase, we focused on the mechanism by which the PGF2α/calcineurin-signaling induces Nurr1 expression. In this respect, Nurr1 the promoter contains a CRE consensus binding site and CREB has been shown to directly
bind to the Nurr1 promoter, and induce Nurr1 expression in synoviocytes (McEvoy et al., 2002). Further, inflammatory mediators such as PGE2, TNFα and IL-1β have also been shown to induce Nurr1 expression through the CREB transcription factor (Ji et al., 2012; McEvoy et al., 2002). Hence, we hypothesized that CREB might be involved in the PGF2α-induced Nurr1 expression during adipogenesis. Using two different CREB mutants that either block the endogenous CREB activity or cannot interact with its co-activator CRTC2, we found that CREB and its association with CRTC2 co-activator are required for the PGF2α to induce Nurr1 expression during adipogenesis. While blocking the calcineurin activity completely inhibits the PGF2α-induced Nurr1 expression, the two different CREB mutants that we used in our studies were unable to completely abrogate the PGF2α-induced Nurr1 expression. While the inadequate expression of these mutants might be a possibility for this incomplete blocked of Nurr1 expression, it is also possible that another transcription factor downstream of calcineurin might also be involved in regulating Nurr1 expression. In this respect, NF-κB is known to be regulated by calcineurin and has been shown to regulate Nurr1 expression in multiple cell types (Ji et al., 2012; McEvoy et al., 2002). Hence, it is possible that Nurr1 expression is regulated by PGF2α through activation of multiple transcription factors downstream of calcineurin. Nevertheless, our findings demonstrate that CREB plays an important role in the PGF2α-induced Nurr1 expression during adipogenesis.

Having established that CREB is involved in the PGF2α-induced Nurr1 expression, we focused on the direct link between calcineurin and CREB in the regulation of Nurr1 expression. It is well-known that CREB activity is regulated by CRTC co-
activator proteins and importantly, these co-activators are dephosphorylated, and activated by calcineurin phosphatase (Screaton et al., 2004). Further, CRTC2 is known to selectively regulate CREB-induced Nur71 expression in multiple cell types (Conkright et al., 2003). In addition, we found that the CREB mutant that cannot interact with CRTC2 was able to significantly inhibit the PGF2α-induced Nur71 expression, thus providing initial evidence for the role of CRTC2 in the regulation of Nur71 expression. Initially, using YFP tagged CRTC2, we found that PGF2α induces a sustained activation and nuclear translocation CRTC2, whereas this sustained activation was completely blocked by pretreatment with calcineurin inhibitor, CsA. Thus, we found that the PGF2α/calcineurin-signaling causes a sustained activation of the CRTC2 co-activator during adipocyte differentiation. Interestingly, MDI treatment alone caused a transient nuclear translocation of CRTC2, although the functional significance of this early transient activation of CRTC2 is not known. Having established that CRTC2 is activated by the PGF2α/calcineurin-signaling, we directly determined its role in Nur71 expression using a shRNA-mediated knockdown approach. We found that the depletion of the endogenous CRTC proteins abrogated the PGF2α-induced Nur71 expression, thereby providing evidence that CRTC proteins are required for the PGF2α-induced Nur71 expression during adipogenesis. Further, the selective activation of a tamoxifen inducible form of a constitutively active CRTC2 was sufficient to induce Nur71 expression and also inhibits adipogenesis. This demonstrates that CRTC2 activation is sufficient to induce Nur71 expression and to inhibit adipogenesis. Collectively, we provide evidence that
FIGURE 26. Schematic model showing that the PGF2α/calcineurin-signaling pathway induces Nurr1 expression via CRTC2/CREB transcriptional complex to inhibit adipogenesis.

The PGF2α/calcineurin-signaling pathway activates CRTC2 co-activator, which in turn activates CREB transcription factor. The CRTC2/CREB transcriptional complex induces Nurr1 expression, which inhibits adipocyte differentiation.
CRTC proteins are required for the PGF2α-induced Nurr1 expression and CRTC2 is sufficient to induce Nurr1 expression.

Though the selective activation of CRTC2 inhibits adipogenesis and CRTC2 plays a crucial role in mediating the inhibitory effects of PGF2α on adipocyte differentiation, surprisingly, we observed that the stable depletion of CRTC expression using the shRNA approach also blocks the normal adipocyte differentiation process. When we determined the expression of key adipogenic transcription factors, we observed that the expression of C/EBPδ, an early adipogenic transcription factor, is substantially decreased in CRTC2 shRNA expressing cells. This is consistent with the previous finding that CRTC2 is recruited to the C/EBPδ promoter to regulate CREB-dependent induction of C/EBPδ (Hallenborg et al., 2012). Further, we also observed that CRTC2 is transiently translocated into the nucleus in response to MDI (Fig. 22A, B), which correlated with the time of expression of C/EBPδ expression during adipogenesis. Thus, we predict that the early transient activation of CRTC2 positively regulates adipogenesis by potentially regulating C/EBPδ expression. Conversely, the PGF2α/calcineurin-signaling causes a sustained activation of CRTC2, resulting in the sustained expression of Nurr1 and inhibition of adipocyte differentiation. Thus, it appears that the temporal activation of CRTC2 is a switch between its positive and negative effects on adipocyte differentiation.

Collectively, we provide evidence that the PGF2α/calcineurin-signaling pathway induces Nurr1 expression via activation of the CRTC/CREB transcriptional complex during adipogenesis. Moreover, we demonstrate that the sustained expression of
endogenous Nurr1 plays a role, at least in part in mediating the inhibitory effects of the PGF2α/calcineurin-signaling pathway on adipogenesis.
CHAPTER V
SUMMARY AND CONCLUDING COMMENTS

PGF2α has long been known to be a potent inhibitor of adipocyte differentiation \textit{in vitro} (Casimir et al., 1996; Miller et al., 1996). Moreover, recent studies have indicated that PGF2α also functions as an endogenous negative regulator of adipogenesis and plays an important role in the regulation of adipose tissue accumulation \textit{in vivo}, where it appears to oppose the development of obesity and its associated co-morbidities (Volat et al., 2012). Given this clear critical physiological role of PGF2α in the negative regulation of adipose tissue development \textit{in vivo}, it is of considerable interest to understand the underlying molecular mechanisms by which PGF2α regulates the adipogenic process. In this regard, our previous studies have demonstrated that PGF2α inhibits adipogenesis through a signaling pathway involving the activation of the Gαq-coupled prostanoid FP receptor, a subsequent increase in the intracellular calcium and the activation of the calcium-dependent phosphatase, calcineurin (Liu and Clipstone, 2007). In this dissertation, we have now extended these prior studies and provide evidence that the PGF2α/calcineurin-signaling pathway acts to inhibit adipogenesis by inducing the expression of the IL-11 cytokine and the Nurr1 nuclear hormone receptor. In the case of IL-11, we demonstrate that IL-11 functions in an autocrine/paracrine fashion and induces a gp130 co-receptor-dependent activation of the STAT1 transcription factor, which we
show is essential for mediating the inhibitory effects of PGF2α on adipogenesis. In the case of Nurr1, we show that Nurr1 plays an important role in mediating the inhibitory effects of PGF2α on adipogenesis and further, demonstrate that PGF2α induces a calcineurin-dependent, sustained activation and nuclear translocation of CRTC proteins, which then interact with CREB to drive Nurr1 expression. Collectively, our studies provide evidence that the PGF2α/calcineurin-signaling pathway inhibits adipogenesis through two independent signaling mechanisms: IL-11/gp130/STAT1- and CRTC/CREB/Nurr1-signaling.

Although we provide evidence that IL-11 and Nurr1 each independently mediate the inhibitory effects of PGF2α on adipogenesis, it is noteworthy that neither depletion of IL-11 and STAT1 using shRNAs, nor blocking the endogenous Nurr1 activity with a dominant-negative Nurr1 were able to completely rescue the differentiation of 3T3-L1 preadipocytes in the presence of PGF2α. These findings contrast with our prior results demonstrating that the inhibition of calcineurin is able to essentially fully abrogate the anti-adipogenic effects of PGF2α (Liu and Clipstone, 2007). While in part these differences may be explained by incomplete knockdown of STAT1 and IL-11 or incomplete expression of dominant-negative Nurr1, as well as potentially the non-cell autonomous actions of the IL-11 cytokine, we feel that it is likely that both the IL-11 and Nurr1 pathways function together collaboratively to mediate the inhibitory effects of PGF2α on adipogenesis. Further, we have previously reported that activation of the PGF2α/calcineurin-signaling pathway in differentiating 3T3-L1 preadipocytes also
induces the HIF-1-dependent expression of DEC1 (Liu and Clipstone, 2008), a transcriptional repressor that is known to inhibit adipocyte differentiation by preventing the expression of PPARγ2 (Yun et al., 2002). Hence, it appears that calcineurin functions as a central node for mediating the inhibitory effects of PGF2α by activating multiple signaling pathways that ultimately collaborate together to effectively inhibit the adipogenic process.

While we demonstrate that exogenous PGF2α acts through calcineurin to inhibit adipogenesis (Liu and Clipstone, 2007), our previous studies showed that blocking the endogenous calcineurin activity enhanced the adipogenic potential of preadipocytes and thus, suggested that calcineurin acts as an endogenous negative regulator of adipocyte differentiation (Neal and Clipstone, 2002). Likewise, PGF2α is transiently synthesized during the very early stage of adipogenesis (Fujimori et al., 2010a) and inhibiting its endogenous synthesis has been shown to enhance adipose tissue development in vivo (Volat et al., 2012). Thus, PGF2α also functions as endogenous inhibitor of adipose tissue development. Hence, it is tempting to speculate that the endogenous inhibitory effect of PGF2α is also mediated through calcineurin-dependent activation of the signaling pathways described in this dissertation. In this regard, we find an endogenous, calcineurin-dependent secretion of IL-11 during the early stages of normal adipogenesis. Further, inhibiting IL-11 signaling using either a dominant-negative gp130 or depleting its downstream effector STAT1, enhances the efficiency of preadipocytes to differentiate into mature adipocytes. Hence, we predict that the endogenous inhibitory effect of the PGF2α/calcineurin-signaling pathway is, at least in part, mediated through activation of
an autocrine/paracrine feedback loop involving IL-11/gp130/STAT1-signaling. In addition, we find that the PGF2α/calcineurin-signaling also activates other signaling pathways including the expression Nurr1 and DEC1, which mediate the inhibitory effects of exogenous PGF2α. Although we do not currently have any direct evidence to support their role in mediating the inhibitory effects of endogenous PGF2α on adipogenesis, we cannot exclude their potential endogenous role and further studies are needed.

Multiple lines of evidence show that adipose tissue expansion through de novo generation of new adipocytes play a protective role from the development of obesity-associated complications by increasing the lipid buffering capacity of adipose tissue and preventing lipotoxicity (Tan and Vidal-Puig, 2008; Virtue and Vidal-Puig, 2010). In this regard, PGF2α is known to restrict adipose tissue expansion in vivo, which results in the development of obesity-associated consequences such as insulin resistance (Volat et al., 2012). In our studies, we find that calcineurin functions as a central node to mediate the inhibitory effects of PGF2α on adipogenesis. Hence, it is tempting to ask whether inhibiting calcineurin activity in vivo specifically in preadipocytes or adipose tissue with clinically available calcineurin inhibitors would enhance adipogenesis, thereby increasing the lipid buffering capacity and providing beneficial effects to obese patients. In this respect, organ transplanted patients who are treated with calcineurin inhibitors to prevent graft rejection are known to develop obesity (LaGuardia and Zhang, 2013). Given our finding that blocking endogenous calcineurin activity enhances adipogenesis (Neal and Clipstone, 2002), it is possible that these patients develop excessive adipose tissue due to increased number of adipocytes. While this effect seems to be beneficial to prevent
lipotoxicity, calcineurin activity is required for the growth and proliferation of insulin secreting pancreatic β-cells (Heit et al., 2006) and the prolonged use of calcineurin inhibitors leads to the development of type 2 diabetes due to dysfunction of pancreatic β-cells (Drachenberg et al., 1999; Weir and Fink, 1999). Hence, while the prolonged oral and parenteral administration of calcineurin inhibitors is more deleterious than beneficial, the selective tissue specific delivery of these drugs to adipose tissue might have better effect with fewer side effects by enhancing adipogenesis and improving metabolic complications in obese patients. Alternatively, other signaling pathways described in this dissertation that are activated downstream of calcineurin could be used as a potential therapeutic target to enhance the adipogenesis.

Finally, while our studies demonstrate that STAT1 and Nurr1 proteins function as downstream effectors of the PGF2α/calcineurin-signaling, the precise molecular mechanism by which they inhibit adipocyte differentiation is not known. Since, these two proteins are known to directly regulate gene expression both positively and negatively (Furukawa et al., 2009; Jacobs et al., 2009; Kim et al., 2013; Liu et al., 2008; Ramana et al., 2000; Sirin et al., 2010), we predict that they likely inhibit adipogenesis by influencing the expression of genes involved in the adipogenic process. In a simple model, both STAT1 and Nurr1 might directly repress the expression of the key adipogenic transcription factor, PPARγ, thereby inhibiting adipogenesis. In this respect, STAT1 has previously been shown to directly bind to the PPARγ promoter in vitro (Hogan and Stephens, 2001). Further, STAT1 is known to interact with co-repressors and inhibit gene expression (Kamitani et al., 2008; Liu et al., 2001). Hence, we predict that
STAT1 could potentially directly bind to the PPARγ promoter, recruit transcriptional co-repressors and inhibit PPARγ expression. In the case of Nurr1, another related nuclear hormone receptor COUP-TFI has been shown to directly bind to the PPARγ locus, where it recruits co-repressor complexes and inhibits adipogenesis (Okamura et al., 2009). Further, Nurr1 has also been shown to interact with co-repressors and inhibit gene expression (Kim et al., 2013; Saijo et al., 2009). Hence, similar to STAT1, it is possible that Nurr1 could directly bind to the PPARγ promoter, recruit co-repressor and inhibit the expression of PPARγ. In contrast to directly inhibiting the expression of key adipogenic transcription factors, it is possible that both STAT1 and Nurr1 might indirectly inhibit adipogenesis by increasing the expression of a gene(s) that itself directly inhibits the expression of key adipogenic transcription factors and thereby, inhibits adipogenesis. However, our microarray analysis did not identify any potential candidate genes. Another possibility is that both STAT1 and Nurr1 could upregulate the expression of a microRNA(s) that inhibits the expression of the key adipogenic transcription factors, PPARγ or C/EBPα. In this regard, it is interesting to note that STAT1 has recently been implicated in promoting the expression of microRNA-27a (Wang et al., 2010), a microRNA that has previously been shown to negatively regulate the expression of the PPARγ mRNA and block adipogenesis when overexpressed in 3T3-L1 preadipocytes (Kim et al., 2010). However, whether this microRNA is induced by STAT1 in response to PGF2α during adipogenesis is not known and thus, requires further studies. While there are multiple possible mechanisms by which STAT1 and Nurr1 could potentially inhibit adipogenesis, the critical genes involved have not been identified.
FIGURE 27. Schematic model showing the signaling pathways involved in mediating the inhibitory effects of PGF2α on adipogenesis.

PGF2α activates the calcium-dependent phosphatase calcineurin via activation of Gαq coupled FP prostanoid receptor. Then, the PGF2α/calcineurin-signaling pathway induces the expression of the IL-11 cytokine, which activates STAT1 transcription factor via gp130 co-activator to inhibit adipogenesis. Further, the PGF2α/calcineurin-signaling pathway also activates CRTC/CREB transcriptional complex and subsequently, induces the expression of the orphan nuclear hormone receptor, Nurr1 to inhibit adipocyte differentiation. Collectively, both IL-11 and Nurr1 function downstream of the PGF2α/calcineurin-signaling pathway to exert its inhibitory effects on adipogenesis.
The identification of the direct *in vivo* targets of Nurr1 and STAT1 will require the use of modern, high throughput techniques such as ChIP-sequencing.

In summary, the work of this dissertation has afforded novel insights into the molecular mechanisms underlying the anti-adipogenic effects of the PGF2α/calcineurin-signaling pathway and has provided evidence for the potential role of the IL-11 cytokine and the Nurr1 orphan nuclear hormone receptor in mediating the inhibitory effects of PGF2α on adipocyte differentiation. Thus, our studies significantly expanded our understanding concerning the molecular mechanisms involved in the PGF2α-mediated inhibition of adipogenesis.
REFERENCES


mouse embryonic fibroblasts via an autocrine mechanism. J. Lipid Res. 52, 1500-1508.


VITA

Damodaran Annamalai, was born in Kanchipuram, Tamil Nadu, India to Annamlai and Kuppammal. He currently resides in Forest Park, IL with his wife, Lavanya and his daughter, Govardhini.

Damodaran completed his Bachelor of Veterinary Sciences in 2002 followed by a Master’s degree in Veterinary Pharmacology and Toxicology in 2004 at Madras Veterinary College, Chennai, India. He then joined Natural Remedies Pvt Ltd, Bangalore, India, where he managed a laboratory animal facility and evaluated plant extracts for their *in vivo* biological activities in a variety of different animal models.

In July 2007, Damodaran joined the PhD program in the Department of Molecular Pharmacology and Therapeutics, Loyola University Chicago. He ultimately joined the laboratory of Dr. Neil Clipstone, where he has studied molecular mechanisms involved in the regulation of adipocyte differentiation. He has presented his research findings in both oral and poster presentations at St. Albert’s Day and has received travel awards to present his work at both Experimental Biology 2013 and the American Society of Cell Biology meeting, 2013. During his time in graduate school, Damodaran has served as both a representative of the Graduate School Council (GSC) and as a student representative to the Department of Pharmacology faculty.

Trained as a veterinarian, Damodaran has received certification from the Education Commission for the Foreign Veterinary Graduates and has recently passed the
North American Veterinary Licensing Examination (NAVLE). After leaving Loyola, Damodaran will enter the residency program in Laboratory Animal Medicine at Oregon Health and Sciences University, Portland, Oregon, where he will pursue advanced clinical training and continue his research interests in the area of obesity and diabetes.