HEDGEHOG SIGNALING PLAYS A CONSERVED ROLE IN INHIBITING FAT FORMATION

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DEDICATION

I would like to thank my advisor, Dr. Jonathan M. Graff, for giving the opportunity to pursue my research interest in his lab. He taught me how to think scientifically and how to become an independent researcher. Because of his constant support and guidance throughout the graduate studies (qualifying examinations, research itself and the writing of the dissertation), I was be able to learn a lot, to get an excellent training as a researcher and to complete the dissertation. Without his patience and persistence, I could not have come this far. I wish to also thank the other committee members: Dr. Jane Johnson, Dr. Edward Wakeland and Dr. Eric Olson, for their time for constructive criticism, advice, expertise, and also for reviewing my thesis. Many thanks to all the people that I interacted with who once were part of Graff lab, specifically Li Meng, Wei tang, Yuan Xu, Jae Myoung Suh, Edgardo Fortuno, Jim McKay, Renee McKay, Kellee Gilmour and Zack Salo. I am grateful for the help from members of the Center for Developmental Biology and the G & D Graduate program. Special thanks to Nancy McKinney, our administrative coordinator, for her counseling and help in my graduate school life. And last but not least, I thank my family and personal friends for their constant support and encouragement.

HEDGEHOG SIGNALING PLAYS A CONSERVED ROLE IN INHIBITING FAT FORMATION

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The involvement of hedgehog (Hh) signaling in cell determination and differentiation in a wide variety of tissues in both invertebrates and vertebrate has been well established. However, relative little is known about its function in formation of adipose tissues. To address this question, *Drosophila* and mammalian models were used to analyze its potential role. Components of the Hh pathway were expressed in the *Drosophila* fat body. Activating Hh signaling specifically in fat body inhibited fly fat formation. Conversely, blocking Hh signaling specifically in fat body stimulated fly fat formation. Analysis in mammalian models suggested the presence of functional Hh signaling in murine developing fat, adult fat and in mammalian adipogenic models. Down-regulation of Hh signaling marked the stage of terminal

differentiation. In 3T3-L1 preadipocyte cell line, addition of recombinant murine sonic Hh (Shh) potently inhibited adipogenic differentiation dose-dependently, resulting in decreased intracellular triglyceride accumulation and reduced mRNA levels of established adipogenic genes. Treatment of KAAD-cyclopamine, an antagonist of Hh signaling, promoted adipogenesis. Activating or blocking Hh signaling genetically produced similar effects as pharmacological treatment. Additional study in multipotent cell lines, NIH3T3 and C3H10T1/2, reinforced the inhibitory role of Hh signaling in adipogenesis. However, the inhibition was effective only when Hh signaling was activated during early stage of adipogenesis. Epistasis tests suggested Hh signaling functioned upstream of PPARγ. Mechanistic studies showed that Hh signaling might act as a molecular switch, likely mediated by anti-adipogenic transcrition factors such as GATA2, to divert preadipocytes as well as multipotent mesenchymal prescursors away from adipogenesis to osteogenesis. My study on the function of Hh signaling in fat formation of both invertebrates and vertebrates suggested that Hh signaling played a conserved role in inhibiting fat formation and highlighted the potential of the Hh pathway as a therapeutic target for osteoporosis, lipodystrophy, diabetes and obesity.

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Suh, J. M.[1], Gao, X.[1], McKay, J., McKay, R., Salo, Z., Graff, J. M. (2006). Hedgehog signaling plays a conserved role in inhibiting fat formation. Cell Metab. *3*, 25-34.

[1] These authors contributed equally to this work.

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aP2, fatty acid binding protein 2

BMI, body mass index

BSA, bovine serum albumin

C/EBP, CCAAT enhancer binding protein

cAMP, cyclic AMP

CREB, cAMP-response element binding protein

CBP, Creb binding protein

CS, calf serum

DEX, dexamethasone

DMEM, Dulbecco's modified Eagle medium

DMI, dexamethasone, methlisobutylxanthine, insulin

DMSO, dimethylsulfoxide

EDTA, ethylenediaminetetraacetic acid

EGF, epidermal growth factor

FBS, fetal bovine serum

FGF, fibroblast growth factor

Glut4, glucose transporter 4

IGF-1, insulin-like growth factor-1

LPL, lipoprotein lipase

MIX, isobutylmethylxanthine

NHANES, National Health and Nutrition Examination Survey

PAGE, polyacrylamide gel electrophoresis

PBS, phosphate-buffered saline

PKA, protein kinase A

PPAR, peroxisome proliferator activated receptor

Pref-1, preadipocyte factor-1

Rb, retinoblastoma protein

RXR, retinoid X receptor

SDS, sodium dodecyl sulfate

T3, triiodothyronine

TNF, tumor necrosis factor

CHAPTER ONE

INTRODUCTION

1.1 The growing prevalence of obesity and the urgent need for studying fat biology

The prevalence of obesity is rising dramatically in recent years and has reached epidemic proportions globally (Popkin and Dock, 1998). In 1997, the World Health Organization (WHO) Consultation on Obesity agreed on an international standard for measuring overweight and obesity, the body mass index (BMI), calculated as weight in kg divided by height in m^2. From this calculation, obesity was defined as having a BMI of >30 kg/m^2, while overweight was delineated as a BMI of 25.0-29.9 kg/m^2 (see Table 1). Furthermore, overweight and obesity tend to be associated with minority, low-income, and undereducated populations, especially in women (Bray et al., 1998; Flegal et al., 1998; Seidell, 1999).

In developed nations such as the United States, the incidence of obesity has been rising ever since health statistics began to be recorded in 1976. According to the data from the National Health and Nutrition Examination Survey (NHANES IV), the number of adults with obesity nearly doubled from 1976 to 1999 (Kopelman, 2000). These dramatic increases have occurred among the three major racial and ethnic groups and include both sexes. It was reported that over 60% of the United States adult population is considered clinically overweight or obese, with a BMI greater than 25 kg/m^2, and at least a third of this

subset is considered obese, with a BMI greater than 30 kg/m^2 (Flegal and Troiano, 2000). The rapid rise in obesity is affecting more children than ever before. According to the NHANES data, the number of overweight children six to eleven years old increased more than three-fold in less than half of a century, which jumped from four percent in 1965 to thirteen percent in 1999. In addition, obesity is not just limited to developed nations. Obesity levels in some lower-income and transitional countries are as high as or higher than those reported for the United States and other developed countries, and those levels are increasing rapidly (Popkin and Dock, 1998).

Table 1 Weight Classification by Body Mass Index (BMI)

BMI = (weight in kilograms) divided by (height in meters × height in meters)

Classification	BMI (kg/m$^{2)}$	Risk of co-morbidities
Underweight	<18.5	Low but risk of other clinical problems increased)
Normal range	18.5 – 24.9	Average
Overweight		
Pre-obese	= 25 – 29.9	Increased
Obese class I	30.0 – 34.9	Moderate
Obese class II	35.0 – 39.9	Severe
Obese class III	= 40.o	Very severe

(From World Health Organization (2000))

Obesity is problematic and costly conditions because of the high incidence of chronic disease that is associated with being obese. Obesity causes or exacerbates a number of health problems, including non-insulin-dependent diabetes mellitus, cardiovascular

diseases (heart disease, stroke and hypertension) and cancer, and in many industrialized countries it is associated with various psychosocial consequences (Foreyt and Poston, 1998; Must et al., 1999; Kopelman, 2000). For example, the Framingham Heart Study reported that adults less than fifty years old have a 2.4-fold increased risk of heart failure in obese women and a 2.0-fold increased risk of heart failure in obese men (Kopelman, 2000). Studies reported by WHO in 2000 indicated that 2%-7% of national health care costs can be ascribed to treatment and control of overweight and obesity in Australia, France, the Netherlands, and the United States, while the highest cost (US$ 46 billion annually) was incurred in the United States, which would equal the total amount currently expended in treating major chronic conditions. Therefore, the prevention and control of obesity need to be taken very seriously throughout the world.

Obesity occurs when energy intake chronically exceeds energy expenditure, resulting in both adipocyte hyperplasia (an increase in cell number) and hypertrophy (an increase in cell size) (Bray et al., 1998). Obese animals have larger and more numerous adipocytes than do their lean counterparts (Marques et al., 1998). These enlarged adipocytes appear to secrete growth factors that promote the proliferation and differentiation of preadipocytes which result in the increase in mature adipocytes, thus ensuring storage potential in an environment of positive energy balance (MacDougald and Lane, 1995). Adipocyte proliferation and differentiation are controlled by distinct signaling pathways and regulated by a defined molecular program (MacDougald and Lane, 1995). A large body of work has focused on the late stages of adipocyte differentiation and much has been learned

(Cowherd et al., 1999; Rosen and Spiegelman, 2000; Smas and Sul, 1995; Cao et al., 1991), however, relatively little is known about the signaling cascades that mediate this process of adipogenesis.

Currently, common treatments for obesity include diet, exercise, behavior modification, surgery, and pharmacotherapy. For people with obesity, the success rate for maintaining weight loss without surgical intervention remains low, especially for those with hyperplastic obesity, an excessive increase in fat cell number (Kral et al., 1977). So far, there are no drug therapies that treat obesity at the level of adipocyte signaling. To our knowledge, inhibition of adipogenic differentiation could reduce excessive adipose tissue and ameliorate obesity associated diseases such as diabetes and hypertension (Marques, et al., 1998). Thus, with the dramatic increase in overweight and obesity of the past several decades, we hope that by studying fat biology we can gain better knowledge of the molecular mechanisms of fat development and identify crucial signaling pathways in adipogenesis, which may provide us greater opportunities in future design of anti-obesity therapies.

1.2 Fat biology in *Drosophila*

The fat body in *Drosophila*, or the adipose tissue functions not only in energy storage and intermediary metabolism but also in the innate immune response. The fat body is also the primary site of diacylglycerol and sugar biosynthesis, which resembles the function of vertebrate liver.

The fat body with other tissues, such as the somatic muscles, the heart, the somatic part of the gonad and most of the visceral muscles, are derived from the *Drosophila* embryonic mesoderm (Hartenstein and Jan, 1992; Hoshizaki et al., 1994; Technau, 1987; Riechmann et al., 1998). The fat body primordium consists of different groups of segmentally repeated cell clusters which are distinguished by their locations within the segment, their time of appearance and their genetic control (Riechmann et al., 1998; Moore et al., 1998; Miller et al., 2002; Vining et al., 2005).

According to what was described by Riechmann, the development of the fat body can be divided into three stages (Riechmann et al., 1998). The primary clusters and secondary clusters of fat body form in the first and second stages, respectively. During the first stage, development of the primary clusters starts when serpent (*srp*) expresses in segmentally repeated clusters within the trunk mesoderm in parasegment (PS) 4-9 at stage 10 (Abel et al., 1993; Rehorn et al., 1996; Sam et al., 1996). In the second stage, two secondary fat body clusters appear near each primary cluster in each parasegment. Rearrangements of the fat body primordium take place at the third stage of fat body development. The primary and secondary clusters finally form the mature fat body through expanding and coalescing. This program of the fat body development was confirmed by analysis of fat related molecular markers (Abel et al., 1993; Hoshizaki et al., 1994).

In the *Drosophila* embryo, along the anterior-posterior axis the mesoderm is divided into alternating domains by two pair-rule genes, *even-skipped* (*eve*) and *sloppy-paired* (*slp*), whose functions are essential for the development of these regions (Grossniklaus et al.,

1992; Cadigan et al., 1994; Riechmann et al., 1998 ; Riechmann et al., 1997). The positional information plays a distinct role in the fat body development. The primordia of the mesoderm localized in different positions of each parasegment are specified into different fates. For example, the progenitors of the fat body, the visceral muscles and the mesodermal glia cells arise from the *eve* domain, while the progenitors of the heart and most somatic muscles arise from the *slp* domain (Borkowski et al., 1995; Azpiazu et al., 1996; Riechmann et al., 1997). In PS4-12, the primordia of the dorsolateral fat body are in the *eve* domain and are activated by *en* and *hh* signaling, while the primordia of the ventral group locate in the *slp* domain and is activated by *wg*. An additional primordium in PS13 is specified by Dpp. This suggests that fat body formed in different domains is regulated by different signaling pathways.

Srp, a member of GATA family of transcription factors, plays an important role in promoting fat body development in flies. The expression of *srp* appears to depend on positive input from *eve*, *en* and *hh* (Azpiazu et al., 1996). It responds to the information for anteroposterior and dorsoventral patterning of the mesoderm (Azpiazu et al., 1996) and is a candidate target gene for specifying fat cells and triggering fat-cell differentiation (Sam et al., 1996; Rehorn et al., 1996). As mentioned above, although it is well established that positional information helps to guide cell-fate choices, little is known about the molecular mechanisms that control the final steps of fat-cell specification and the irreversible commitment to differentiate to a specialized cell type of fat body. Further definition of the fat body formation awaits analysis of the appropriate signaling pathways.

1.3 Adipocyte biology in vertebrates

Adipocytes or fat cells are cells that have differentiated and become specialized in the synthesis and storage of fat. Adipocytes are generally believed to originate from common pluripotent progenitors, known as mesenchymal stem cells, which can also give rise to variety of other cell types, such as myocytes and osteoblasts.

In mammals, adipocyte precursor cells give raise to two major cell populations with two distinct types of terminally differentiated adipocytes: white and brown, which composed white adipose tissue (WAT) and brown adipose tissue (BAT). White adipocytes are unilocular, storing excess energy in the form of triglyceride and distributing energy to the organism when needed, whereas brown adipose tissue (BAT) has the ability to dissipate energy through adaptive thermogenesis (Lowell and Spiegelman, 2000; Rosen et al., 2000). Brown adipocytes are multilocular and rich in characteristic mitochondria expressing the uncoupling protein 1 (UCP-1). Genetic ablation of BAT in mice causes obesity without hyperphagia, demonstrating the important energy dissipating role of this tissue (Lowell et al., 1993).

1.4 The physiological roles of adipocytes

Adipocytes have a number of physiological functions in the body with the most commonly recognized role being as a storage organ that maintains proper energy balance. They provide a massive energy reserve of calories in the form of lipids and mobilize energy

sources in response to hormonal stimulation (MacDougald and Lane, 1995).

In the past, adipocytes were thought to be merely a passive energy storing vessels. Since leptin has been identified as a specific fat cell-derived hormone (Zhang et al., 1994), a large body of work has clearly established that adipose tissue is a multifunctional organ that produces and secretes various compounds and signaling molecules termed adipokines and that interacts extensively with other organs in overall physiological and metabolic control. To date, more than 50 adipokines have been reported to be produced and released by adipocytes (Trayhurn and Wood 2004). They regulate feeding, energy metabolism, reproduction, immunity, and hematopoiesis, many of which are key regulators of chronic disease (Hamilton et al., 1995, Hotamisligil et al., 1995; Lefebvre et al., 1998; Morrison and Farmer, 1999; Trayhurn and Wood 2004).

Among the secretory products of adipocyte, fatty acids are quantitatively the largest products. It is well known that fatty acids themselves play a number of key roles in metabolism as a major metabolic fuel. They are normally secreted into the circulation for the purpose of absorption by other tissues such as the liver and muscle tissue. In these tissues, free fatty acids can be catabolized to produce energy or metabolized into other cellular compounds such as eicosanoids and phospholipids. However, elevations of free fatty acids can stimulate insulin secretion both in vitro and in vivo which may lead to hyperinsulinemia (Grill and Qvigstad, 2000). On the other hand, if free fatty acids concentrations remain consistently elevated, they prevent glucose-stimulated insulin secretion leading to the development of insulin resistance.

Another adipokine produced by adipocytes is TNF-α which was among the first secretory products from adipocytes that were originally described 11 years ago (Hotamisligil et al., 1995; Kern et al., 1995). TNF-α has distinct effects on adipose tissue including induction of insulin resistance, induction of leptin production, stimulation of lipolysis, suppression of lipogenesis. Therefore, TNF-α has been the subject of much research recently. One theory behind TNF-α's effect is a reduction of maximum insulin-stimulated glucose transport in insulin-sensitive tissues through alterations in insulin signaling, along with a reduction of glucose transporter synthesis (for review, see Qi and Pekala, 2000). Within the insulin signaling pathway, TNF-α is thought to interfere with insulin receptor substrate-1 (IRS-l) activity thereby preventing GLUT-4 transporters from reaching the cell surface where glucose is absorbed.

TNF-α plays a distinct role in limiting increase in fat mass. For example, it affects adiposity by decreasing BAT mass via p38-mediated apoptosis (Valladares et al., 2000). This reduction in BAT mass decreases thermogenic capacity, resulting in a positive energy balance and an increased risk of obesity. A similar regulation might take place in WAT, where TNF-α is also able to induce apoptosis (Prins et al., 1997). However, the signaling pathways mediating these TNF-α actions are not known. TNF-α can also induce adipocyte dedifferentiation, which was examined by exposing the cells to TNF-α (Ron and Habener, 1992). Taken together, these effects of TNF-α adipokine produced by adipocytes all tend to decrease adipocyte volume and number thereby limiting increase in fat mass.

Adipose tissue also secretes plasminogen activator inhibitor-1 (PAI-1) compound that

is the major physiological inhibitor of the tissue plasminogen activator and uroplasminogen activator in the regulation of fibrinolytic balance. PAI-1 inhibits the fibrinolytic activity of plasmin, thus increasing the risk for embolism or coronary artery disease (CAD) (Birgel et al., 2000). Studies found that the size of the adipose tissue, in particular the visceral adipose tissue, is closely associated to development of metabolic syndrome and cardiovascular diseases, and the greater the fat cell size and the adipose tissue mass, the greater is the contribution of adipose production to circulating PAI-1 (Juhan-Vague and Alessi, 1997; Loskutoff and Samad, 1998; Samad, and Loskutoff, 1996; Samad et al., 1997; Loskutoff et al., 2000). It is also noteworthy that preadipocyte is also an important production site for PAI-1 (Crandall et al., 1999).

Adipose tissue also expresses all components of the renin–angiotensin system (Cassis et al., 1988; Karlsson et al., 1998). Angiotensinogen and the enzymes expressed by adipose tissue are converted in the kidney to angiotensin II which stimulates prostacyclin production in a preadipocyte cell-line. Prostacyclin is well known as a potent promoter of adipose differentiation leading to increased adiposity (Darimont et al., 1994; Jones et al., 1997). In addition, angiotensin II may lead to the development of hypertension through increasing sodium retention (Tamura et al., 1994; Zorad et al., 1995; Jones et al., 1997).

Adipose tissue also secretes both androgens and estrogen, which when elevated can lead to problems such as polycystic ovary disease and infertility (Nestler, 2000) as well as induce certain reproductive cancers such as breast and endometrial cancer (Yoo et al., 2001).

Leptin is one of most important adipokines synthesized and secreted mainly by adipocytes. As mentioned previously, the identification of leptin (Zhang et al., 1994) led to the recognition that adipose tissue is an endocrine organ with adipocytes being major endocrine cells. Leptin is a potent cytokine that regulates energy expenditure, food intake, and the activity of the sympathetic nervous system. Leptin is a 16 KDa adipokine which mainly functions to signal the brain to inhibit food consumption (Havel, 2000). Along with insulin, it sends a negative feedback signal to the hypothalamus to inhibit food intake. When energy intake and/or adipose tissue mass decreases, leptin levels decrease which produces a stimulatory signal in the central nervous system (CNS) to increase food intake. Similarly, if energy intake and/or adipose tissue mass increase, leptin levels increase and subsequently signal the hypothalamus to limit food intake. In addition, increased circulatory leptin levels can stimulate energy expenditure by increasing body temperature and physical activity (Pelleymounter et al., 1995). The importance of leptin has been demonstrated in both humans and animals through hyperphagia, reduced energy expenditure and obesity that is associated with both leptin receptor defects in *db/db* mice and Zucker fatty rats as well as leptin deficiency in *ob/ob* mice (Havel, 2000). In addition to the function that regulates body weight by inhibiting food intake and stimulating energy expenditure, as mentioned above, leptin also has a number of other actions. In particular, leptin is a pleiotropic hormone whose multiple effects include regulation of endocrine function, reproduction, and immunity (Harris 2000; Trayhurn and Beattie, 2001).

1.5 Cell culture models for studying adipocyte development

Much of our current understanding of the molecular regulation of adipogenesis comes from in vitro studies of two types of cell lines: 1. multipotent stem cell lines, such as C3H10T1/2 and Balbc/3T3 cell lines and 2. preadipocyte cell lines, such as 3T3-L1, 3T3-F442A and Ob1771 cell lines (Green and Kehinde, 1974; Negrel et al., 1978). The former, which has not committed to the adipocyte lineage, can give rise to several cell types, including adipocytes, myocytes and chondrocytes, when treated with 5-azacytidine, an inhibitor of DNA methylation. In contrast, the preadipocyte cell lines have already undergone commitment. Upon reaching confluence, exposure to dexamethasone, methlisobutylxanthine and insulin leads to adipogenic differentiation of these cells to fully mature adipocytes.

To study adipogenesis in cell culture, we could have established primary cultures from rodent or human adipose tissue. Although primary preadipocytes may serve as a more physiologically relevant model of adipogenesis in vivo than immortalized cell lines, the isolation of preadipocytes from adipose tissue requires considerable effort, and these cells have a limited lifespan. Importantly, they are not homogeneous in their physiological function. It has been shown that rat adipose tissues from various anatomical sites exhibit difference in preadipocyte content and lipogenic activity (Caserta et al., 2001; Cousin et al., 1993). Depot-specific differences in fat metabolism also characterize human adipocytes (Lefebvre et al., 1998). Additionally, the proliferative and differentiative potential of human preadipocytes may reflect the weight status of the donor: preadipocytes isolated

from omental tissue of the massively obese have a significantly higher rate of proliferation in vitro than those from lean individuals (Roncari, 1990). In contrast, preadipocyte cell lines, such as 3T3-L1 and 3T3-F442A, have defined characteristics, are homogeneous, and are not known to differentiate into any other cell type. In addition, these cells respond to hormones during differentiation in culture in a similar fashion to observations made with similar agents that induce adipose tissue growth in vivo, including morphological changes associated with lipid accumulation, cessation of cell growth, expression of many lipogenic enzymes, and establishment of sensitivity to most or all of the key hormones that impact on this cell type. Therefore, these model cell lines provide a useful tool to study the adipocyte differentiation process (Gregoire et al., 1998; MacDougal and Lane, 1995). Of the preadipocyte models, 3T3-L1 cell line is one of the most widely used preadipocyte models to study the adipogenesis process.

1.6 Adipgenesis: a well defined program

The adipogenic differentiation program of 3T3-L1 cells mimicks hyperplasia of adipocytes in obesity (Shepherd et al., 1993; Gnudi et al., 1996). 3T3-L1 preadipocyte is a non-transformed cell line which is a continuous substrain of Swiss albino 3T3 murine cells developed through clonal expansion (MacDougald and Lane, 1995). Upon stimulation with the appropriate hormones, these cells can convert from a preadipose to an adipose-like phenotype. Differentiation of 3T3-L 1 preadipocytes is a well defined program that can be generally divided into four distinct stages: 1. Proliferation at preconfluence; 2. Growth

arrest at confluence; 3. Mitotic clonal expansion; and 4. Terminal differentiation (Cowherd et al., 1999; MacDougald and Lane, 1995). During the differentiation of 3T3-L1 preadipocytes, there is a coordinate expression pattern of specific preadipocyte and adipocyte markers associated with each of these stages (Fig 1). This section will outline the molecular events required for 3T3-L1 adipogenesis.

1.6.1 Proliferation at preconfluence

At preconfluent stage, 3T3-L1 preadipocytes resemble fibroblasts and replicate and proliferate in culture until they form a confluent monolayer. Preadipocyte Factor 1 (Pref-1) and C/EBPα undifferentiated protein (CUP) have been identified to be expressed in preadipocytes. Their expression levels fall upon stimulation of differentiation. Pref-1, an EGF-repeat containing transmembrane protein, is a cell surface protein that appears to be inhibitory of adipocyte differentiation and maintain preadipocytes in the undifferentiated state (see line A in Fig 1) (Smas and Sul, 1993; Smas et al., 1994; Smas and Sul, 1997).

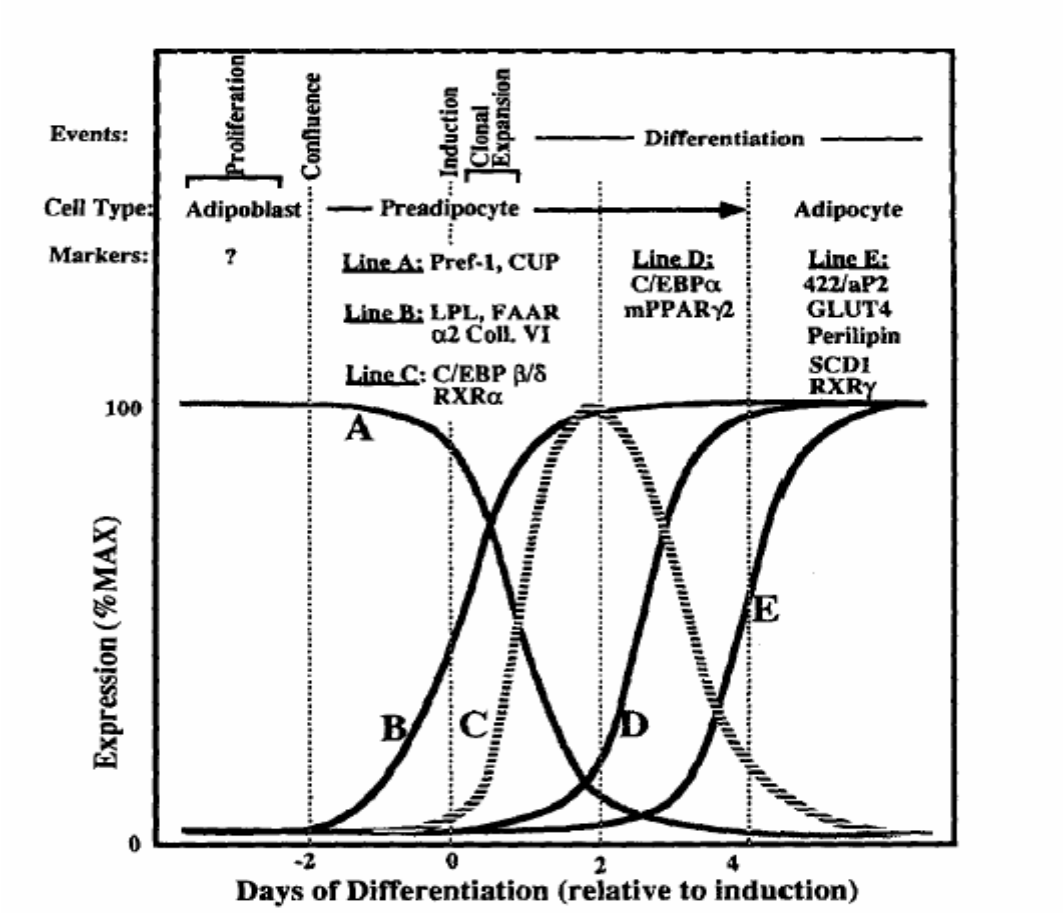

Figure 1 Stages in the adipocyte development program illustrating the events and temporal expression of various preadipoeyte and adipoeyte markers (Adopted from (MacDougald and Lane, 1995)). Preadipocyte differentiation is a well defined program that can be generally divided into four distinct stages: 1. Proliferation at preconfluence; 2. Growth arrest at confluence; 3. Mitotic clonal expansion; and 4. Terminal differentiation.

1.6.2 Growth arrest at confluence

At confluent stage, preadipocytes arrest at the G_0/G_1 cell cycle boundary because of cell contact inhibition. They begin to express the early markers of adipocyte differentiation which are not expressed in replicating preadipocytes at the preconfluent stage. Blocking cell cycle with pharmacological agents in actively growing preconfluent cells will also stimulate expression of these early markers (Amri et al., 1986). It appears that growth arrest is necessary for early marker expression.

The cell-cell contact induces the expression of lipoprotein lipase (LPL), the mouse equivalent of human $\alpha 2$ chain of Type VI collagen, and FAAR (fatty acid-activated receptor) (see line B in Fig 1) (Cornelius et al. 1988, Dani et al. 1990). LPL appear rapidly at confluence and remains highly expressed in fully differentiated adipocytes. Type VI collagen genes also appear rapidly in density-arrested cells but declines to 50% of maximal induction by day 16 after confluence (Dani et al., 1990). FAAR appears to mediate fatty acid transcriptional effects during adipogenesis (Amri et al., 1995).

1.6.3 Mitotic clonal expansion

Mitotic clonal expansion phase is after cell/cell interaction and growth arrest and precedes the adipogenic gene expression program. It refers to the three-day period during which confluent growth-arrested preadipocytes undergo cell division in response to hormonal stimulation. Following cessation of growth, the cells are induced to differentiate by exposure to the “adipogenic cocktail” consisting of DEX, MIX and insulin (DMI).

These growth arrested preadipocytes will synchronously reenter the cell cycle and undergo at least one round of cell division**.** However, postconfluent mitosis does not always lead to differentiation. Some mitogens, such as FGF and PDGF, promote postconfluent mitosis but fail to induce differentiation. Thus, DNA replication per se appears to be necessary but not sufficient for induction of differentiation. Presumably, DNA replication and the accompanying changes in chromatin structure increase the accessibility of *cis-elements* to *trans-acting* factors which activate (or derepress) transcription of the gene(s) that gives rise to the adipocyte phenotype (MacDougald and Lane, 1995).

Numerous studies have demonstrated that inhibiting mitotic clonal expansion prevents adipogenesis (Yeh et al., 1995b; Reichert and Eick, 1999; Tang et al., 2003). It is shown that mitotic clonal expansion is virtually a prerequisite for terminal differentiation, although there is some debate about the requirement for clonal expansion (Qiu et al., 2001). In fact, under conditions where mitotic clonal expansion does not occur, or occurs minimally, i.e. in the presence of DEX and MIX, the cells do not differentiate to the same extent. It is possible that DNA replication in these cells alters the accessibility of promoter elements to transactivating factors that control differentiation by functioning as positive or negative regulators.

During the early stage of adipogenesis that coincides with mitotic clonal expansion (0-24 hours), C/EBPβ and C/EBPδ are expressed in response to MIX and DEX, respectively (Cao et al., 1991; Yeh et al., 1995b), and retinoid X receptor α and γ (RXRα and RXR γ) are induced (Chawla and Lazar, 1994) (see line C in Fig 1).

1.6.4 Terminal differentiation

Following clonal expansion, preadipocytes exit the cell cycle and enter a quiescent phase referred to as the G_D state of growth arrest. This second state of quiescence is permissive for subsequent differentiation (Scott et al., 1982a, b). The growth arrest is required for subsequent differentiation. It is also noteworthy that even after differentiation has been initiated, the preadipocytes retain the ability to dedifferentiate and re-enter mitosis. Loss of these characteristics can be achieved by blocking cell-cell contact or exposing the cells to inhibitors of differentiation like tumor necrosis factor α (TNFα) (Ron and Habener, 1992) or retinoic acid (Hoerl et al., 1984). However, once the cells have exited G_D Phase and reach a specific point, they can no longer undergo mitosis or dedifferentiation, thus the cells have become committed to terminal differentiation (Wier and Scott, 1986; Wang and Scott, 1993).

Differentiating cells begin to express adipocyte-specific genes (MacDougald and Lane, 1995). Among these genes, two important transcription factors are expressed, PPARγ and C/EBPα (see line D in Fig 1), followed by the coordinate activation of adipose-specific genes including aP2, glycerol phosphate dehydrogenase, adipsin, Glut4 and stearoyl-CoA desaturase 1 (see line E in Fig 1). In addition, the cells lose their fibroblastic morphology due to a down-regulation of cytoskeletal proteins, actin, tubulin and vimentin (Spiegelman and Farmer, 1982; Bernlohret al., 1984; Cook et al., 1985), and they begin to accumulate small cytoplasmic lipid droplets.

Terminal differentiation is maintained, at least in part, through the expression of C/EBPα and PPARγ. C/EBPα and PPARγ act synergistically to activate transcription of genes that produce the adipocyte phenotype (Tontonoz et al., 1994b).

1.7 Adipocyte-related genes

Adipogenesis involves cooperative interaction between members of the CCAAT/enhancer-binding protein (C/EBP) and peroxisome proliferator-activated receptor (PPAR) families, the primary adipogenic transcription factors. There are other factors that function to suppress fat cell differentiation. This section provides a brief review of pro-adipogenic factors and anti-adipogenic factors used in our experiments, including PPARs and C/EBPs, GATA factors, GILZ and preadipocyte factor-1 (Pref-1).

1.7.1 Pro-adipogenic factors

1.7.1.1 The C/EBP family

C/EBPs belong to the basic leucine zipper (bZIP) family of transcription factors. They share similar structural characteristics including an activation domain located in the N-terminus, a basic DNA binding region located in the C-terminus, and a leucine zipper domain which is responsible for the dimerization potential of these proteins (Hurst 1994). Before binding DNA, C/EPBs must dimerize with C/EBP family members or with other transcription factors, such as members of the NF-κB and Fos/Jun families (Vinson et al.,

1993). The C/EBP family has six members: C/EBPα, C/EBPβ, C/EBPγ, C/EBPδ, C/EBPε and C/EBPζ. C/EBPβ and δ are both expressed in liver, lung, adipose, and intestine. Expression pattern of C/EBPα is similar to C/EBPβ and δ, with additional measurable expression in adrenal gland, peripheral blood mononuclear cells, and placentas. C/EBPγ and ζ are expressed ubiquitously. C/EBPε expresses highly in promyelocyte and late myeloblast-like cell lines (Lekstrom-Himes and Xanthopoulos 1998). Of the six members of the C/EBP family that have been identified, adipose tissue expresses C/EBPα, C/EBPβ, C/EBPγ, C/EBPδ, and C/EBPζ.

The C/EBP family of transcription factors have a profound impact on the regulation of adipocyte differentiation. They express in a distinct temporal pattern during adipogenesis. C/EBPβ and C/EBPδ express early and transiently in response to hormonal stimulation and play an early role in differentiation. cAMP and glucocorticoid are responsible to induce the expression of C/EBPβ and C/EBPδ, respectively. The timing of this activity coincides with cell-cycle progression through S phase (Tang et al., 1999). Upon activation, C/EBPβ and C/EBPδ synergistically promote the expression of PPARγ and C/EBPα, two of the central regulators of adipogenesis (Wu et al., 1996; Yeh et al., 1995a). Although embryonic fibroblasts lacking either C/EBPβ or δ showed slight reductions in adipogenic potential, cells lacking both C/EBPβ and δ exhibit severe defects in adipocyte development (Tanaka et al., 1997). Of the two isoforms, C/EBPβ appears to dominate, not only in duration of expression but also in degree of activity. C/EBPβ homodimers and C/EBPβ-C/EBδ heterodimers appear to be the preferred C/EBP complexes for activation of PPARγ gene

expression (Wu et al., 1996). C/EBPβ is also the more potent isoform: ectopic expression of C/EBPβ is sufficient to induce the differentiation of 3T3-L1 cells without the addition of hormonal inducers; C/EBPδ, on the other hand, still requires the prodifferentiative agents (Yeh et al., 1995a). C/EBPβ may also play a role in fate determination. When ectopically expressed in NIH-3T3, C/EBPβ (but not δ) is able to determine these cells to the adipose lineage (Wu et al., 1996; Yeh et al., 1995a).

C/EBPα plays a central role in the development of adipocytes. Its expression is induced during the terminal phase of differentiation. It induces the expression of many adipocyte specific genes through binding to the C/EBP-binding sites on their promoter, including aP2, GLUT-4, steroyl-CoA desaturase, PEPCK, insulin receptor, and UCP (Christy et al., 1989, 1991; Kaestner et al., 1990; McKeon and Pham, 1991; Park et al., 1993; Park et al., 1999). Inhibition of C/EBPα function by antisense technique blocks differentiation in 3T3-L1 preadipocytes (Lin and Lane, 1992). On the other hand, constitutive expression of CEBPα promotes adipocyte differentiation in the absence of hormonal inducers (Freytag et al., 1994; Lin and Lane, 1994). C/EBPα-null mice have dramatically reduced fat accumulation in WAT and BAT pads (Wang et al., 1995), which further illustrates the importance of C/EBPα in adipogenesis (Wang et al., 1995).

1.7.1.2 The PPAR family

Nuclear receptors are ligand-activated transcription factors that activate target genes by binding specific DNA sequences, called response elements, and regulate gene expression in response to small lipophilic compounds such as testosterone, estrogen,

retinoic acid, and vitamin D. PPARs were first discovered by Issemann and Green (Issemann and Green, 1990). These factors have a structure similar to other members of the nuclear hormone receptor superfamily and are activated by ligands such as the peroxisome proliferators, clofibric acid, prostaglandins and thiazolidinedione antidiabetic drugs. Accordingly, these factors were called peroxisome proliferator–activated receptors (PPARs). The functional domains of the PPARs consist of N-terminal, DNA-binding (DBD) and ligand-binding (LBD) domain. PPARs, like other members of the nuclear receptor superfamily, bind to ligand and this causes a conformational change in the LBD. Ligand-bound PPAR then forms a heterodimeric complex with other transcription factors, such as 9-cis-retinoic acid receptor (RXR) and these heterodimers regulate transcription of various genes.

There are three identified subtypes, (PPAR -α, -δ, and -γ) and are encoded by three separate genes (Dreyer et al., 1992; Lemberger et al., 1996; Tontonoz et al., 1994a). PPARα is highly expressed in liver and plays an important role in the oxidation of fatty acids in the liver, which is a crucial adaptive response to nutritional challenges such as fasting (Sher et al., 1993). PPARδ is more ubiquitously expressed. PPARγ is expressed in spleen, retina, hematopoietic cells (Lemberger et al., 1996), and epithelial cells of the colon, prostate, mammary gland (Jiang et al., 1998; Kubota et al., 1998; Lefebvre et al., 1998; Mueller et al., 1998; Saez, 1998), and most abundantly and specifically in adipose tissue (Tontonoz, 1994a). Activators of PPARs promote adipose differentiation of preadipocytes (Chawla et al., 1994), myoblasts (Teboul et al., 1995), and the multipotent C3H10T 1/2

cells, and ectopic expression of certain PPARs in the presence of their activators can induce differentiation of NIH-3T3 fibroblasts (Forman et al., 1995; Brun et al., 1996). The ability of ectopically expressed PPAR isoforms to induce adipogenesis has been directly compared in NIH-3T3 cells (Brun et al., 1996). PPARγ was by far the most active and strongly promoting adipogenic differentiation of NIH-3T3 cells when supplemented with an appropriate ligand. PPARα, while less robust, can also promotes adipogenesis, whereas ectopic PPARδ expression does not promote adipose differentiation. The appearance of PPARγ in several adipose cell lines, including 3T3-L1 and 3T3-F442A, precedes that of several markers of the adipose differentiated state, such as adipocyte lipid binding protein (aP2), phosphoenolpyruvate carboxykinase (PEPCK) and C/EBPα, which indicates its role in transcriptional regulation of adipogenesis (Tontonoz et al., 1994a, b, 1995a).

PPARγ plays a crucial role in the function of many, and perhaps most, fat-cell-specific genes. PPARγ binding is absolutely required for the function of the fat-selective enhancers for the aP2 and PEPCK genes in cultured fat cells (Tontonoz et al., 1995a). In addition, PPARγ regulates the expression of several other genes that coordinate fatty acid uptake and storage, such as adipsin, lipoprotein lipase, acyl-CoA synthetase, and fatty acid synthase (Schoonjans et al., 1997).The in vitro analysis of the PEPCK gene has been extended in vivo, where activation of this promoter in fat was shown to be dependent on a PPARγ binding site, whereas expression in other tissues was not (Devine et al., 1999). Ectopic expression of PPARγ in non-adipogenic Swiss 3T3 and NIH-3T3 fibroblasts triggers the adipogenic program (Hamm et al., 1999).

It was known that PPARγ and C/EBPα appear to induce expression of each other and act synergistically to promote adipocyte differentiation. However, ectopic expression of either C/EBPα or PPARγ in NIH 3T3 cells fails to stimulate adipogenesis in the absence of inducing agents; ectopic expression of both C/EBPα and PPARγ, on the other hand, stimulates differentiation readily with or without inducing agents (Tontonoz et al., 1994b). Of the two, PPARγ appears to have a leading role in the adipogenic hierarchy: ectopically expressed C/EBPα could not rescue adipogenesis in PPARγ-null fibroblasts (Rosen et al., 2002), but exogenous PPARγ induced adipogenesis in fibroblasts from mice deficient in C/EBPα (Wu et al., 1999). Interestingly, the ability of PPARγ to promote adipogenesis is not limited to fibroblastic cells. Myoblastic cell lines can also be converted to adipocytes, particularly when the cells co-express C/EBPα in addition to PPARγ (Hu et al., 1995).

PPARγ exists in two protein isoforms that are created by alternative promoter usage and alternative splicing at the end of the gene; PPARγ2 contains 30 additional amino acids at the N terminus compared with PPARγ 1 (Fajas et al., 1997; Zhu et al., 1995). The two PPARγ isoforms differ in tissue distribution: PPARγ1 is the more ubiquitous isoform, while PPARγ2 appears primarily in fat (Vidal-Puig et al., 1997).

In human tissues, PPARγ is expressed highly in fat, lower in heart and skeletal muscle, and lowest in liver, pancreas, and kidney (Mukherjee et al., 1997). Additionally, PPARγ also has expression in human bladder, ileum, and spleen (Guam et al., 1997). Patients with rheumatoid arthritis exhibit enhanced expression of PPARγ in macrophages, the synovial lining layer, fibroblasts, and endothelial cells (Kawahito, et al., 2000). Kitamura et al.

detected the expression of PPARγ in six human colon cancer cell lines (Kitamura et al., 1999). Both isoforms have been also detected in skeletal muscle, with PPARγ1 the dominant one (Mukherjee et al., 1997). In mouse tissues, the patterns of PPARγ1 and PPARγ2 expression reflect those found in human tissues. Interestingly, mature adipocytes express high levels of both isoforms, but the stromal vascular fraction contains only PPARγ1 mRNA (Vidal-Puig et al., 1996). Recently, it has been shown that PPARγ2, and not PPARγ1, is required for adipogenesis (Ren et al., 2002).

1.7.2 Anti-adipogenic factors

1.7.2.1 GATA factors

GATA are antiadipogenic factors. Memebers of the GATA family of transcription factors share highly conserved zinc finger DNA binding domains and bind specifically to a consensus DNA sequence (A/T)GATA(A/G) (Evans and Felsenfeld, 1989; Tsai et al., 1989; Weiss and Orkin, 1995). They have been implicated in a variety of developmental processes, including the differentiation and proliferation of adipocyte precursors in invertebrates and vertebrates. The Gata transcription factor family consists of six members (Gata1-6). Gata-1, the founding member of the GATA protein family, positively regulates transcription of most erythroid-specific genes by binding to the promoter regions of such genes (Orkin, 1992). Since its discovery, five other GATA proteins have been identified in vertebrates (Arceci et al., 1993; Morrisey et al., 1996; Morrisey et al., 1997; Yamamoto et al., 1990) and orthologs exist in invertebrates as well (Abel et al., 1993; Ramain et al., 1993;

Rehorn et al., 1996). The GATA family is divided into two groups, the haematopoietic (Gata1, 2 and 3) (Weiss and Orkin, 1995; Cantor and Orkin, 2002; Mullen et al., 2001; Patient and McGhee, 2002) and the heart and gut group (Gata4, 5 and 6) (Molkentin, 2000). The GATA members share their highest homology in the Zn-finger DNA-binding domain, while outside the DNA-binding domain, they are quite different.

GATA members function as transcription factors in a broad range of settings and have been demonstrated to regulate both cell differentiation and cell fate determination. Murine Gata2 and Gata3 are specifically expressed in white adipocyte precursors and their downregulation sets the stage for terminal differentiation (Tong et al., 2000). Ectopic GATA expression in 3T3-L1 and 3T3-F442A cells results in dramatic decreases in genes such as PPARγ and C/EBPα, as well as late adipocyte markers such as aP2 and adipsin. Along with this altered gene profile is full inhibition of lipid accumulation, as the preadipocytes expressing GATA-2 or-3 are locked into a fibroblast-like appearance. It was demonstrated that Gata-2 and -3 suppress adipocyte differentiation through at least two pathways: inhibition of PPARγ expression by binding to its promoter, and interference with C/EBP function through protein-protein interaction (Tong et al., 2000; Tong et al., 2005). Gata3-deficient embryonic stem cells exhibit an enhanced capacity to differentiate into adipocytes, and defective Gata2 and Gata3 expression is associated with obesity (Tong et al., 2000). Thus, Gata2 and Gata3 are potential preadipocyte markers and play important roles in the regulation of adipocyte differentiation.

Interestingly, the *Drosophila serpent* (*srp*) gene, which also belongs to the GATA

family of transcription factors, is critical for fat body formation (Abel et al., 1993; Rehorn et al., 1996). Expression of *srp* with UAS/Gal4 system in the mesoderm induces the formation of ectopic fat cells. Therefore, it appears that GATA family of transcription factors is involved in fat development of both invertebrates and vertebrates. As mentioned in section 1.2, in addition to the *srp* gene, the *Drosophila* fat body expresses many other genes that share structural or functional homology with genes found in mammalian adipose tissue (Riechmann et al., 1998; Moore et al., 1998; Miller et al., 2002; Vining et al., 2005). This encourages us to further investigate whether the signal cascades that function in fat body formation in *Drosophila* also play a role in the formation of adipose tissues in vertebrates.

Shh signaling may play a role upstream of Gata2 (Craven et al., 2004). Similar to Gata2, expression of Smo-M2, a constitutively active form of the Shh receptor Smo, induced the development of 5-HT neurons and mimiced the effect of Shh signaling. Only cells that expressed Smo-M2 ectopically expressed Gata2 and only cells that ectopically expressed Gata2 become 5-HT positive. In addition, when Gata2 function was blocked by DN-G2, a dominant-negative form of Gata2, cells that expressed Smo-M2 failed to differentiate into 5-HT neurons, indicating that Gata2 serves as a mediator between Shh and 5-HT development. This evidence shows that Hh may acts upstream of GATA.

1.7.2.2 GILZ

Glucocorticoid induced leucine zipper (GILZ) belongs to the leucine-zipper family of transcription factors. GILZ contains a leucine zipper, a C-terminal proline-rich and acidic

region, an N-glycosylation site, and several sites for threonine and serine phosphorylation. Northern blot analysis detected highest GILZ expression in brain, lung, spleen, skeletal muscle, and in all hematopoietic cell lineages examined. Lower expression was found in heart and kidney, and no expression was found in liver and pancreas. Western blot analysis found complete concordance between mRNA and protein expression, suggesting that GILZ expression is regulated at the transcriptional level. GILZ binds to a C/EBP tandem DNA element in the PPARγ2 promoter as a sequence-specific transcriptional repressor. It inhibits the transcription of the PPARγ2 gene and antagonizes glucocorticoid (GC) function by inhibiting GC-induced adipogenesis. The expression of downstream adipocyte differentiation marker genes (for example, LPL and adipsin) is all inhibited in GILZ-expressing 3T3-L1 cells (Shi et al., 2003).

GILZ is not only able to inhibit adipogenesis through repression of the PPARγ2 gene and glucocorticoid (GC) function, but also functions downstream of Shh (Ingram et al., 2002). GILZ was identified in a microarray screen in the Shh responsive cell line, C3H10T1/2, designed to search for downstream target genes regulated by the Hh pathway. Northern Blot analysis further validated GILZ as a target gene of Shh.

1.7.2.3 Pref-1

Pref-1, a member of the EGF-like family of proteins, also has anti-adipogenic activity. Pref-1 mRNA, though abundant in 3T3-LI preadipocytes, disappears during differentiation (Smas et al., 1993). Constitutive expression of pref-1 inhibits differentiation blocking the expression of C/EBPα and PPARγ (Smas et al., 1997). Dexamethasone promotes 3T3-L1

adiponesis by down-regulation of pref-1 gene expression (Smas et al., 1999). In vivo function of pref-1 was examined in transgenic mice by specifically expressing in adipose tissue the full ectodomain corresponding to the large cleavage product of Pref1 (Lee et al., 2003). The transgenic mice had a substantial decrease in total fat pad weight, reduced expression of adipocyte markers and adipocyte-secreted factors in adipose tissue. Pref1 transgenic mice with a substantial loss of adipose tissue exhibited hypertriglyceridemia, impaired glucose tolerance, and decreased insulin sensitivity. Therefore, there is inhibition of adipogenesis by Pref1 in vivo and the resulting impairment of adipocyte function leads to the development of metabolic abnormalities.

1.8 Hedgehog signaling is highly conserved pathway

Developmental pathways first elucidated by genetic studies in *Drosophila melanogaster* have been found to be conserved in vertebrates. Indeed, the TGFβ, Wnt, Notch, receptor tyrosine kinase, and Hedgehog pathways are used repeatedly in early and later development within both invertebrates and vertebrates (Gerhart, 1999). Among them, the Hedgehog (Hh) signaling pathway is one of the most prominent ones. It plays a crucial role in embryonic development of many tissues and cell differentiation from invertebrates to vertebrates (Ingham and McMahon, 2001).

1.8.1 Hh signaling in *Drosophila*

Hh signaling pathway has been most extensively studied in *Drosophila*. Hh was

identified originally in *Drosophila melanogaster* in a genetic screen for mutations disrupting the polarity of the segments of the cuticle (segment polarity genes) (Nusslein-Volhard and Wieschaus, 1980). The name hedgehog is derived from the appearance of the hh mutant *Drosophila* embryo, which is covered with bristles and, in this sense, resembles the small mammals known as hedgehogs. In view of this mutant phenotype, hh is included in the segment polarity class of genes, which are those that appear to pattern the anterior/posterior axis of each segment of the larval body (Nusslein-Volhard and Wieschaus, 1980). Cloning of the hh gene (Lee et al., 1992; Mohler and Vani, 1992; Tabata et al., 1992) revealed that it coded for a secreted protein, which suggested that intercellular cell signaling was involved in patterning the *Drosophila* larval cuticle. During the years that followed the discovery of the hh gene, it was shown that hh plays a central role in numerous processes of *Drosophila* embryogenesis, including patterning the wing (Mohler, 1988; Basler and Struhl, 1994; Tabata and Kornberg, 1994), leg (Diaz-Benjumea et al., 1994), and eye discs (Heberlein et al., 1995), as well as being a regulator of several other processes, including germ-cell migration (Deshpande et al., 2001), and development of the optic lamina (Huang and Kunes, 1996; Huang and Kunes, 1998), gonad (Forties et al., 1996; Zhang and Kalderon, 2000), abdomen (Struhl et al., 1997), gut (Pankratz and Hoch, 1995), and tracheal system (Glazer and Shilo, 2001).

The Hh protein is synthesized as an inactive ~45 kD precursor that is first autocatalytically cleaved to produce a ~19 kD N-terminal (Hh-N) active polypeptide and a C-terminal (Hh-C) fragment (Lee et al., 1994; Porter et al., 1996). Upon autocleavage, a

molecule of cholesterol becomes covalently attached to the C-terminus of Hh-N (Porter et al., 1996). This cholesterol modification mediates controlled release and movement of Hh from the sending cell (Ingham and McMahon, 2001), perhaps by localizing the protein to lipid rafts, membrane microdomains with specialized signaling functions (Rietveld et al., 1999). The N-terminal region of the cholesterol modified Hh is further modified by addition of palmitate that is essential for its signaling activity (Amanai and Jiang, 2001; Chamoun et al., 2001; Lee and Treisman, 2001; Micchelli et al., 2002; Pepinsky et al., 1998). A key issue in Hh signaling is to understand how the lipid-modified Hh is released from its site of synthesis and subsequently moves through a field of cells. The mechanism by which N-Hh acts at long range is still unclear. It is known that such movement requires the activity of Tout-velu (*Ttv*), which is a type II transmembrane (TM) protein. The genetic analysis of *Ttv* in the wing imaginal disc elegantly demonstrated impaired Hh diffusion in the absence of *Ttv* activity (Bellaiche et al., 1998).

Two distinct membrane proteins are implicated in the receipt of the Hh signal and in the activation of the Hh signaling pathway. One is Patched (Ptc), a 12-pass transmembrane protein (Nakano et al., 1989; Hooper and Scott, 1989) and another is Smoothened (Smo), a 7-pass transmembrane protein, which is suppressed by Ptc in the absence of Hh (Alcedo et al., 1996; van den Heuvel and Ingham, 1996). Ptc is a negative regulator of the pathway required to limit Hh diffusion and to repress induction of Hh target genes (Chen and Struhl, 1996; Johnson and Scott, 1997). Binding of Hh ligand inactivates Ptch, de-repressing Smo and resulting in positive Hh pathway signaling (Ingham and McMahon, 2001). Smo

transduces Hh signaling into nuclei through activation of the Cubitus interruptus (Ci) protein, which induces the transcription of Hh target genes.

So far, all functions of Hh are mediated by the transcription factor Ci, which can act both as a repressor and as an activator of target genes (Methot and Basler, 1999). Ci is regulated by phosphorylation, proteolytic cleavage, and subcellular localization (Ingham and McMahon, 2001). Regulation of Ci occurs in a cytoplasmic complex containing Ci, the kinesin-like protein Costal-2 (Cos2), the serine-threonine kinase Fused (Fu), and the Suppressor of Fused (Su(Fu)) protein (Robbins et al., 1997; Sisson et al., 1997; Lefers et al., 2001). In the absence of Smo function, full-length Ci (Ci-155) is phosphorylated by Protein Kinase A (PKA) and tethered to microtubules by Cos-2 (Johnson et al., 1995; Ohlmeyer and Kalderon, 1998; Wang et al., 2000). The activities of both PKA and Cos-2 lead to the proteolytic processing of Ci to a shorter form (Ci-75) which contains the zinc finger DNA-binding domain and an N-terminal repression domain (Aza-Blanc et al., 1997). This truncated form of Ci (Ci75) dissociates from microtubules and translocates into the nucleus and represses Hh target genes including Dpp (Aza-Blanc et al., 1997) and Hh (Methot and Basler, 1999). Modest levels of Hh signaling cause the dissociation of the Ci-Cos2 complex from microtubules and the simultaneous inhibition of Ci cleavage. Hh signaling, in addition to preventing Ci-155 proteolysis, dissociates Ci-155 from the complex and allows its transport to the nucleus (Chen et al., 1999). Full-length Ci binds to Su(Fu) in the cytoplasm, which retain Ci in the cytoplasm (Methot and Basler, 1999). It is only with maximal activation of Smo that Fused acts upon Su(fu) and leads to the dissociation of

Su(fu) from full-length Ci (Ohlmeyer and Kalderon, 1998; Methot and Basler, 1999). Once in the nucleus, Ci associates with the Creb binding protein (CBP) transcriptional co-activator and activates the transcription of target genes. It is shown that the truncated form of Ci acts as a transcriptional repressor of Hh pathway genes, while in the presence of Hh the full length Ci acts as a transcriptional activator of target genes.

1.8.2 Hh signaling in vertebrates

A major excitement derived from research in *Drosophila* was the realization that many central signaling pathways had been highly conserved throughout evolution. Vertebrate homologs of many segment polarity genes, such as hh, have been identified, and in most cases, a single gene in *Drosophila* corresponds to a family of related homologs in vertebrates (Hammerschmidt et al., 1997). Although there is only one Hh in *Drosophila* that regulates different aspects development, vertebrate species proved to have several hedgehog genes (Echelard et al., 1993; Krauss et al., 1993; Riddle et al., 1993). There are three types of mammalian Hh proteins, Sonic hedgehog (Shh), Indian hedgehog (Ihh) and Desert hedgehog (Dhh). It is known that the initiation of signaling is similar to that in the *Drosophila* system: Hh protein binds to Ptc and relieve its inhibition of Smo. In addition, there are vertebrate homologs of Su(Fu), Fu, CBP, and PKA that are involved in Hh signaling. The key transcriptional mediators of Hh signaling in vertebrates are three Gli transcription factors (Gli1~3), which are believed to divide the *Drosophila* Ci activator and repressor functions among themselves. Fig 2 shows the hedgehog (hh/Shh) signaling

pathway in both the *Drosophila* (e.g. hh) and vertebrate (Shh). Many (or all) of the components of Hh signaling known in *Drosophila* have been identified in vertebrates. In summary, Hh pathways are highly conserved between vertebrates and invertebrates.

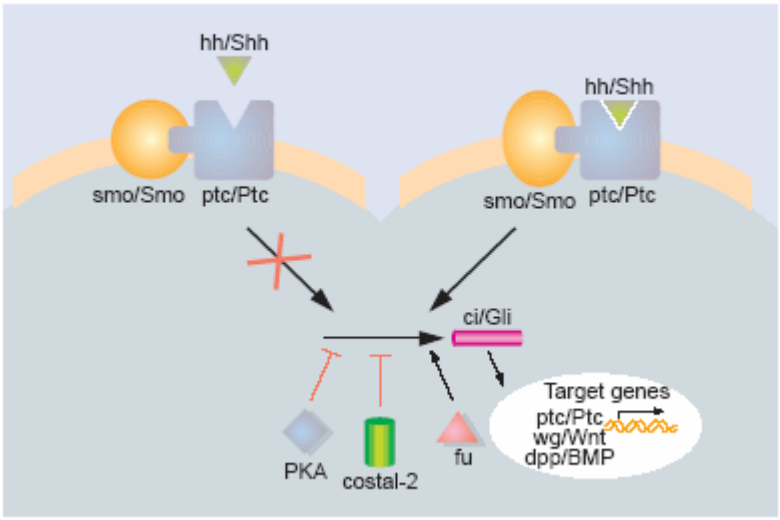

Figure 2 The Sonic hedgehog (hh/Shh) signaling pathway. The pathway as shown has been largely deduced from *Drosophila* work, although many of the basic features are present in vertebrates. The *Drosophila* (e.g. hh) and vertebrate (e.g. Shh) homologs are indicated, although the functional equivalence in vertebrates has not been conclusively established for some of the factors. Patched (Ptc) binds to and is probably the receptor for hh. Smoothened (Smo), another transmembrane protein, initiates the intracellular cascade. In the absence of Shh, Ptc antagonizes the actions of Shh by inhibiting Smo (left). Following Shh binding to Ptc, Smo is released from repression, and signal transduction occurs (right). Other components identified in *Drosophila* exert positive [fused (fu)] or negative [costal-2, protein kinase A (PKA)] influences. The pathway culminates in the transcription factor cubitus interruptus (ci) in *Drosophila*, or the GLI factors in vertebrates. In response to Shh, transcription of Ptc, wingless (wg), and decapentaplegic (dpp) are increased. The human homologs of the latter two genes are the WNT family and the members of the transforming growth factor b family, which encode the bone morphogenetic proteins (BMPs). Human homologs have not been identified for all of the components of the pathway. (Adopted from (Ming et al., 1998))

1.8.3 Hedgehog signaling in osteogenesis

The conserved Hh signaling has been shown to be involved in many important developmental processes in vertebrate, including osteogenesis (Ingham, and McMahon, 2001, Lanske, et al., 1996, Kinto, et al., 1997, Parmantier, et al., 1999, Pola, et al., 2001, Vortkamp, et al., 1998).

Misexpression experiments in chick first identified Ihh as a regulator of chondrocyte differentiation (Vortkamp et al., 1996). It is produced by chondrocytes in the early stage of terminal differentiation and acts as a morphogen to regulate growth of adjacent proliferative chondrocytes. It can also regulate the rate of differentiation of chondrocytes through its stimulation of parathyroid hormone-related protein (PTHrP). It recruits early progenitors toward the osteoblast lineage and induces early osteoblasts to differentiate into mature osteoblasts. By providing crucial local signals from prehypertrophic and hypertrophic chondrocytes to both chondrocytes and preosteoblasts, *Ihh* couples chondrogenesis to osteogenesis in endochondral bone development (Chung et al., 2001).

Shh, another member of the conserved Hh family, is able to induce ectopic bone formation in vivo when injected in mesenchyme in a manner similar to BMP-2. Shh is also able to induce alkaline phosphatase activity (ALP), a marker of osteoblast differentiation in the osteoblast cell line MC3T3-E1 in vitro (Nakamura et al., 1997; Yuasa et al., 2002), and also increases selectivity in the differentiation of multipotent mesenchymal cells into the osteoblast lineage (Spinella-Jaegle et al., 2001; Wu et al., 2004). In addition, Shh and BMPs synergistically induced mRNA expression of ALP and osteocalcin (OC) in

C3H10T1/2 cells and murine primary osteoblast cells (Yuasa et al., 2002).

Ablation of the function of either Shh or Ihh gene in mice causes different forms of skeletal defect, indicating that both are essential for skeletal development but they play different roles in this process. Shh-deficien mice have abnormalities of early skeletal development with severe growth retardation, lack of vertebrae, and distal limb structures (Chiang et al., 1996). This suggests a role for Shh in regulation of skeletal patterning. Ihh-deficient mice have short limbs but they are considerably more developed than those of Shh deficient animals. The absence of mineralized bone structures in Ihh-deficient animal indicates a role for Ihh in the coordination of multiple cellular events during endochondral bone development including chondrocyte proliferation and differentiation as well as osteoblast differentiation (St-Jacques et al., 1999). Mutations in the Ihh gene have been linked to two inherited skeletal developmental defects: brachydactyly type A-1 and acrocapitofemoral dysplasia, clearly implicating Ihh as a key regulator of skeletal development in humans (Gao et al., 2001; Hellemans et al., 2003).

Hypothesis:

The ability to store energy as fat is a fundamental property that has been conserved from invertebrates to vertebrates. It provides a massive energy reserve that can be mobilized upon conditions of nutritional deficiency. Abnormalities in fat accumulation produce pathological states in human. The incidence of overweight and fat-related diseases, such as obesity and type II diabetes, has risen dramatically throughout the world. Therefore,

understanding the molecular events that govern the determination and differentiation of adipose tissue are of potential clinical importance for developing therapeutic strategies for disease treatment.

Adipocytes are generally believed to originate from common mesodermal progenitor cells that can also give rise to variety of other cell types, such as myocytes and osteoblasts (Taylor and Jones 1979). Although a large body of literature has demonstrated a well-defined program for the late stage of adipocyte differentiation, relative little is known about the commitment of the pluripotent stem cells into the adipocyte lineage. Development of vertebrate adipose tissue occurs at multiple, anatomically distinct sites in a stereotypical fashion, which suggests that it is under the influence of developmental cues (Cinti, 2000; Rosen and Spiegelman, 2000). These cues might be from developmental signalling pathways, such as Wnt, BMP and Hh, which have been conserved from invertebrates to vertebrates (Cadigan and Nusse, 1997; Graff. 1997; Ingham and McMahon, 2001; Lall and Patel, 2001).

Recent studies on Hh function in osteogenesis (Kinto et al., 1997; Bitgood and McMahon, 1995) have found Shh and BMPs synergistically induced osteogenesis in multipotent mesenchymal cell line C3H10T1/2 (10T1/2). At the same time, fewer adipocytes were found, suggesting that Shh might play an inhibitory role in the regulation of adipogenesis. However, other data reported in literature supported a pro-adipogenic role for Hh signaling, which is conflicting with the anti-adipogenic role of Hh seen in 10T1/2 cells. Since the potential role of Hh on adipogenesis has been inferred primarily from the

modest reduction in adipocyte formation, a byproduct of its effect on osteogenesis observed only in sufficiency tests, it could produce spurious results (Graff et al., 1994; LeSueur et al., 2002; LeSueur and Graff, 1999; Peters et al., 1999; Slack, 1991; Spinella-Jaegle et al., 2001; van der Horst et al., 2003; Wu et al., 2004). Therefore, key tests, including assessing necessity in adipogenic conditions, are needed.

We hypothesize that Hh signaling plays a conserved role in inhibiting adipogenesis from invertebrates to vertebrates. To test this, throughout the rest of this dissertation, we will examine the role of Hh signaling in adipocyte development by sufficiency and necessity tests in both invertebrate and vertebrate model systems and explore the underlying mechanism by searching for the downstream target genes.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Cell model

C3H101/2 cells are multipotent stem cells, which were isolated from a line of C3H mouse embryo cells (Reznikoff et al., 1973). When treated with 5-azacytidine, these cells can generates several cell types, including adipocytes, myocytes and chondrocytes. NIH-3T3 is another multipotent stem cell line, which was established from NIH Swiss mouse embryo cultures (Jainchill et al., 1969). Under appropriate condition, it can be converted to mature adipocytes (Wu et al., 1995). 3T3-L1 preadipocyte is a non-transformed cell line, which is a continuous substrain of Swiss albino 3T3 murine cells developed through clonal expansion (Green and Meuth, 1974). These cells can be converted from a preadipose to adipose-like phenotype when appropriately stimulated. Lastly, these cells are capable of differentiating in culture in response to agents that induce adipose tissue differentiation in vivo (MacDougald and Lane, 1995).

2.2 Adipocytes cell culture

C3H10T1/2 cells, NIH-3T3 and 3T3-L1 cells were purchased from American Type Culture Collection (Rockville, MD). C3H10T1/2 cells were cultured with α-MEM (Invitrogen) supplemented with 10% heat inactivated fetal calf serum, 10 units/ml

penicillin and 10 μg/ml streptomycin in 5% CO_2 at 37°C. NIH3T3 cells and 3T3-L1 cells were cultured in DMEM (Invitrogen) supplemented with 10% heat inactivated fetal calf serum, 10 units/ml penicillin and 10 μg/ml streptomycin in 5% CO_2 at 37°C. Cells were passaged before confluence and discarded after 10 passages. Media changes were performed every other day during cell maintenance and adipogenesis. 3T3-L1 cells were induced to form adipocytes as described (MacDougald and Lane, 1995; McKay et al., 2003). Briefly, cells were seeded at a density of 3.3 x $10^3/cm^2$ in plates and grown to confluence at 37°C in DMEM with 10% fetal bovine serum (FBS). At 2 days postconfluence (designated "day 0") cell differentiation was induced with 1 μg/ml insulin in DMEM containing 10% FBS and further maintained in induction media until analysis. For C3H10T1/2 and NIH-3T3 adipogenesis, post-confluent cells (day 0) were induced with a mixture of 250 nM dexamethasone, 0.5 mM isobutyl-methyl-xanthine and 1μg/ml insulin in DMEM containing 10%FBS. On day 2 (C3H10T1/2) or day 4 (NIH-3T3) this media was replaced and maintained in DMEM containing 10% FBS and 1μg/ml insulin until analysis. For pharmacological treatment, recombinant Shh (R&D systems) was dissolved in 2% bovine serum albumin (BSA) and added to the post-confluent, differentiating cultures at indicated time points. An equal volume of 2% BSA was added to the cells as control. Shh was added to cells at a final concentration of 300ng/ml unless indicated otherwise. KAAD-cyclopamine (Toronto Research Chemicals Inc.) was dissolved in DMSO and added to the post-confluent, differentiating cultures at indicated time points. An equal volume of DMSO was added to the cells as control.

KAAD-cyclopamine was added to cells at a final concentration of 3.6μM. Media was changed at two-day intervals and fresh Shh or KAAD-cyclopamine was added at each media change until the day of harvest. Each treatment combination per experiment was conducted in triplicate and repeated at least once (e.g., n=6) unless otherwise indicated.

2.3 Retroviral plasmids and infections

SmoA1 cDNA was provided by P. A. Beachy (Taipale et al., 2000). To construct pLNCX2-SmoA1, SmoA1 was digested with HindIII and SalI and the fragment was subsequently subcloned into pLNCX2 retroviral vector (Clontech). DNGli was prepared as described previously by ApaI digestion of full-length Gli2 cDNA (Sasaki et al., 1999) and subsequently cloned into pLNCX2. The Gata2 retroviral expression construct was made by subcloning the murine Gata2 cDNA into pLNCX2 vector. DNGATA was made by site direct mutagenesis using PCR to introduce KRR mutation (amino acids KRR in positions 304–306 changed to AAA) into the murine Gata3 cDNA expression vector Gata3-RV described previously (Ranganath et al., 1998). Recombinant viral packaging was achieved by transfection of the plasmid into Phoenix packaging cells (cultured in DMEM with 10% FBS in 5% CO_2) using Lipofectamine 2000 (Invitrogen) (Pear et al., 1993). Viral supernatants were supplemented with 8 μg/ml polybrene and added to cells for infections for 24–36 hr. Cells were selected with 50μg/ml neomycin (Sigma), expanded, and seeded for differentiation experiments.

2.4 Extraction of total RNA and RT-PCR

Total RNA from flies, mouse perigonadal fat pads or cultured cells was extracted by the Trizol isolation method (Invitrogen). The first strand cDNA was generated by using the oligo (dT) primer in the reverse transcription system using M-MLV-reverse transcriptase (Invitrogen). Subsequently, the expression of various genes was examined either by semiquantitative PCR (McKay et al., 2003) or real-time PCR using SYBR Green Master Mix reagent (Applied Biosystems, 7500 Real-Time PCR System). Semiquantitative PCR was carried out in a reaction mixture containing 5 µl of the above first-strand cDNA, 10x PCR buffer, $MgCl_2$, 50 pmol of each primer and Taq DNA polymerase (Invitrogen). The PCR products specific for each cDNA were analyzed by electrophoresis on a 1 % agarose gel. DNA was visualized by ethidium bromide staining. Two concentrations of cDNA template were used to demonstrate that the reaction conditions were semi-quantitative. Real-time PCR values for gene expression were normalized over ß-actin expression. For the primer sets used, see Table 2.

2.5 Oil Red O staining

Lipid droplets in differentiated adipocytes were stained with Oil Red O and extracted stain was quantified as described previously (McKay et al., 2003; Ramirez-Zacarias et al., 1992). Briefly, monolayer cells were washed three-times with PBS and then fixed for 10 min with 10% formalin. Fixed cells were incubated with freshly prepared Oil Red O

solution (four parts water mixed with six parts 0.5% Oil Red O in isopropanol) for 1h at room temperature, and washed several times with water to remove excess stain. Stained lipid droplets in cell monolayers were visualized by light microscopy and photographed. Oil Red O was extracted from stained cells with isopropanol and absorbance (600nm) was measured to quantify stain.

Table 2 Primer list.

Gene	Sense	Antisense
Ci (*Drosophila*)	5'-TCGATGACTAGCTTGCCAGA-3'	5'-TGTGGTTCAGGGGGAAATAA-3'
Smo(*Drosophila*)	5'-CAGGTGGCATTGGAGAACTT-3'	5'-CAAAGCCACATCCAGCTCTT-3'
Ptc(*Drosophila*)	5'-GAGCATCCAGACCGCATATC-3'	5'-GTTGCTGGACTTCCACGACT-3'
Smo(mouse)	5'-TATGGCTCTTCCTGAAAGCACAC-3'	5'-AAGCTCTGTCGGAGACTAACACTG-3'
Gli1	5'-TTCACCCTGCCATGAAACTTTC-3'	5'-AGAGTCCAGAGCGTTACACACCTG-3'
Gli2	5'-TGGGTTGCTGTGGACTAGGAATAG-3'	5'-TCCATGCCAAATACCTGGAGAG-3'
Gli3	5'-ATGGGCTTCAGTCAGCAAGACAG-3'	5'-AGGAGCTGTGGGAAAGGTTCTG-3'
Ptc1	5'-TTCTGCTTCGGTGACTGTTGC-3'	5'-TCTCCTCACATTCCACGTCCTG-3'
Ptc2	5'-TTGCTGGGTCTGCTCATGCTTG-3'	5'-ACAGTCATGGAGGTAGTCACTCTGG-3'
HPRT	5'-CTTGCTCGAGATGTCATGAAG-3'	5'-GTTTGCATTGTTTTACCAGTG-3'
Pref-1	5'-CCTGGGCGTGCTCACCAGCC-3'	5'-GCCTCCTTGTTGAAGGTGG-3'
Adipsin	5'-GGGCTGTGGAGGCTAGCTAGG-3'	5'-CCATTGCCACAGACGCGAGAGC-3'
aP2	5'-GAGATTTCCTTCAAACTG-3'	5'-CATAAACTCTTGTGGAAG-3'
Gata2	5'-ACACACCACCCGATACCCACCT-3'	5'-TAGCCCATGGCAGTCACCATGC-3'
Gata3	5'-AGAAGGCATCCAGACCCGAAAC-3'	5'-ACTTGGAGACTCCTCACGCATGTG-3'
Gilz	5'-TCGTGAGCTGCTTGAGAAGA-3'	5'-GGAGCCAAAAACAAACTGGA-3'
C/EBPα	5'-CGGAATCCCGATGGAGTCGGCCGACTTC-3'	5'-CCGCTCGAGTCACGCGCAGTTGCCCATG-3
PPARγ	5'-CCACCAACTTCGGAATCAGCTC-3'	5'-GCAACCATTGGGTCAGCTCTTG-3'
CKIT	5'-CAAATGGGAGTTTCCCAGAA-3'	5'-CACCAGCTCCCAATGTCTTT-3'
SCA1	5'-GACGGAGGATTTCATCCAGA-3'	5'-CCCCAACAGAGAGTTTGGAA-3'
CD34	5'-GCATTGGTCACCTCTGGAGT-3'	5'-TTCTGTGTCAGCCACCACAT-3'
CD45	5'-CAAACAGAAGCTTCCCAAGG-3'	5'-GGATAGATGCTGGCGATGAT-3'
Osx	5'-CTTAACCCAGCTCCCTACCC-3'	5'-AGAGCGAGTGAACCTCTTGC-3'
ALP	5'-CGGACATCATGAGGGTAAGG-3'	5'-GAGACATTTTCCCGTTCACC-3'
Runx2	5'-CTAAGCTTCCACCATGCTTCATTCGCCTCAC-3'	5'-CAGGAAGTTGGGACTGTCGG-3'
PTHR	5'-TGCTTGCCACTAAGCTTCG-3'	5'-TCCTAATCTCTGCCTGCACC-3'

2.6 Triglyceride content.

To quantify triglyceride levels, flies or cells were lysed in 0.5% SDS/PBS and triglyceride content was measured using the Infinity Triglyceride Reagent (Sigma) following manufacturer's instructions. This procedure employs enzymatic hydrolysis of

glycerol and fatty acids. The glycerol is then measured by enzyme coupled reduction of a dye that absorbs light at 500 nm and can be quantified spectrophotometrically. To normalize triglyceride content, protein concentrations were measured using the BCA protein assay kit (Pierce). This assay measures the reduction of Cu^{2+} to Cu^{1+} by protein in an alkaline medium thereby forming a tetradentate-Cu^{1+} complex. The Cu^{1+} ions then chelate with two molecules of bicinochoninic acid (BCA) which absorbs light at 562 nm and can be quantified spectrophotometrically.

2.7 Cell number

On the indicated day of harvest, cells were first washed with PBS and removed from culture plate by trypsinization. After centrifuged at 500 x g, the supernatant was removed and the remaining cell pellet was resuspended in PBS and the number of cells was counted using a hemocytometer plate.

2.8 BrdU labeling

Post-confluent cells were induced to differentiate with appropriate hormonal stimulation. Cells were incubated with growth media containing 10 mg/ml BrdU for two hours at designated time points. The the cells were washed twice with PBS, fixed in 4% paraformaldehyde for 10 min at room temperature. Fixed cells on the coverslips were then treated with 1.5 M HCl, permeabilized with 0.05% TritonX-100 in PBS, and then blocked with 2% BSA and 3% goat serum in PBS for 45 min at room temperature. Blocked cells

were incubated for 1h with a mouse monoclonal anti-BrdU primary antibody (1:100 dilution). After washing with PBS, the cells were finally incubated for 1 h in the dark with an FITC-conjugated secondary antibody (1:200 dilution) containing 0.1 mg/ml DAPI at room temperature. The cells were then washed twice for 5 min with PBS and mounted for immunofluorescence microscopy analysis. For quantitation, BrdU positive cells were counted from 10 random fields and averaged for analysis.

2.9 Preparation of total cell lysate and Western blotting

Monolayer cells were washed twice with ice-cold PBS, scraped into 0.2 ml of lysis buffer (50 mM HEPES [pH 7.5], 150 mM NaCl, 10% glycerol, 100 mM NaF, 0.2 mM Na-orthovanadate, 0.5% NP-40, 1.5 mM $MgCl_2$, 1 mM EGTA, 1 mM dithiothreitol, 1 μg/ml leupeptin, 10 mM benzamidine, 1 μg/ml pepstatin A, 10.5 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride), and incubated for 20 min on ice with intermittent vortexing. Protein concentrations were determined with BCA protein assay kit. For Western blotting, cell extracts (usually containing ~25μ g of protein) were subjected to 10% SDS-polyacrylamide gel electrophoresis. Resolved proteins were blotted onto a PVDF membrane (Immobilon-P, Millipore). After blocking with 2% non-fat dried milk in 1x TTBS (Tween/Tris-buffered saline) containing 25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween, and 0.001% thimerosal for 2 h at room temperature, membranes were incubated with a monoclonal Rb antibody (BD biosciences) for 2 h at room temperature. Chemiluminescence was produced with ECL Kit (Amersham Corp.).

2.10 *In Situ* Hybridization.

Female C57B6 mice, obtained from Jackson Laboratories (Bar Harbor, ME), were maintained and mated as previously described (Jaskoll and Melnick, 1999); plug day = day 0 of gestation. Pregnant females were killed by cervical dislocation on embryonic days 14.5 and 15.5 (E14.5 and E15.5). Embryos were dissected in cold phosphate-buffered saline (PBS) and staged as described (Theiler, 1989). E14.5 and E15.5 embryos were collected, fixed in 4% paraformaldehyde, dehydrated through graded alcohols, cleared in xylene, and embedded in paraffin. Paraffin sections of E14.5 and E15.5 embryos were hybridized with ^{35}S-labeled sense and antisense probes, as described previously (Hui et al., 1994). Near adjacent sections were used in most cases to allow a more accurate comparison of expression patterns.The following RNA in situ probes were used as described: Smo (Zhang et al., 2001), *Ptc1* (Goodrich et al., 1997), Gli1~3 (Hui et al., 1994).

2.11 Fly experiments

Fly cultures and crosses were grown on standard fly medium at 25°C, unless otherwise indicated. The wild-type control stock used throughout this work is *w1118* and obtained from the Bloomington Stock Center. UAS-hh, UAS-smo, UAS-ci^{-3p} and UAS-DNCi have been previously described (Chen and Struhl, 1998; Wang et al., 1999; Wang et al., 2000; Wang and Jiang, 2004). All UAS alleles were maintained over a cyo balancer to make homogeneous stocks. Specifically, ci^{-3p} is identical to wild type Ci except that 3 PKA sites

have been mutated to make it constitutively active. DNCi has an internal deletion that removes aa 346–440 from full-length Ci to make it a strong dominant-negative form. Tissue specific activation or inhibition of the Hh pathway was carried out using the *Gal4/UAS* system (Brand et al., 1994). Flies with desired genotype were generated according to Fig 3. Briefly, males of the genotype w^{118}; e7b/cyo-GFP was crossed to w^{118}; UAS-hh/cyo or w^{118};UAS-smo/cyo or w^{118};UAS-ci-3p, or w^{118};UAS-DNCi. Male progeny with GFP expression in the gut were crossed to w^{118};DCG-Gal4; DCG-GFP. The progeny with GFP expression in larval fat body were subject to analysis. 3rd instar larval fat bodies were dissected in PBS under a microscope and lipid content of fat bodies was analyzed by Nile Red staining as described (McKay et al., 2003) and triglyceride assays as described above. Fat body gene expression levels were analyzed by semi-quantitative RT-PCR as described above. Sequences of used primer sets were listed in Table 2.

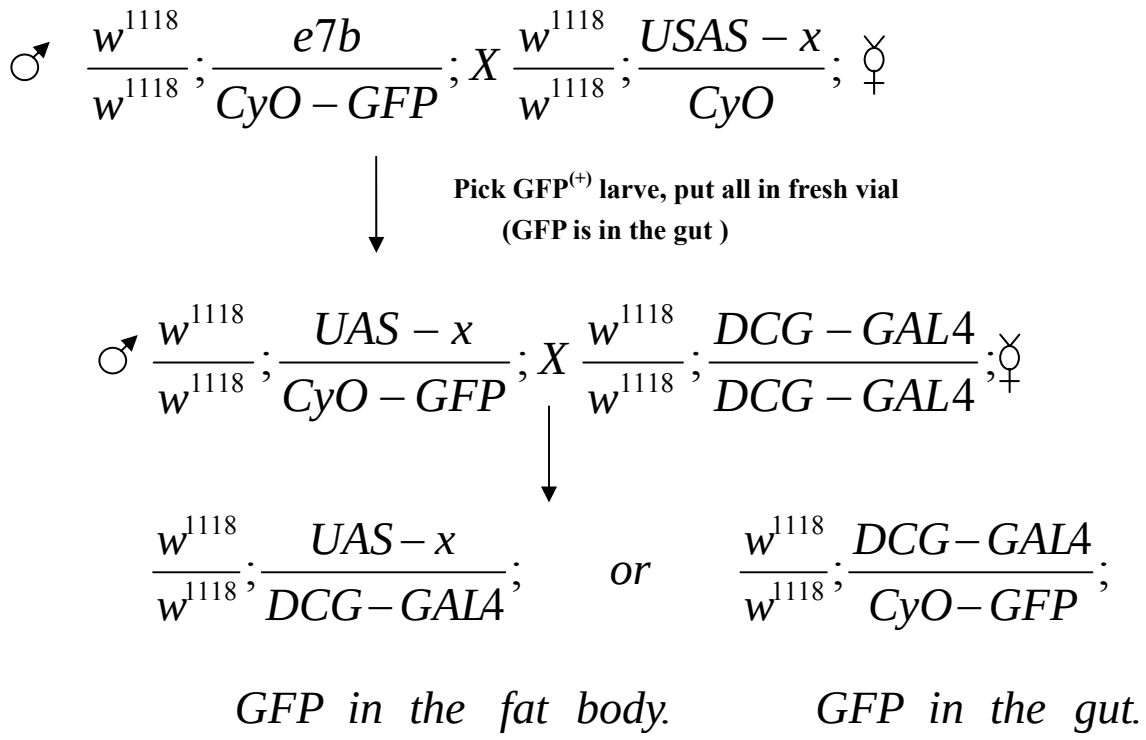

Figure 3 Schemes of fly crosses**.**

Fly crosses performed to generate desired transgenic flies. x= hh, smo, ci^{-3p} and UAS-DNCi

2.12 Mouse studies.

Pure inbred C57BL/6J mice and pure inbred C57BL/6J Ob/Ob mice were purchased from the Jackson labs. Mice were housed in a 12:12 light:dark cycle and chow and water were provided *ad libitum*. For diet-induced obesity we mated the inbred C57BL/6J mice and then randomized 5-week old C57BL/6J littermates to four months of either normal (4% fat, Teklad) or high fat chow (60% fat, Research Diets). After four months on the appropriate diet, RNA was extracted from identical fat depots from all mice in the cohorts and molecular analyses were done as described above. Ob/Ob genetically obese mice and

matched controls were fed normal chow and at six months of age, gene expression was analyzed in identical fat depots explanted from all mice in the cohort as described above. Veterinary care was provided by the Division of Comparative Medicine. All animals were maintained under the guidlines of the U.T. Southwestern Medical Center Animal Care and Use Committee according to current NIH guidelines.

CHAPTER THREE

RESULTS

3.1 Hh signaling blocks fat formation in *Drosophila melanogaster*

In *Drosophila*, excess energy is stored in intracellular lipid droplets of specialized organ called the fat body, which has analogous function to the adipose tissue in vertebrates (Hoshizaki et al., 1994). Since enhancer trapping has been proved to be useful in identifying genes that are expressed in specific spatial and developmental patterns (O'Kane and Gehring, 1987), a broad two-component enhancer trap screen (minimal promoter-Gal4, UAS-GFP) was performed in our lab to screen for genes important in fat-cell metabolism and development. We screened P[*GAL4*] *enhancer trap* strains in *Drosophila* for expression in the fat body by mating them with a *UAS-GFP* strain (Brand and Perrimon, 1993). Plasmid rescue was used to recover genomic DNA flanking the P[*GAL4*] elements to identify the inserted genomic locus. Of the examined enhancer trap lines that expressed GFP in the larvae fat body, one line had P-element inserted into the 5' UTR region of the *smo* gene, a seven transmembrane receptor that transduced Hh signals. This suggested Hh signaling might be implicated in the development of fly fat body.

3.1.1 Hh signaling components are expressed in *Drosophila* fat body

To examine the potential role of Hh signaling in fly fat biology, I first seek to examine

whether the Hh signaling components are expressed in the developing fat body. RT-PCR was used to achieve this goal. To mark the fat body, I utilized a fat body specific DCG-GFP reporter developed in the lab by coupling the DCG promoter/enhancer region to GFP. The *DCG* gene codes for a basement membrane type IV collagen chain (Cecchini et al., 1987), and is expressed in larval fat body and adult fat cells. Thus it could serve as a good cell marker for developing fat cells during fat body formation. Larval fat body was dissected out under the fluorescent microscope according to GFP expression. RT-PCRs with specific primer sets for smo, ptc and ci were performed with RNA extracted from the explanted fat body. As shown in Fig 4A, expression of all three genes was detected in the larval fat body, which further suggested the role of Hh signaling in fat body formation.

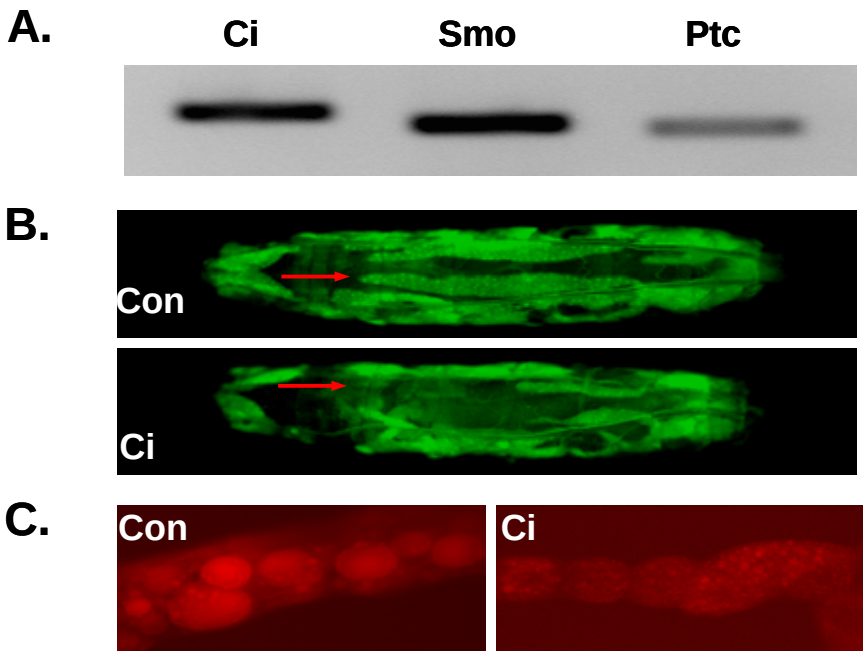

Figure 4 The Hh pathway blocks *D. melanogaster* fat formation.

A) The fat body was dissected from wild-type *D. melanogaster* 3rd instar larvae under the fluorescent microscope according to GFP expression and the expression of the indicated components of the Hh pathway was assessed by RT-PCR. **B-C**) An activated form of *ci*, the Hh transcription factor, was specifically expressed in the fat body with the Gal4-UAS system and fly fat formation was evaluated with a fat body-specific GFP reporter (B, red arrows indicate loss of dorsal fat), Nile Red, a fat specific stain (C), or molecular analysis of explanted fat bodies.

3.1.2 Tissue-specific activation of Hh signaling gives lower fat content

Targeting gene expression in a temporal and spatial fashion has proven to be one of the most powerful techniques for addressing gene function in vivo. To examine the potential cell autonomous function of Hh signaling in fly fat formation, I activate Hh signaling specifically in fat body by generating transgenic animals carrying ci^{-3p}, a constitutively active form of ci (Wang et al., 2000), under the control of the Gal4/UAS system (Brand and Perrimon, 1993). Overexpression of ci^{-3p} in the fat body was achieved using a DCG-Gal4 line that directs Gal4 expression in fat body cells. To do this, a homogenous stock of UAS-ci^{-3p}/Dcg-Gal4 was generated by crosses shown in Fig 3. Both the wild type and transgenic flies were also introduced with the DCG-GFP reporter to visualize fat body. Animal with transgenic ci^{-3p} activity developed into larvae with dramatically reduced size of fat body (Fig 4B), as evidenced by the GFP expression of fat body reporter.

To determine whether Hh signaling also affect lipid deposition in fat body, I explanted the fat bodies from both wild type and transgenic flies and stained them with Nile red, which labels neutral lipids. ci^{-3p} mutant fat body cells showed significant reduction in the size of lipid droplets (Fig 4C). ci^{-3p}-dependent difference of organismal triglyceride content was further assessed by triglyceride assay. Third instar larvae expressing ci^{-3p} accumulated 39% less storage fat compared to control larvae, as shown in Fig 5.

Next, I examined expression of established fat body specific markers. Overexpression of ci^{-3p} decreased the expression of HLH106 (fly SREP homolog), and fly fatty acid

synthase (Rosenfeld and Osborne, 1998) (Fig 6). This suggested that Hh signaling was able to block the fly fat formation at molecular level. I also activate Hh signaling in fly fat body by overexpressing hh and smo using Gal4/UAS system. The transgenic flies overexpressing either gene produced similar outcomes as ci transgenesis establishing the generality of the effects of the Hh signaling on fly fat formation.

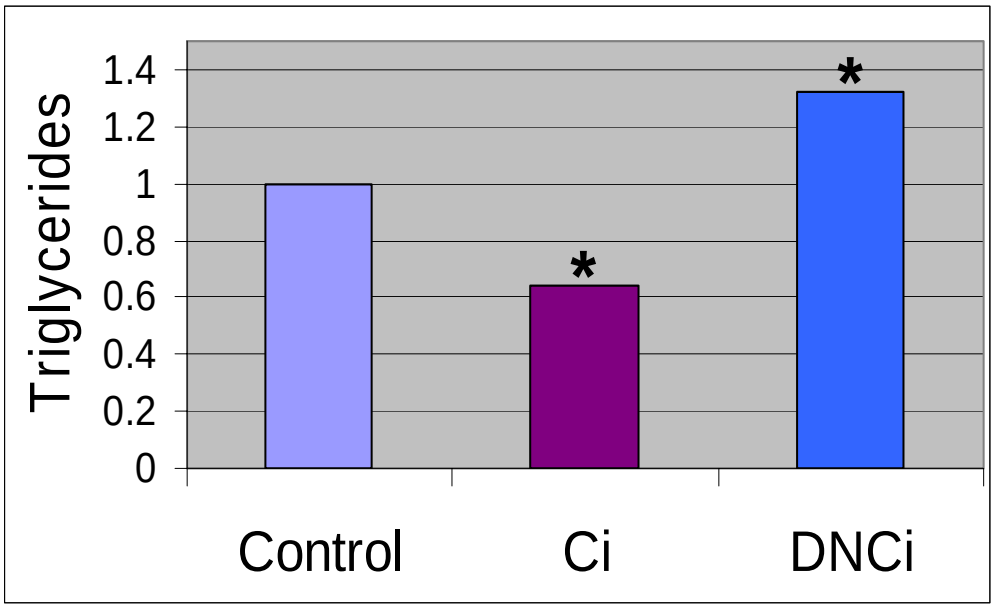

Figure 5 The Hh pathway blocks *D. melanogaster* triglyceride accmulation.

The fat body was dissected from 3rd instar larvae of *UAS-ci^{-3p}/DCG-Gal4* genotype under the fluorescent microscope according to GFP expression and subject to triglyceride quantitation. It shows that active ci inhibits fly triglyceride formation and that dominant negative ci (DNCi) increases fly triglyceride formation. *$p<0.05$

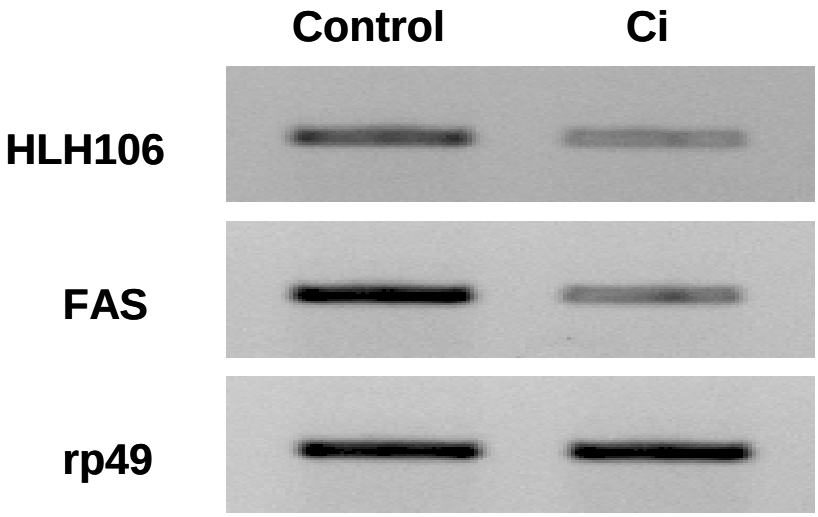

Figure 6 The Hh pathway downregulates the expression of fat body specific marker genes.

The fat body was dissected from 3rd instar larvae of *UAS-ci^{-3p}/DCG-Gal4* transgenic flies under the fluorescent microscope according to GFP expression and the expression of the indicated components of the Hh pathway was assessed by RT-PCR. *HLH106* (fly SREBP) and *fas* (fly fatty acid synthase) are fat body markers and *rp49* serves as a loading control.

3.1.3 Tissue-specific blockade of Hh signaling causes obesity

To complement the sufficiency results in previous experiments, I performed necessity tests to block Hh signaling in fly fat body. This was achieved by fat body specific overexpression of a dominant negative ci (DNCi) (Wang and Jiang, 2004). DNCi transgenic larvae developed an obese phenotype, with enlarged fat body and increased fluorescent intensity of the GFP fat body reporter. DNCi transgenesis also significantly increased lipid deposition, as evident by 28% increase in triglyceride content compared to control flies (Fig 5). Flies carrying hh, smo, ci^{-3p} and DNCi generally show good viability and can grow to their adulthood. When encountered with starvation, the obese Dcg-Gal4: UAS-DNCi flies are more starvation resistant than control flies, whereas the lean ci^{-3p}, hh, smo flies are starvation sensitive (not shown). Therefore, both the sufficiency and necessity results suggested that Hh signaling cell autonomously inhibits fly fat formation.

3.2 Components of Hh signaling are dynamically expressed in mammalian fat

To extend the finding in invertebrate model system to vertebrate model system, I first seek to examine whether components of Hh signaling pathway are expressed in mammalian fat. RT-PCRs of RNA from E14.5 embryonic fat pad and subcutaneous fat of adult mice were performed with gene-specific primers. I detected the expression of many of the Hh pathway components, including Smo, the negative regulatory receptors Ptc1 and

Ptc2, and the Gli family of Hh transcription factors (ci homologs) in embryonic fat and adult fat (Fig 7).

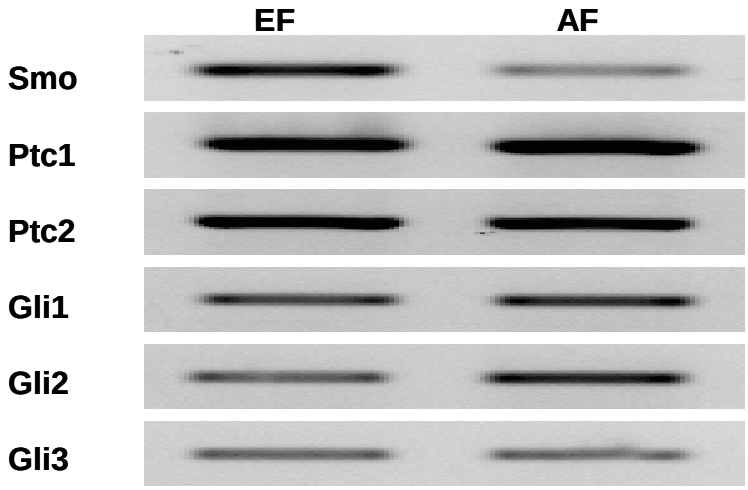

Figure 7 Shh pathway components are expressed in murine fat.

E14.5 embryonic murine fat anlagen (EF) and adult white adipose depots (AF) were explanted, RNA extracted, and semi-quantitative RT-PCR was performed on the indicated components of the Hh pathway.

To further characterize the spatial expression pattern of Hh cascade components during mouse embryonic fat development, I performed *in situ* hybridization on parasagital sections from E14.5 and E15.5 embryos with gene-specific probes. As shown in Fig 8, expression of Ptc1 and Smo was clearly detected in embryonic fat pad. With Ptc2 and Gli1~3 probes, no significant hybridization signals above background could be detected in embryonic fat pad of embryos from either day. Expression of these genes was clearly detected in many other mesoderm-derived structures, such as the mesenchyme of the gut and craniofacial mesenchyme, consistent with the previously reported expression patterns (Hui et al., 1994). This lack of signal for Ptc2 and Gli1~3 is probably because these genes are expressed at low level in embryonic fat pad at these stages, and the sensitivity of *in situ* hybridization in my hand is not as high as RT-PCR.

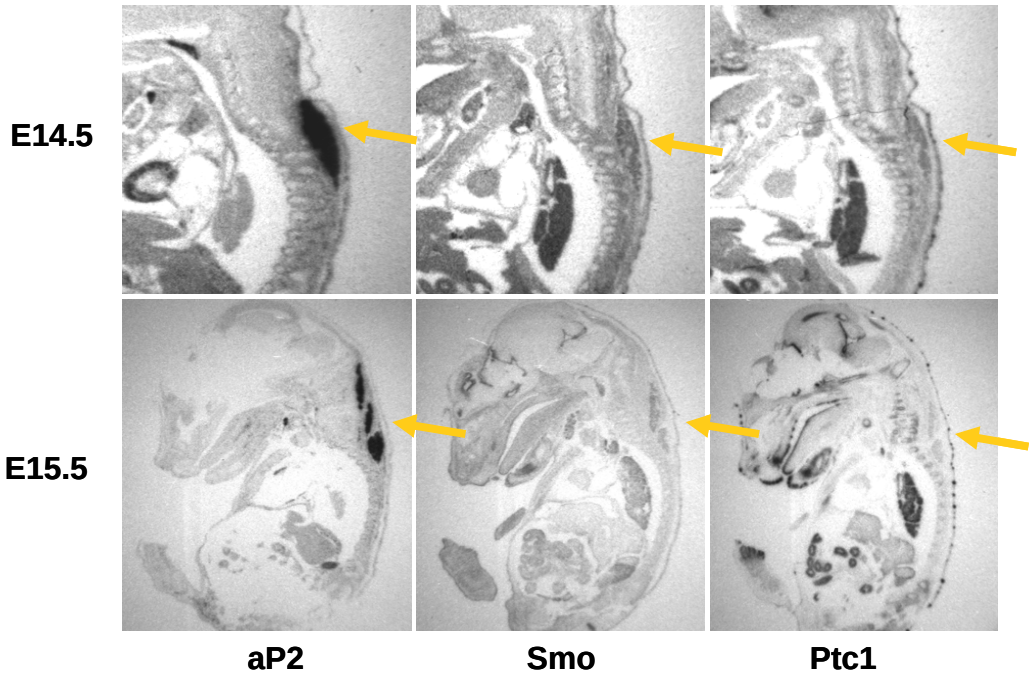

Figure 8 Hh signaling components are expressed in embryonic fat pad.

In Situ hybridization of 14.5-dpc and 15.5 parasagital embryo sections with antisense aP2, Smo, and Ptc1 as indicated. Yellow arrow showed gene expression in embryonic fat pad.

Development of preadipocyte cell culture models has made it tractable to study adipocyte development and physiology. 3T3-L1 cell line is one of the most extensively characterized and widely used cell models (MacDougald and Lane, 1995). To investigate the role of Hh signaling in 3T3-L1 adipogenic differentiation, I first surveyed the expression pattern of various hedgehog signaling components at different stages of adipocyte differentiation. 3T3-L1 cells were induced to adipogenic differentiation with MDI. Fully mature adipocytes develop in an 8-day culture period. RNA was harvested from 3T3-L1 cells at day 0, immediately before induction, as well as 4 days and 8 days after induction. Gene expression of Hh signaling components was determined by semi-quantitative RT-PCR. As shown in Fig 9, expression of active regulator of Hh pathway, Smo, and transcription factor Gli1~3 had already dramatically decreased by day 4 post-induction. In contrast, expression of ptc2, a negative regulator of Shh pathway, was up-regulated. Another negative regulator ptc1 expressed at similar level during induction. PPARγ is upregulated upon induction, which was used as a positive control for adipogenesis. This dynamic expression profile suggests that Hh pathway might function to block mammalian adipogenesis.

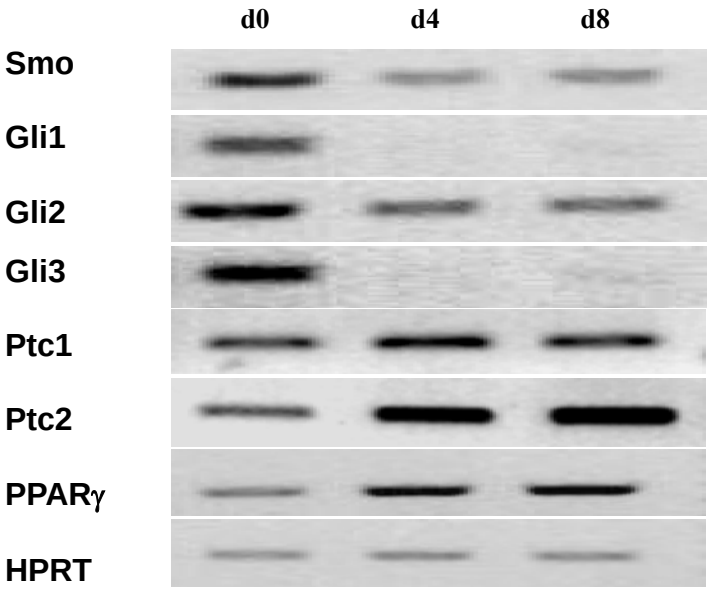

Figure 9 The Hh components express dynamically during adipocyte formation.

3T3-L1 cells were incubated in adipogenic induction media, RNA extracted on days 0, 4, and 8, and semi-quantitative RT-PCR was done for the indicated transcripts. Expression levels of the positively acting Hh pathway components (Smo, Glis) decrease during adipogenesis, while the levels of the negatively acting components (Ptc1, Ptc2) increase. HPRT serves as a loading control.

3.3 Hh signals inhibit mammalian adipogenesis

3.3.1 Recombinant hedgehog blocks 3T3-L1 adipogenic differentiation

To investigate effects of hedgehog on adipocyte differentiation, I cultured 3T3-L1 cells for 14 days in the presence of vehicle or Sonic Hh protein (Shh) at 300ng/ml and subsequently analyzed them microscopically (Fig 10A top), with Oil Red O staining (Fig 10A bottom), and triglyceride content of the cultures (Fig 10B). Shh at this dosage could sufficiently activate Hh signaling in 3T3-L1 cells, as indicated by elevated expression of Hh responsive gene Gli1 (Fig 10C) (Marigo et al., 1996). By day 14 post-induction, a large percentage of the control cells had differentiated into adipocytes, as evidenced by morphological changes visualized under the light microscope and lipid accumulation indicated by Oil Red O staining. In contrast, Shh treated cells failed to differentiate. Continuous addition of Shh starting from day 0 significantly decreased adipogenic differentiation of 3T3-L1 cells. Triglyceride content of treated cultures was only about 60% of the control cells. Shh inhibited formation of adipocyte in 3T3-L1 cells in a dose-dependent manner (Fig 11). By day 14 of induction, adipogenic differentiation in 3T3-L1 cells treated with 10ng/ml Shh has little difference from that of vehicle treated cells. The inhibition effect became more apparent as the level of Shh increased in the cultures (Fig 11).

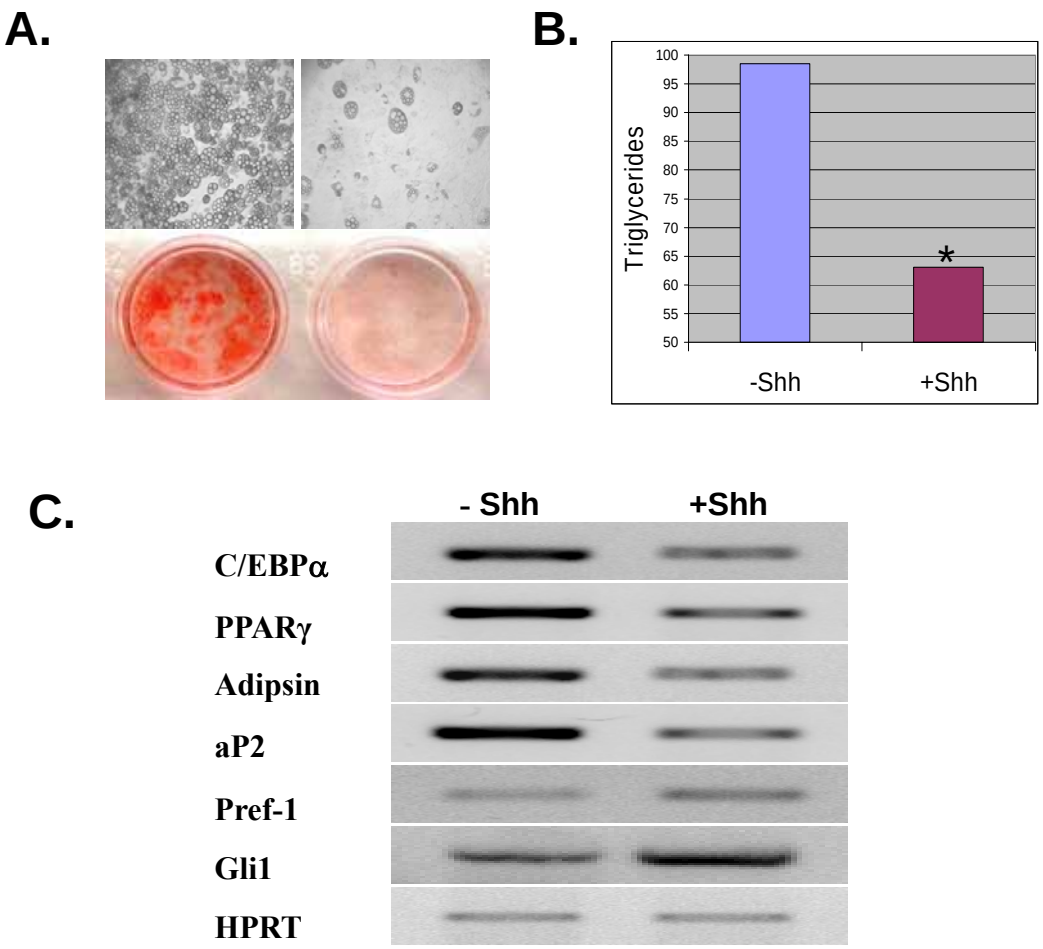

Figure 10 The Hh cascade inhibits 3T3-L1 adipogenesis.

A, B) 3T3-L1s were incubated in adipogenic induction media in the presence of vehicle (-Shh) or Shh protein and fat formation was assessed based upon morphology (A top), Oil Red O staining (A bottom), and triglyceride quantitation (B). *$p<0.01$ **C)** RNA was extracted from induced 3T3-L1s treated with vehicle or Shh and semi-quantitative RT-PCR was done for the indicated transcripts. C/EBPα and PPARγ are adipogenic transcription factors; adipsin and aP2 mark differentiated adipocytes. Pref-1 is a preadipocyte marker. HPRT serves as a loading control. Gli1 is a positive control for Hh pathway activation.

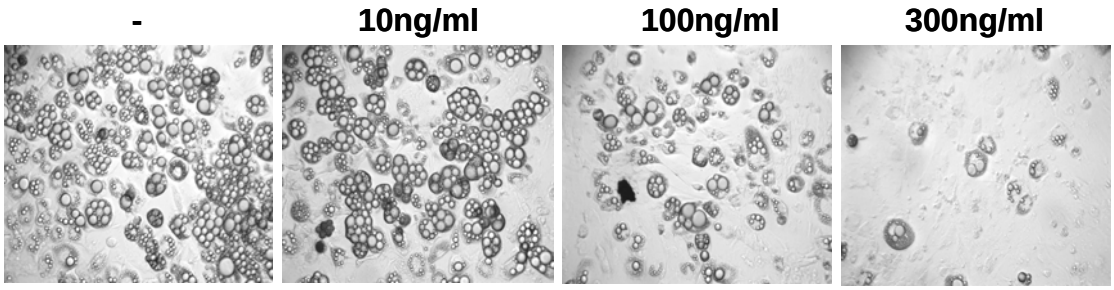

Figure 11 The Hh cascade inhibits 3T3-L1 adipogenesis dose-dependently.

3T3-L1s were incubated in adipogenic induction media in the presence of vehicle (-) or Shh protein at indicated concentration and fat formation was assessed based upon morphology.

Molecular analysis indicated that Shh treatment decreased the expression of established adipogenic differentiation markers, C/EBPα and PPARγ, and terminally differentiation markers, aP2 and adipsin (Rosen and Spiegelman, 2000). In contrast, mRNA expression of Pref-1, whose expression inversely correlates with adipogenesis (Sul et al., 2000), was up-regulated by Shh treatment. This suggested that Hh signaling was able to block the adipogenic program at molecular level (Fig 10C).

3.3.2 SmoA1 expression blocks 3T3-L1 adipogenic differentiation

SmoA1 is an activated form of the Hh receptor Smo. It has been shown that expression of SmoA1 alone is sufficient to activate Hh signaling (Taipale et al., 2000). I first establised stable cell lines expressing SmoA1 or GFP control by retroviral transduction followed by

neomycin selection, then induced them into adipogenesis by 10% fetal bovine serum with insulin. At day 14 post-induction, a significant fraction of the 3T3-L1-GFP cells differentiated into fat-laden cells with the typical morphology of cultured adipocytes, whereas lipid-containing cells were not detected in the 3T3-L1-SmoA1 cells (Fig 12A).

When I examined the molecular markers, I found that infection of SmoA1 retrovirus reduced the expression of PPARγ, aP2 and adipsin. As a positive control for Hh activation, Gli expression was up-regulated by SmoA1 expression (Fig 12B).

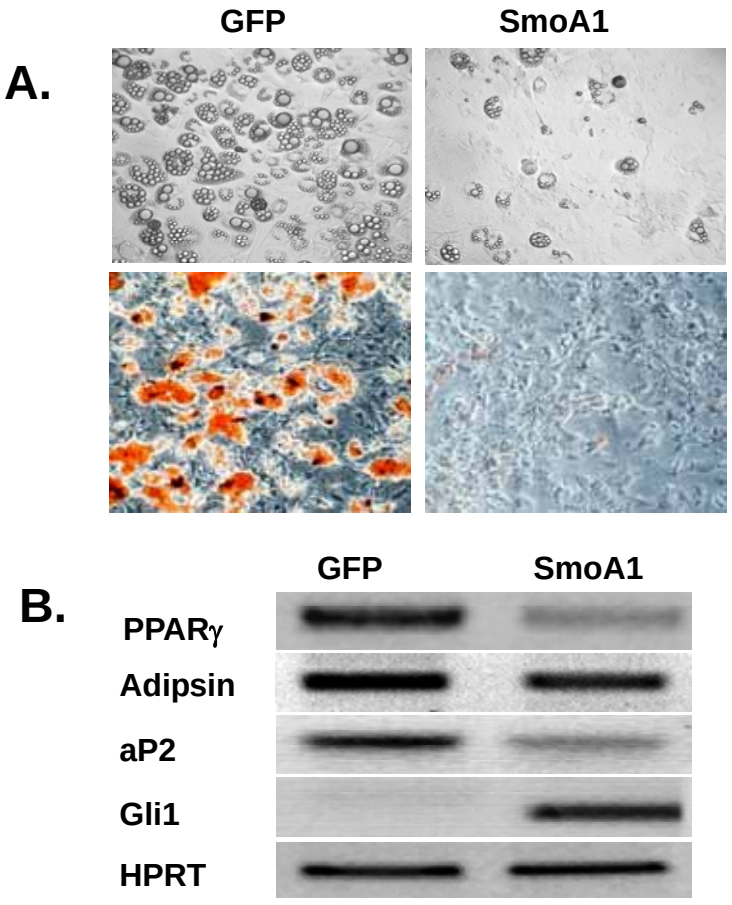

Figure 12 The Hh cascade inhibits 3T3-L1 adipogenesis.

A) 3T3-L1s were infected with a retrovirus expressing either GFP or SmoA1, an activated form of the Hh receptor Smo. Smo A1 reduced fat formation as scored either morphologically (top) or by Oil Red O staining (bottom). **B)** RNA was extracted from induced 3T3-L1s infected with GFP or SmoA1 expressing virus and semi-quantitative RT-PCR was done for the indicated adipocyte marker genes transcripts. HPRT serves as a loading control.

3.3.3 Hh signals also block adipocyte formation in NIH3T3 cells

I next tested whether activation of Hh signaling can inhibit adipocyte differentiation in NIH3T3 cells, a line with lower adipogenic potential. Under appropriate condition, NIH3T3 can also be induced to form adipocyte at a reduced rate compared to 3T3-L1 cells. I also evaluated whether Shh protein could decrease adipocyte formation in NIH-3T3 cells. NIH3T3 cells were induced and incubated with vehicle or Shh and adipocyte development was assessed microscopically on day 10 of induction. As observed in the 3T3-L1s, Shh decreased fat formation in NIH3T3 cells (Fig 13A). To further test this, I developed stable NIH3T3 cell lines carrying either control GFP or SmoA1 and induced them to adipogenic differentiation. Microscopy and Oil Red O staining showed that SmoA1 also reduced adipogenesis in NIH3T3 cells (Fig 13B). Expression of adipocyte marker genes, aP2, adipsin and PPARγ, were significantly lower in NIH-SmoA1 cells than in NIH-GFP control cells (Fig 13C).

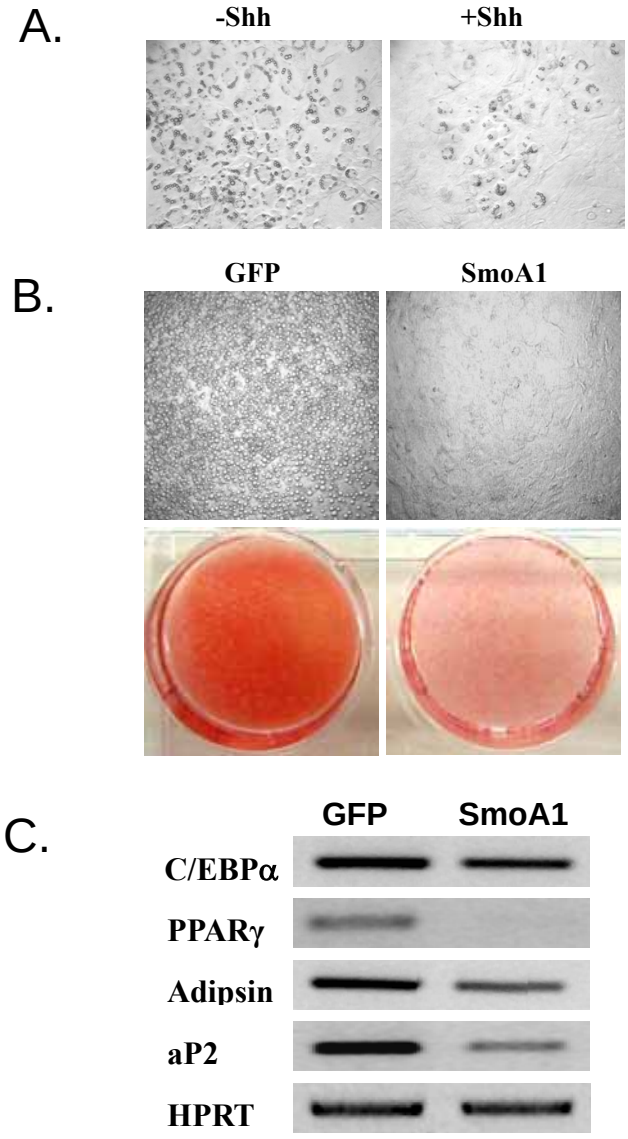

Figure 13 The Hh cascade inhibits NIH-3T3 adipogenesis.

A) NIH-3T3s were incubated in adipogenic induction media in the presence of vehicle (-) or Shh protein and fat formation was assessed based upon morphology. **B)** NIH-3T3s were infected with a retrovirus expressing either GFP or SmoA1. Fat formation was ananlyzed either morphologically (top) or by Oil Red O staining (bottom). **C)** RNA was extracted from induced NIH-3T3s infected with GFP or SmoA1 expressing virus and semi-quantitative RT-PCR was done for the indicated transcripts.

3.4 Inhibiting Hh signaling increases mammalian adipogenesis

3.4.1 KAAD-cyclopamine promotes 3T3-L1 adipogenic differentiation

To complement the sufficiency results in previous experiments, I performed necessity tests to block Hh signaling in 3T3-L1 cells. The Hh effect could be completely blocked by the alkaloid cyclopamine, which has previously been demonstrated to inhibit hedgehog signal transduction in target cells. KAAD-cyclopamine is one of the most specific and selective cyclopamine derivatives (Chen et al., 2002; Taipale et al., 2000). To block the function of endogenous Hh signaling, I treated 3T3-L1 cells with either vehicle or KAAD-cyclopamine from day 0 of adipogenic induction. By day 14 post-induction, a much larger percentage of KAAD-cyclopamine treated cells differentiated into adipocytes (Fig 14 top). Nile red staining also showed more lipid accumulating cells by drug treatment (Fig 14 bottom).

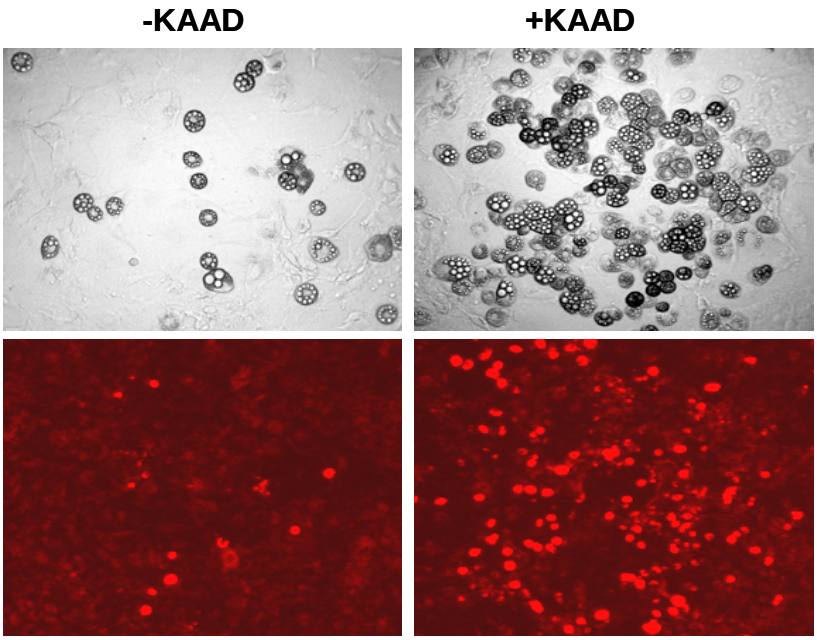

Figure 14 Inhibiting the Hh pathway stimulates 3T3-L1 adipogenesis.

3T3-L1s were incubated in adipogenic induction media in the presence of vehicle (-KAAD) or KAAD-cyclopamine, a Hh inhibitor. Fat formation was analyzed morphologically (top) and with Nile Red staining (bottom).

3.4.2 Gli2-ΔC4 expression promotes 3T3-L1 adipogenic differentiation

In addition, I inhibited Hh signalling by overexpressing Gli2-ΔC4, a dominant-negative form of the Hh transcription factor Gli2. It was made by C-terminal truncation of Gli2, and previously shown to strongly repress Hh pathway (Sasaki et al., 1999). Cell lines stably expression GFP control or Gli2-ΔC4 were made by transfection followed by neomycin selection. Both cell lines were then induced to differentiate until day 10. As assessed by light microscopy and Oil Red O staining (Fig 15A), a significantly higher percentage of Gli2-ΔC4 expressing cells underwent adipogenesis, as compared to the control cell line, accompanied by up-regulation of adipogenic marker genes, C/EBPα, PPARγ, aP2 and adipsin (Fig 15B). These data suggest that blocking Shh signalling promotes adipogenesis.

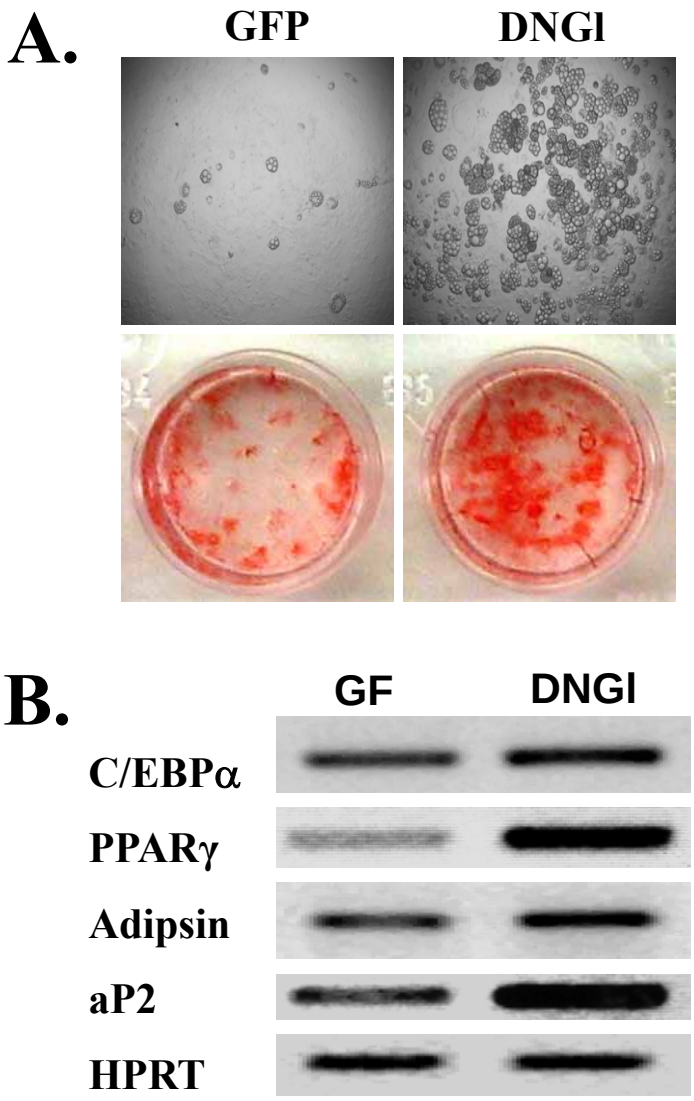

Figure 15 Inhibiting the Hh pathway stimulates 3T3-L1 adipogenesis.

A) 3T3-L1s were infected with a retrovirus expressing either GFP or DNGli, a dominant negative form of Gli2, a Hh transcription factor. DNGli increased fat formation as assessed morphologically (top), by Oil Red O staining (bottom). **B)** RNA was extracted from induced 3T3-L1s infected with GFP or DNGli expressing virus and semi-quantitative RT-PCR was done for the indicated transcripts.

3.5 Hh inhibits fat formation early in adipogenesis

3T3-L1 adipogenic differentiation can be divided into various periods (Rosen et al., 2000). To determine during which phases hedgehog was able to inhibit adipocyte differentiation, I treated 3T3-L1 cells with either vehicle or Shh at two-day intervals during induction and the effect on adipocyte formation and triglyceride content was measured after a 14-day culture period. Shh decreased adipocyte formation and lipid accumulation (Fig 16A and B). Maximal inhibition of adipogenesis was observed by Shh addition during the first three days of induction, with triglyceride content over 15 fold lower in Shh treated culture than control culture. Adding Shh from day 5 was much less effective, as indicated by triglyceride quantitation. Addition of Shh during later stages of induction did not affect adipocyte differentiation. These data suggested that Hh predominantly affected early differentiation events.

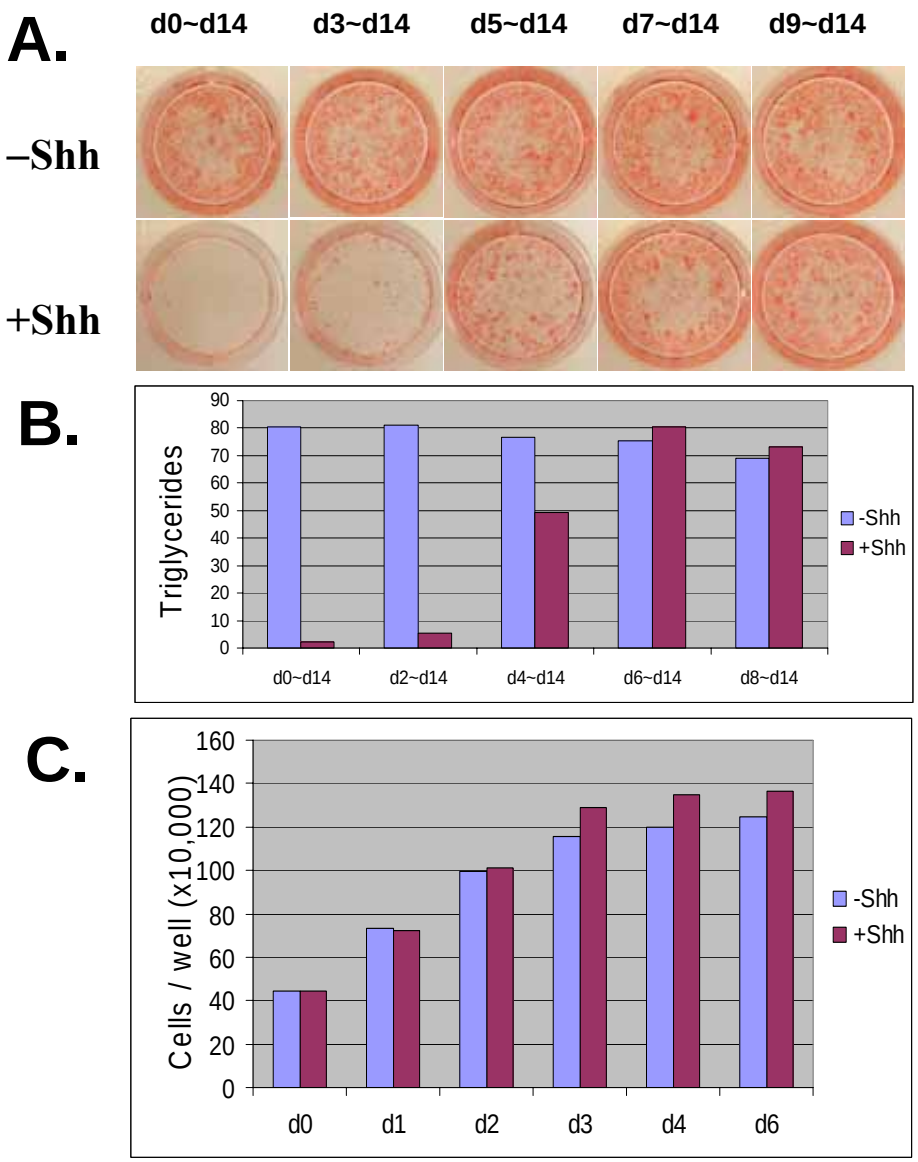

Figure 16 The Hh pathway acts early during 3T3-L1 adipogenesis, independently of the cell cycle.

A, B) 3T3-L1s were incubated in adipogenic induction media in the presence of vehicle (-Shh) or Shh at indicated time points and fat formation was scored with Oil Red O staining (A) and triglyceride quantitation (B). **C)** 3T3-L1s were incubated in adipogenic induction media in the presence of vehicle (-Shh) or Shh protein and cellular proliferation was evaluated with cell counts (C). No statistically significant changes were detected at any time point in either assay.

3.6 Hh signaling does not promote proliferation during 3T3-L1 adipogenesis

In cultured cell models, addition of prodifferentiative hormonal regimen upon induction is immediately followed by one or two rounds of cell division know as clonal expansion (Tang et al., 2003). Since Hh can sometimes act as a powerful mitogen to stimulate proliferation (Paladini et al., 2005; Wechsler-Reya and Scott, 2001), it may perturb clonal expansion stage of 3T3-L1 cells differentiation, in turn, inhibited adipogenesis. To test this possibility, I first estimate proliferation by cell count. 3T3-L1 cells were continuously incubated with vehicle or Shh during induction. Cell number was counted on each successive day. No significant difference was seen in cell count (Fig 16C). The small and statistically insignificant change observed in the latter time points was not detected in all experiments. To further investigate this, I used a quantitative BrdU assay to label DNA synthesis (Gratzner, 1982). No significant difference was seen in the production of BrdU positive cells (Fig 17A). To further confirm this, I quantified and analyzed BrdU incorporated cells from multiple independent fields in multiple experiments. Again, no statistically significant difference was found (Fig 17B). In addition, the fact that expression level or activity of Rb was also not altered by Shh addition further supports that Hh does not inhibit adipogenesis by stimulating proliferation in 3T3-L1 differentiation (see Fig 23 below).

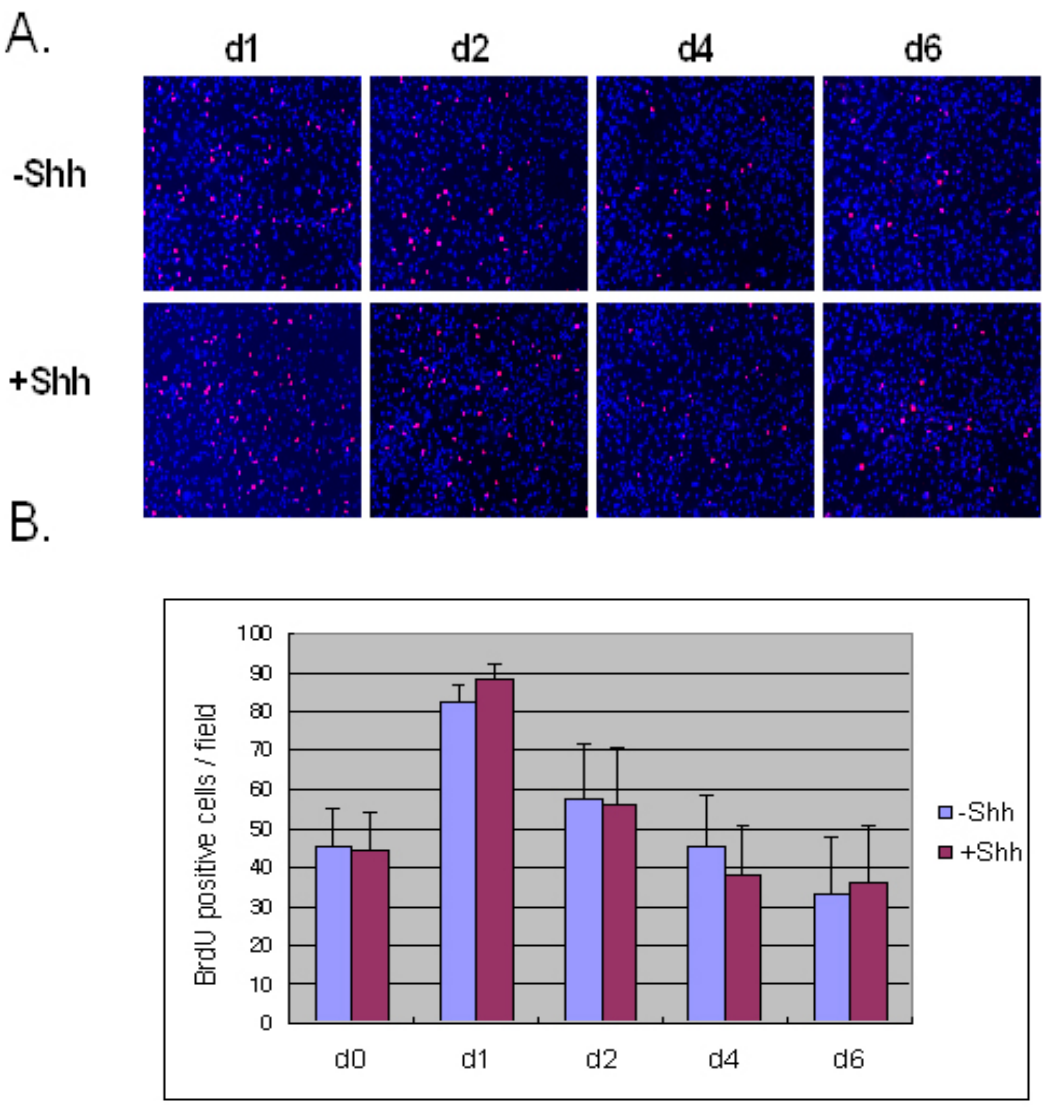

Figure 17 The Hh pathway acts early during 3T3-L1 adipogenesis, independently of the cell cycle.

A, B) 3T3-L1s were incubated in adipogenic induction media in the presence of vehicle (-Shh) or Shh protein and cellular proliferation was evaluated with a quantitative BrdU assay. No statistically significant changes were detected at any time point in either assay. BrDu positive cells are pink; DAPI staining in blue (A). Error bars represent SEM.

3.7 Hh signaling upregulates the expression of bone markers in 3T3-L1 cells

Shh can inhibit 3T3-L1 adipogenesis through two possible mechanisms: either by (1) blocking adipogenic differentiation and keep 3T3-L1 cells in undifferentiated preadipocyte state or (2) alternating their fate to a different cell lineage. The first possible mechanism was supported by the Hh-dependent increase in the expression of preadipocyte marker gene Pref-1 (Fig 10C). However, close morphological examination of Shh treated 3T3-L1 cells rather supported the second mechanism. Shh treated cells had an elongated spindle-like morphology, which was different from the more extended and flatter morphology of the 3T3-L1 preadipocytes. The second possibility seems less likely since 3T3-L1 cell is generally viewed as a preadipocyte cell line, which has already undergone commitment into adipocyte. However, since Shh plays a critical role in fate determination of many cell lineages, including neuronal cells, osteoblasts, skin cells, and hematopoetic cells (Goodrich and Scott, 1998; Hammerschmidt et al., 1997), the second mechanisms is deserved to investigate. To test this, I treated 3T3-L1 cells with either vehicle control or Shh during adipogenic differentiation. RT-PCR was performed to analyze expression of a group of marker genes specific for muscle, blood and bone lineage. No significant change was detected in the expression of muscle and blood marker. In contrast, two bone-specific transcription factors, Osterix (Osx) and Runx2 (Cbfal MASN splice variant), which are indispensable for osteoblastic differennation were upregulated by Shh addition. In addition, Shh treatment also enhanced expression levels of type I PTH/PTHrP receptor (PTHR) and

alkaline phosphatase (ALP), two markers of osteogenic differentiation (Fig 18). These data suggested that Shh signaling was able to switch the committed preadipocyte 3T3-L1 cells to osteogenic lineage.

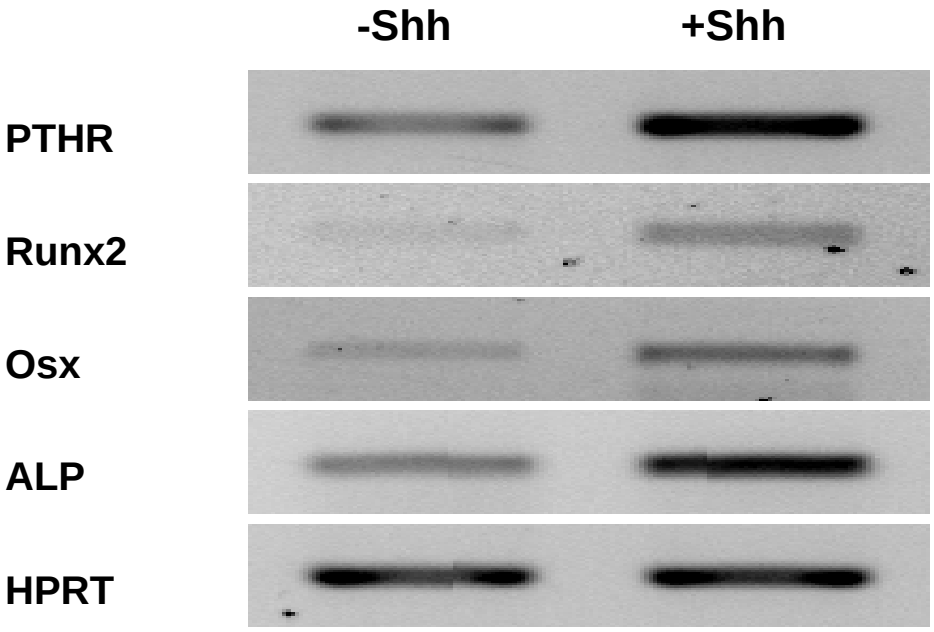

Figure 18 Hh signals induce the expression of bone markers in 3T3-L1s.

3T3-L1s were induced to form adipocytes in the presence of vehicle (-Shh) or Shh and the levels of the indicated osteogenic markers were assessed with semi-quantitative RT-PCR. HPRT serves as a loading control.

3.8 Hh signaling inhibits fat formation in multipotent mesenchymal stem cells

As stated above, I observed Hh signaling increased expression of markers of the osteogenic lineage in 3T3-L1 cells. It is difficult, however, to assess the extent of this event in preadipocyte model since it was not normally progress along the osteogenic pathway. Multipotent mesenchymal stem cell lines, such as 10T1/2 cells, are another type of cell lines widely used in adipocyte study. These cell lines can undergo commitment and differentiate into adipocytes and cells of multiple other mesodermal fates, such as myocytes and chondrocytes, which provide us a useful tool for understanding the determination process of adipocyte development. To examine the role of Hh pathway in cell fate specification, 10T/12 cells were induced to adipogenic differentiation in the presence of vehicle control or Shh. Adipocyte differentiation was assessed by microscopic examination and molecular analysis. Shh treatment dramatically decreased adipocyte formation (Fig 19A). PPARγ and aP2, two adipogenic differentiation markers, were downregulated by Shh treatment. Cultures treated with Shh had more than 80% lower PPARγ and aP2 expression than vehicle control by real-time PCR analysis (Fig 19B), indicating that activation of Hh pathway in 10T1/2 cells inhibits adipogenesis. Conversely, KAAD-cyclopamine was used to block Hh pathway in 10T1/2 cells during adipogenic induction. Morphology showed that KAAD-cyclopamine treatment promoted adipogenesis (Fig 20). In addition, Runx2 and ALP, two osteogenic markers, were up-regulated by Shh addition (Fig 21). These data suggested that Hh pathway retained its anti-adipogenic and pro-osteogenic property in 10T1/2 cells.

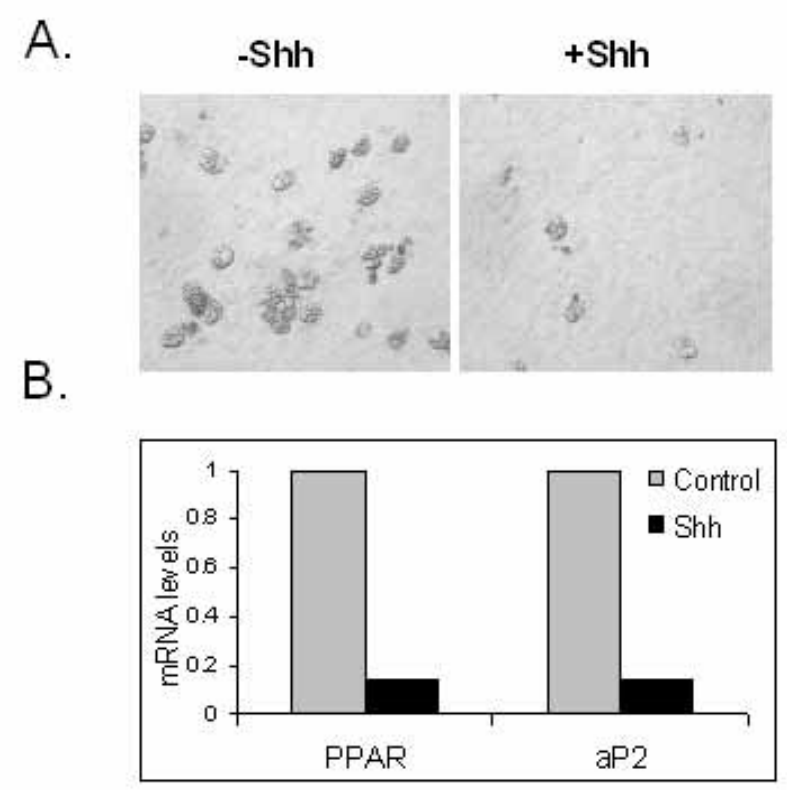

Figure 19 Hh signaling is anti-adipogenic in 10T1/2.

10T1/2 multipotent mesenchymal cells were incubated in adipogenic induction media in the presence of vehicle (-Shh) or Shh, which inhibited adipogenesis as scored morphologically (A) or with real-time PCR analysis (B).

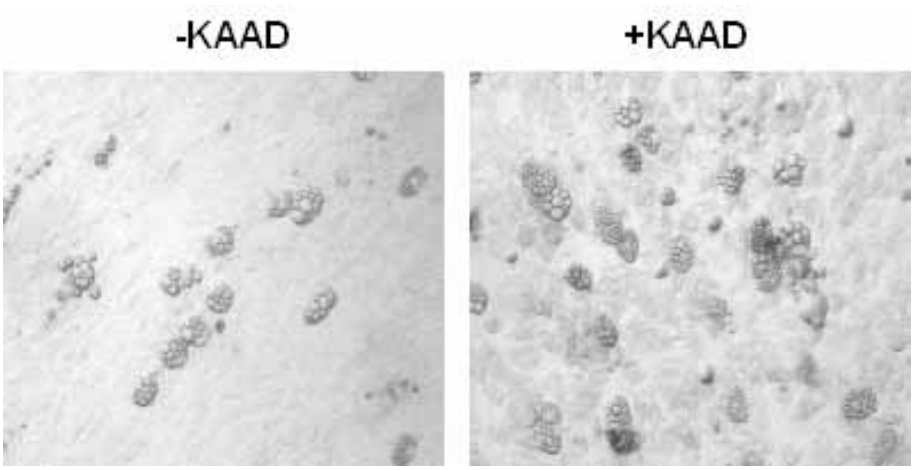

Figure 20 Inhibiting the Hh pathway stimulates 10T1/2 adipogenesis.

10T1/2s were incubated in vehicle (-KAAD) or KAAD (Hh antagonist) and morphology showed that KAAD stimulated adipogenesis.

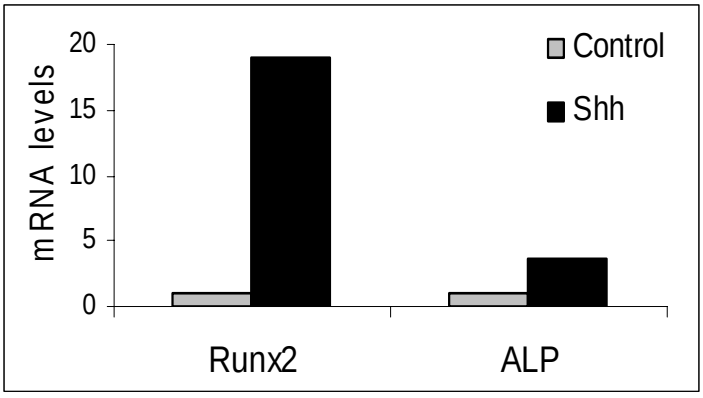

Figure 21 Hh signals induce the expression of bone markers in 10T1/2.

10T1/2s were incubated in adipogenic induction media with vehicle or Shh and osteogenic markers were assessed with real-time PCR. ß-actin serves as a loading control for real-time PCR (C, E).

3.9 Hh signaling functions upstream of PPARγ

PPARγ, a master regulator of adipocyte gene transcription discovered in fat, plays a crucial role in the regulation of adipogenesis (Devine et al., 1999; Dreyer et al., 1992; Schoonjans et al., 1996; Tontonoz et al., 1994a, c; Tontonoz et al., 1995b). In 3T3-L1 cells, it is firstly expressed around day 3 of induction, which coincides with the effective time window of Shh (Fig 16A and B). In addition, activation of Hh signaling lead to downregulation of PPARγ expression (Fig 10C, Fig 12B and Fig 13C), which suggested that Hh pathway may function earlier than PPARγ in regulating adipocyte differentiation. If so, I hypothesized that expression of PPARγ should be able to rescue adipogenic differentiation of Hh signaling activated cells. To examine this hypothesis, I first infected NIH3T3-SmoA1 cells with retroviruses carrying the genes for control GFP or PPARγ. After selection, cells were induced to differentiate. Two weeks later, as expected, GFP expressing control NIH3T3-SmoA1 cells failed to differentiate into adipocytes. In contrast, fully differentiated adipocytes were seen in the NIH3T3-SmoA1 cells expressing PPARγ, as evident by microscopic examination (Fig 22 top). To further test it, I established stable 3T3-L1 cell lines carrying either control GFP or PPARγ. Next, both stable cell lines were induced to adipogenic differentiation in the presence of vehicle control or Shh. As showed previously (Fig 10A), Shh treatment inhibited adipogenesis in 3T3-L1-GFP cells. In contrast, adipocyte differentiation in 3T3-L1-PPARγ cells was not affected by Shh addition (Fig 22 bottom). These results suggest that Shh signaling functions upstream of PPARγ.

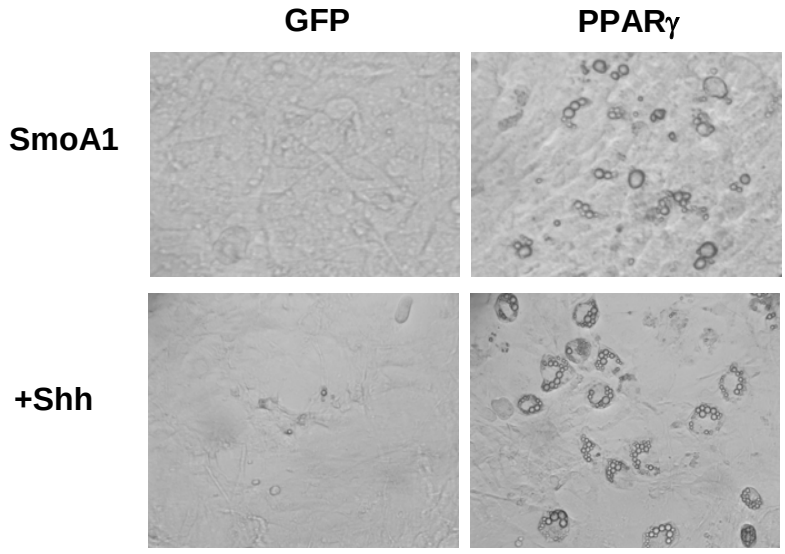

Figure 22 Hh signaling functions upstream of PPARγ.

NIH-3T3 cells expressing SmoA1, an activated form of the Hh receptor Smo, were infected with a virus encoding either GFP or PPARγ and PPARγ rescued the SmoA1 blockade of adipogenesis (top).

3T3-L1s were infected with a virus encoding either GFP or PPARγ and Shh protein was added throughout induction. PPARγ reversed the Shh-dependent inhibition of adipogenesis (bottom).

3.10 Hh pathway requires Gata to inhibit adipogenesis

To understand how Hh pathway elicits its inhibitory effect on adipogenesis through PPARγ, literature was reviewed to search for Hh responsive candidate genes that play a role in adipogenesis upstream of PPARγ. Four candidate genes were identified, which were Rb, Gata2, Gata3 and Gilz.

Retinoblastoma protein RB is a negative regulator of the cell cycle, which plays a crucial role in adipocyte differentiation (Fajas et al., 2002). Hh signaling can inactivate Rb activity by increasing level of the hyperphosphorylated Rb (Duman-Scheel et al., 2002; Kenney and Rowitch, 2000). To investigate whether Hh signaling regulated Rb activity in 3T3-L1 cells, I examined the expression and phosphorylation of Rb by Western blot. To do this, 3T3-L1 cells were continuously treated with either vehicle or Shh during induction. Western blot was performed on cell lysate harvested from time 0 of induction and every four hours after until 28 hours. The hyperphosphorylated Rb (ppRb) was identified by reduced mobility in comparison with the faster-migrating hypophosphorylated Rb (pRb). Consistent with the previous report, Rb phosphorylation was first deteted after 16hrs of induction (Fig 23 and Tang et al., 2003). However, no significant difference was seen in the level of expression or phosphorylation of Rb. This result suggested that Hh does not inhibit adipogenesis by regulation of Rb.

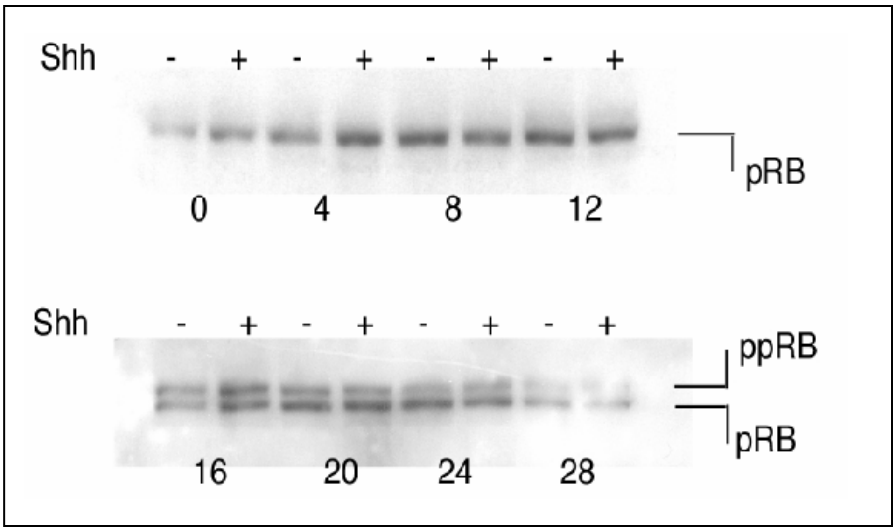

Figure 23 Hh signaling does not affect Rb phosphorylation in 3T3-L1.

3T3-L1s were incubated in adipogenic induction media in the presence of vehicle (-) or Shh (+), cell lysates were harvested at the indicated hours after Shh addition, and subjected to SDS-PAGE and Western blotting with anti-Rb antibodies. The phosphorylated and inactive Rb protein migrates slower than non-phosphorylated Rb and the levels of both forms are unaffected by Shh.

Gata2 and Gata3, two newly identified preadipocyte genes, inhibit adipogenesis by controlling the transition from preadipocytes to adipocytes (Tong et al., 2000). In 3T3-L1 cells, both transcription factors can suppress PPARγ function by direct binding on its promoter. Therefore, Gata2 and Gata3 can serve as two candidate genes downstream of Hh signaling for its inhibition on adipogenesis. *Glucocorticoid Induced Leucine Zipper* (*GILZ*), one of the characteristic members of TSC-22 family, is another good candidate gene. It was identified by a microarry study as a downstream target of Shh signaling

(Ingram et al., 2002). In mammalian cell culture models, GILZ suppresses adipocyte differentiation upstream of PPARγ (Shi et al., 2003). To test whether Hh signaling blocks adipogenesis by increasing the expression of the anti-adipogenic transcription factors Gata2, Gata3 and GILZ, 3T3-L1 cells were induced to differentiate in the presence of vehicle control or Shh. Two weeks later, when inhibition of adipocyte differentiation was fully observed, expression of Gata2, Gata3 and GILZ was examined by RT-PCR. Shh addition significantly elevated the expression level of all three genes (Fig 24). I then examined whether Hh signaling regulated their expression during early adipogenesis, the period when Shh treatment had its maximal effect. To test this, 3T3-L1 cells were incubated with vehicle control or Shh during adipogenic differentiation. Total RNA was harvested from the time-point indicated on Fig 25, and real-time RT-PCR was carried out. Shh significantly increased expression of Gata2 at both 12 and 24 hours. In contrast, only a moderate increase was detected in Gata3 expression at 12 hour and no significant change in GILZ expression was found. This suggested that GATA factors might serve as mediators for Hh-dependent blockade of adipogenesis.

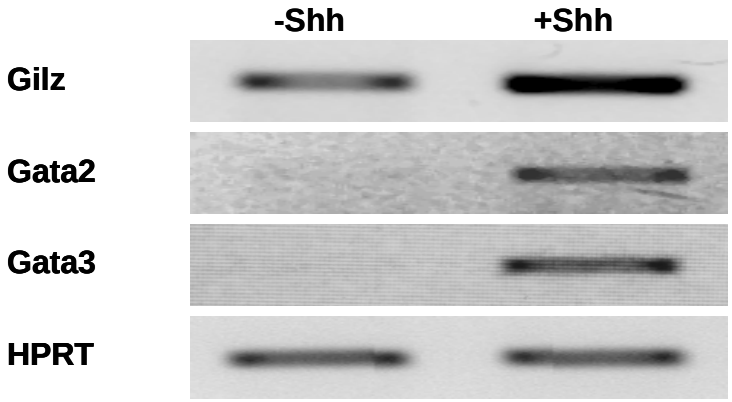

Figure 24 The Hh signaling induces expression of Gilz, Gata2 and Gata3.

3T3-L1 cells were induced to form adipocytes in the presence of vehicle or Shh and the levels of the indicated anti-adipogenic transcription factors were assessed with semi-quantitative RT-PCR. HPRT serves as a loading control.

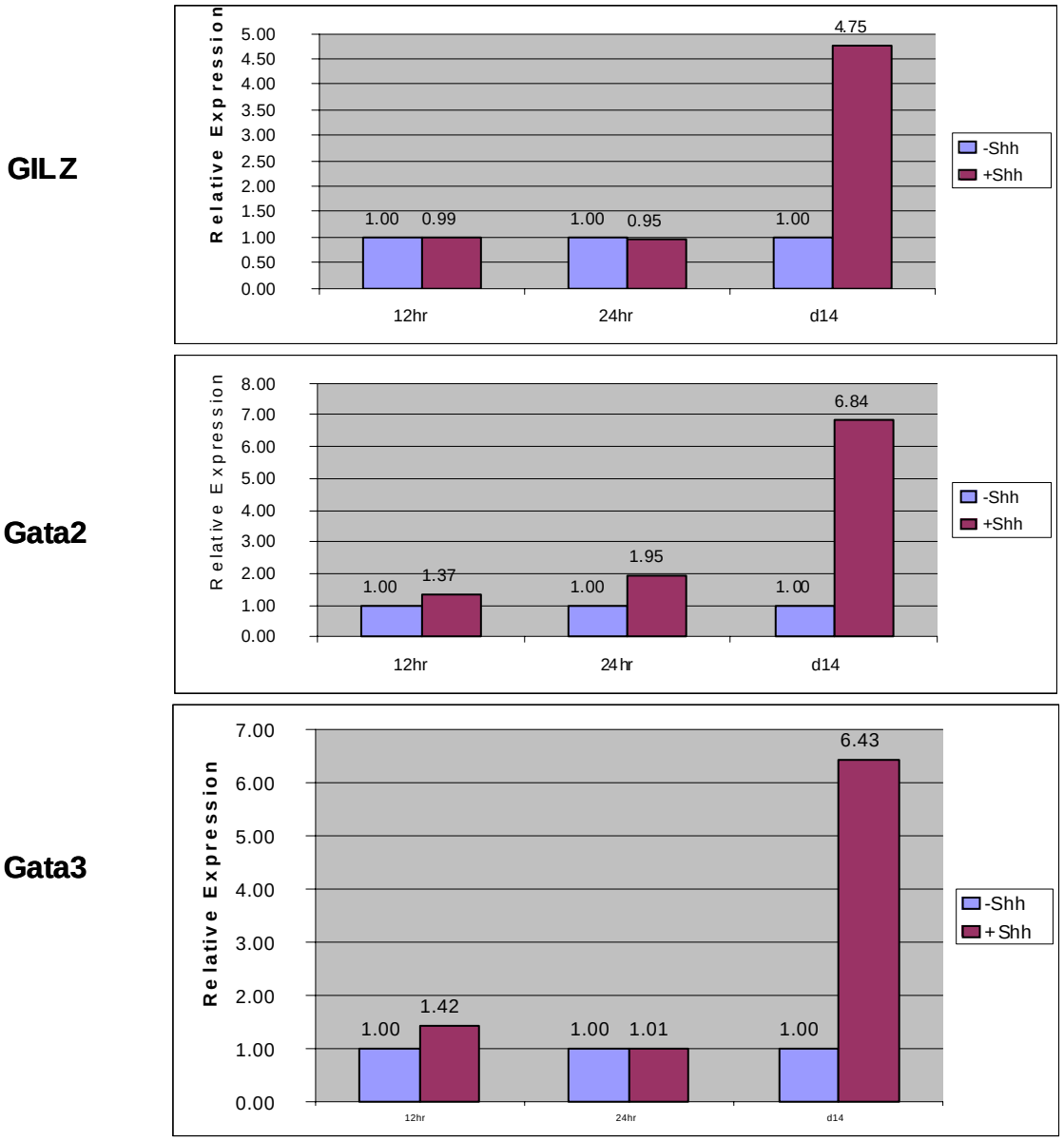

Figure 25 The Hh signaling induces expression of Gata early in adipogenesis.

3T3-L1 cells were induced to form adipocytes in the presence of vehicle or Shh and the expression levels of Gilz, Gata2 and Gata3 at indicated time points were assessed with real-time PCR. Analysis of relative expression showed that expression of Gata factors was induced by Shh addition early in adipogenesis.

To further determine the epistatic relationship between Hh signaling and Gata2, I first infected 3T3-L1 cells with retroviruses carrying the genes for control GFP or Gata2. After selection, both stable cell lines were induced to differentiate in the presence of vehicle control or KAAD-cyclopamine. Adipocyte differentiation was examined by Oil-Red O staining of the lipid droplets in the cells. Two weeks later, as expected, KAAD-cyclopamine treatment promoted adipogeneis in the control 3T3-L1-GFP cells. In contrast, no significant change in adipocyte formation was found by KAAD-cyclopamine addition. Either treated with vehicle control or KAAD-cyclopamine, 3T3-L1-Gata2 cells failed to differentiate into adipocytes (Fig 26A). To complete the epistasis test, 3T3-L1 cells were infected with a control GFP virus or a virus containing a dominant negative Gata3 (DNGATA) that blocks activity of Gata2 and Gata3 (Smith et al., 1995). After selection, both stable cell lines were induced to adipogenic differentiation in the presence of vehicle control or Shh. As showed previously (Fig 10), Shh treatment inhibited adipogenesis in 3T3-L1-GFP cells. In contrast, adipocyte differentiation in 3T3-L1-DNGATA cells was not affected by Shh addition (Fig 27). Epistasis test was also performed in 10T1/2 cell. KAAD-stimulated adipogenesis could be reversed by overexpression of Gata2.

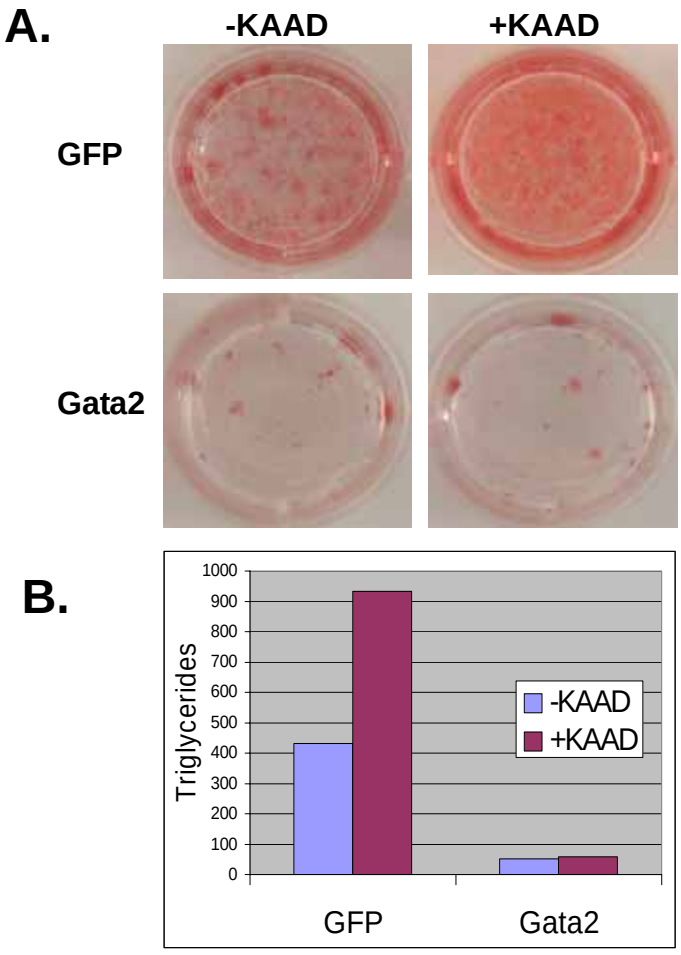

Figure 26 The Hh cascade requires Gata factors to inhibit fat formation.

A, B) 3T3-L1s infected with a retrovirus expressing either GFP or Gata2 were incubated in adipogenic induction media containing either vehicle (-KAAD) or KAAD (Hh antagonist). Based upon Oil Red O staining (A) and triglyceride quantitation (B), Gata2 inhibits adipogenesis even when the Shh pathway was blocked.

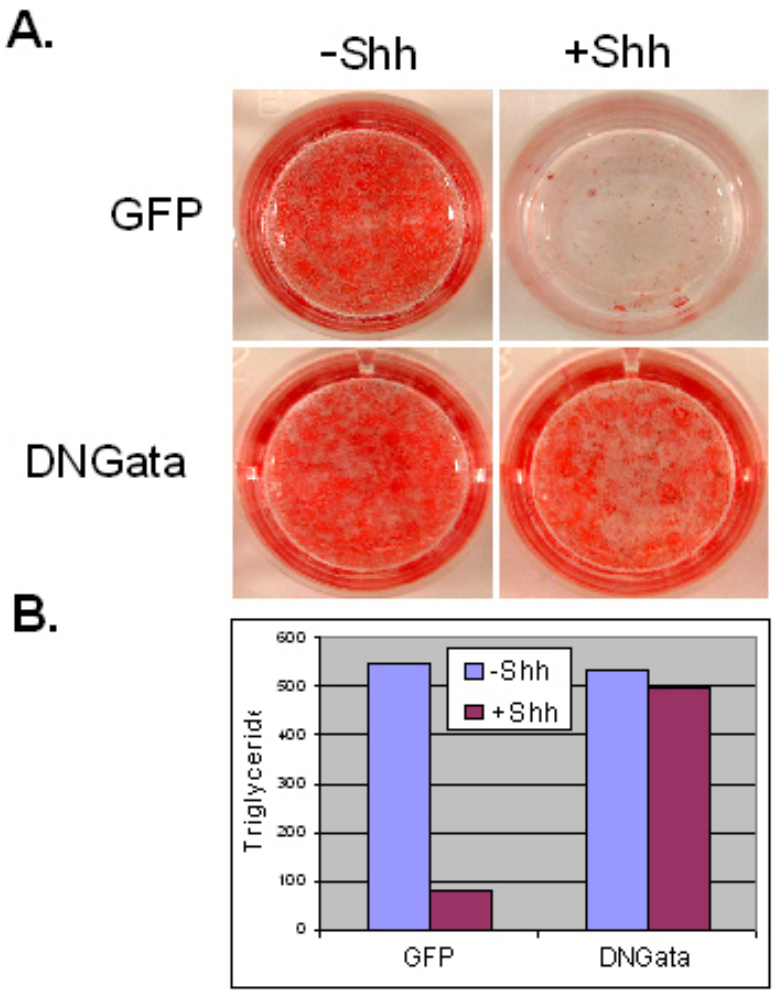

Figure 27 The Hh cascade requires Gata factors to inhibit fat formation.

A, B) 3T3-L1 cells were infected with a GFP or a dominant negative Gata (DNGata) virus, incubated in adipogenic induction media containing either vehicle or Shh, and adipogenesis was assessed by Oil Red O staining (A) or triglyceride quantitation (B), both show that Gata inhibition reverses the Shh-dependent adipogenic blockade.

These results suggest that Gata2 functions downstream of Shh and its expression is required for Hh signaling for its inhibitory role in adipogenesis. Since GATA factors possess similar function as Shh signaling in inhibiting adipogenesis, it is reasonable to hypothesize that they also promote osteogenesis as Shh signaling does. To test this, 10T1/2 cells were infected with retroviruses containing GFP or Gata2 (Fig 28). At mRNA level, overexpression of Gata2 during induction stimulated expression of the osteogenic transcription factors Runx2 and Osx (Fig 28B), at the same time reduced the expression level of adipogenic differentiation markers PPARγ and aP2 (Fig 28C).

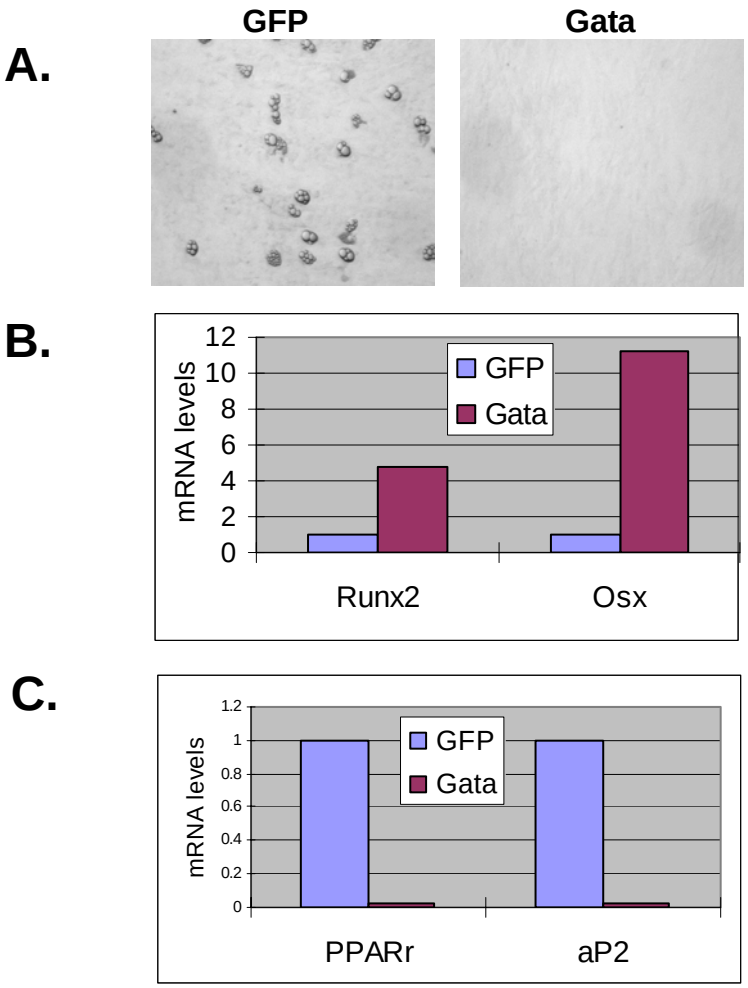

Figure 28 The Hh signaling inhibits adipogenesis and promotes osteogenesis via Gata factors

A, B) 10T1/2 multipotent mesenchymal cells were infected with a virus encoding either GFP or Gata, incubated in adipogenic induction media and adipogenesis was scored microscopically (A) and molecularly (B). **C)** C3H10T1/2s expressing GFP or Gata were adipogenically induced and RNA was extracted. Real-time PCR showed that Gata induced the expression of the osteogenic transcription factors, Runx2 and Osx. ß-actin serves as a loading control for real-time PCR (B, C). *$p<0.01$

3.11 Hh signaling is reduced in mouse models of obesity

As shown in mammalian cell culture models and *in vivo Drosophila,* blocking Hh signaling promotes adipogenesis. Conceivably, increased adiposity might be associated with defects in the expression and/or function of Hh signaling components. To test this, potential changes in expression of Hh signaling components were investigated in adipose samples from two independent models of obesity, Ob/Ob and diet-induced obesity model. Real-time PCR demonstrated a dramatic reduction in the adipose expression of Smo, Gli1, Gli2 and Gli3 in both models, in comparison to matched control littermates (Fig 29). These data suggested that Hh signaling may be implicated in fat homeostasis in vivo and in the pathogenesis of diseases of fat.

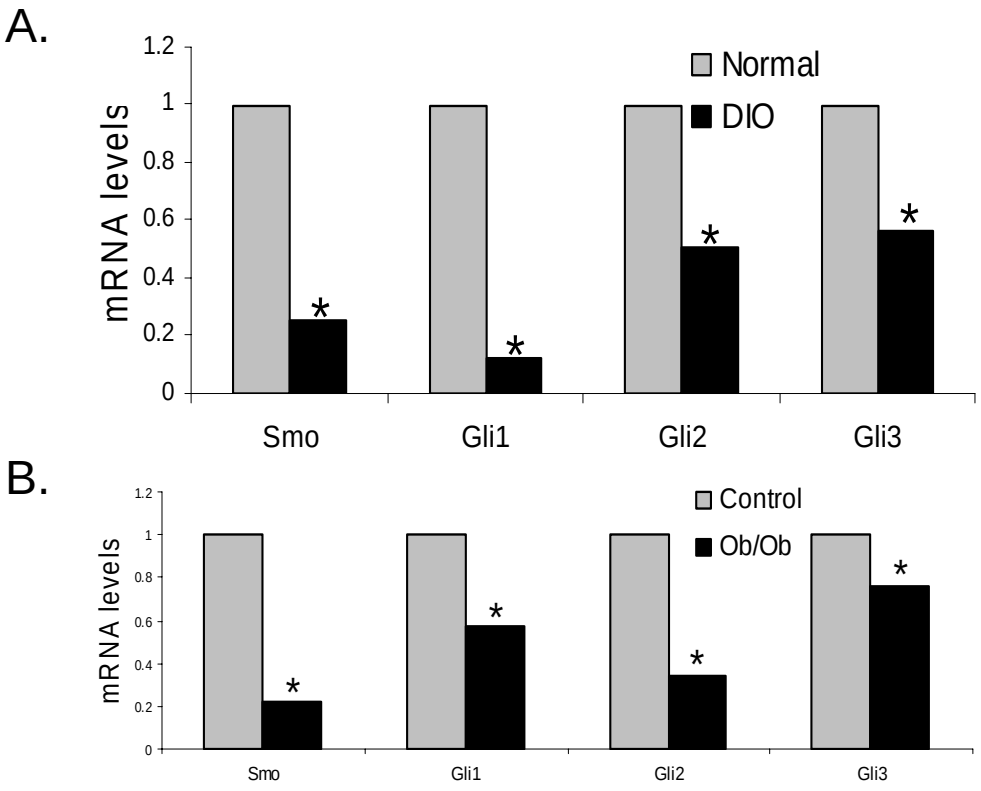

Figure 29 Murine obesity regulates the Hh pathway.

A) Matched littermates were fed four months of normal or high fat chow (DIO) and adipose depot gene expression was analyzed with real-time PCR, which demonstrated a statistically significant decrease in Smo, Gli1, Gli2, and Gli3 expression in DIO fat. n = 4. **B)** Smo and Gli1, Gli2, and Gli3 levels are reduced in genetically obese (Ob/Ob) fat depots compared to controls. n = 4. ß-actin serves as a loading control for real-time PCR. *P<0.05

CHAPTER FOUR

DISCUSSION

4.1. *Drosophila melanogaster* as a model system to study fat biology

4.1.1 Conservation of fat biology between invertebrate and vertebrate

Homeostatic regulation of fat storage is a fundamental property of both vertebrates and invertebrates (Spiegelman and Flier, 2001). In organisms as different as humans and *Drosophila*, fat storage provides a massive energy reserve that can be mobilized upon conditions of nutritional deficiency, which is key to an individual's survival during food deprivation. In addition, it also provides physiological fuel that is required for various aspects of biological life cycles including migration, hibernation and metamorphosis. When caloric intake exceeds expenditure, excess energy is directed into pathways leading to triacylglycerol (TAG) synthesis and stored as intracellular lipid droplets of specialized organs called the adipose tissue in mammals or the fat body in *Drosophila*. By synthesis and secretion of various compounds and signaling molecules, both adipose tissue and fat body play not only an energy-storing role, but also regulate reproduction and immunity, etc (Hamilton et al., 1995; Hotamisligil et al., 1995; Lefebvre et al., 1998; Morrison and Farmer, 1999). In human, abnormalities in fat storage produce pathological states such as obesity and type II diabetes (Campbell and Dhand, 2000).

It has been shown that the sequence and function of genes that play essential roles in life cycles of organisms have been greatly conserved throughout evolution. Most human

disease-associated genes are present in the genome of *Drosophila* (Kornberg and Krasnow, 2000; Rubin et al., 2000). Therefore, it is not surprising that the ability to store fat has been conserved over a wide evolutionary distance. For example, the regulatory mechanisms of TAG mobilization have great similarities between mammals and *Drosophila*. In mammals, upon energy demand, β-adrenergic receptors are activated by binding of agaonists and subsequently stimulate production of cAMP and activation of protein kinase A (PKA) through the stimulatory GTP-binding protein (G-protein) (Clifford et al., 2000; Egan et al., 1992; Sztalryd et al., 2003). Activation of PKA through post-translational modification acutely activates hormone-sensitive lipase (HSL) and perilipin through phosphorylation, which causes a dramatic increase in storage fat lipolysis (Anthonsen et al., 1998; Clifford et al., 1997; Londos et al., 1999). In *Drosophila*, adipokinetic hormone (AKH) plays a key role in regulating TAG mobilization (Lee and Park, 2004). Similar to mammalian storage fat lipolysis, it starts with AKH binding to a cell membrane-bound Drm-AKH receptor (the *Drosophila* AKH receptor), which, in turn, changes conformation and interacts with a G-protein (Park et al., 2002; Staubli et al., 2002). This leads to subsequent activation of the cAMP second messenger signaling pathway, increased intracellular cAMP and activation of PKA (Asher et al., 1984; Arrese et al., 1999). Activated *Drosophila* TAG lipase Brummer mobilizes the organismal fat and produces demanded energy (Gronke et al., 2005). As *Drosophila* is an excellent model for many biological processes, it seemed plausible that it might also prove to be a powerful system for analyzing the mechanisms of fat storage. Therefore, here in our lab, we utilized *Drosophila* as a useful genetic model

system for discovery of new genes and signaling pathways important in fat biology.

4.1.2 The inhibitory role of Hh signaling in *Drosophila*

First, we did an unbiased enhance trap screen designed to find genes whose expression pattern is specified in the developing fat body cells. In this screen, P-element insertion was identified into the gene encoding Smo, a Hh receptor. We also found that other components of the Hh pathway were expressed in the fly fat body. Then, we decided to explore the role of Hh signaling in fat formation first in *Drosophila*.

4.1.3 Importance of *Drosophila* as a model to study Hh signaling

Drosophila is an excellent model system in which to study the function and action of Hh signaling. First, the Hh signaling pathway was originally identified and has been most extensively studied in *Drosophila* (reviewed in Ingham and McMahon, 2001). Second, all of the Hh signaling components have vertebrate homologs, although in most cases, a single gene in *Drosophila* corresponds to a family of related homologs in vertebrates (Hammerschmidt et.al., 1997). Third, not only the underlying mechanism for Hh signaling pathway is quite similar between invertebrates and vertebrates, but also their functions are highly conserved. Much of what is known about Hh signaling in vertebrates has been inferred from studies in *Drosophila*. For example, Hh signaling regulates embryo morphogenesis in both invertebrates and vertebrates. Disruption of Hh signaling in developing vertebrate embryos can lead to defects analogous to segment polarity

abnormalityes in *Drosophila* (Wicking et al., 1999). Therefore, characterizing the action and function of Hh signaling in invertebrate gains us good mechanistic and functional insights in vertebrates. Finally, *Drosophila* has been well studied and characterized both genetically and developmentally for nearly a hundred years, and is easily manipulated using a wide variety of experimental techniques. For example, the creation of the UAS/Gal4 system in *Drosophila* provides us one of the most powerful tools to target gene expression in a temporally and spatially controlled fashion for addressing gene function in vivo (Brand and Perrimon, 1993).

4.1.4 Hh signaling blocks *Drosophila* fat formation

To determine whether the Hh cascade may play a role in fly fat formation, we undertook UAS/Gal4 system to express Hh signaling components specifically in the developing fat body. We found that expressing an activated form of the Hh transcription factor Ci reduced fly fat accumulation and expression level of fat body markers. Expressing other positive regulator of the Hh signaling pathway in fat body, such as Hh and Smo, produced similar lean phenotype, which suggested the generality of the effects of the Hh pathway on fly fat formation. Blocking Hh signaling with DNci, a dominant-negative form of ci, induced an obese phenotype in *Drosophila*, which further substantiated the inhibitory role of Hh signaling in fly fat formation.

As previously discussed, the ability to store fat is highly conserved between invertebrates and vertebrates. In addition, the conservation between the function of Hh

signaling in invertebrates and vertebrates further increases the likelihood that we can make connections to mammalian fat biology. Therefore, we predicted that the inhibitory role of Hh signaling might be conserved in mammalian fat biology. Our subsequent findings in the more traditional systems, 3T3-L1 preadipocytes and C3H10T1/2 multipotent stem cells, confirmed this hypothesis.

4.2 The inhibitory role of Hh signaling in mammalian fat

4.2.1 3T3-L1 cells express Hh signaling components and respond to Shh

So far, much of our current understanding of the molecular regulation of adipogenesis comes from in vitro studies of pre-adipocyte cell lines such as 3T3-L1 and multipotent stem cell lines such as C3H10T1/2 (MacDougald and Lane, 1995; Rangwala and Lazar, 2000). To extend the data from *Drosophila* to mammals, we first analyzed the role of Hh signaling in adipogenic differentiation of the murine preadipocyte cell line 3T3-L1. A systematic survey of mRNA expression of Hh components showed 3T3-L1 cells expressed the mediators for a functional Hh signaling pathway. We showed that the positive regulators Smo, Gli1~3 were present in 3T3-L1 cells, as well as the negative regulators Ptc1 and Ptc2. In addition, analysis of Hh signaling components over the course of 3T3-L1 adipocyte differentiation revealed that the Hh signaling components were expressed in a dynamic pattern. Interestingly, the expression of positive regulators Smo, Gli1~3 was highest in growing and confluent cells, but decreased upon adipogenic induction (Fig. 9), while the expression of negative regulator Ptc2 was up-regulated during differentiation,

which is consistent with the idea that a decrease in Hh signaling is required for adipogenesis to occur. The presence of a function Hh signaling pathway was also reflected by the fact that 3T3-L1 cells responded to the recombinant Shh. Addition of Shh to 3T3-L1 cells induced the expression of Gli1, whose expression has been shown to be regulated by Hh activation (Marigo et al., 1996).

4.2.2 Hh signaling inhibits adipogenesist

Our data provided evidence that the inhibitory role of Hh signaling is conserved in mammalian adiogenic differentiation. Addition of recombinant Hh potently inhibited adipogenic differentiation of 3T3-L1 cells dose-dependently as determined by decreased Oil Red O staining and reduced mRNA expression of established adipogenic marker genes. In addition, the inhibition of adipogenesis by Shh addition is a specific result of activation of Hh signaling since activating Hh signaling genetically by a constitutively active Smo receptor also produced the same effect. In contrast, blocking Hh signaling, either pharmacologically by KAAD-cyclopamine or genetically by a dominant-negative Gli2, strongly promoted adipogenesis, which further supported the inhibitory role of endogenous Hh signaling in adipocyte differentiation. Taken together, out data support that Hh signaling functions as a molecular switch: when it is on, adipogenesis is repressed; when it is off, adipogenesis is enhanced.

To begin to elucidate how Hh signals negatively regulate fat formation, we first examined the effect period of Shh signaling. The use of recombinant Shh permits analyses

of the time frame during which Shh signaling inhibits the differentiation program. For this inhibition, only addition of Hh during the first 3 days of adipogenic differentiation was effective, while addition during later phases had no effect, suggesting that this effect is time-sensitive. This Shh-sensitive period is consistent with the time window of mitotic clonal expansion, one of the early events of adipogenic program, in which pre-adipocytes undergo one or two rounds of cell division. Proper cell cycle control is closely linked to adipogenic potential. Interestingly, although Hh signaling is well known to have potent pro-proliferative activity and stimulate cell division in many cell types, including normal and malignant cells (Bailey et al., 2000; Bak et al., 2003; Kenney and Rowitch, 2000; McMahon et al., 2003), it did not affect the clonal expansion in 3T3-L1 cells, as assayed by cell count and Brdu staining. We also examined the expression and activity of Rb, because Rb is one of the central regulators of cell cycle and critical for proper mitotic clonal expansion. The fact that Hh signaling did not alter expression or activity of Rb further substantiated that Hh signaling does not regulate cellular proliferation during adipogenesis. This lack of effect on cell culture is consistent with the fly data; the Hh effects observed in flier were elicited independently of cellular proliferation as we activated the Hh pathway in post-mitotic fat body cells. It also supported that Hh signaling does not regulate adipogenesis via Rb. Since the Hh-dependent inhibition of adipogenesis did occur early during adipogenic differentiation, Hh signaling is likely to affect early events after mitotic clonal expansion.

PPARγ, a master regulator of adipocyte lineage, is necessary and sufficient to

differentiate adipocytes and control the function of many, and perhaps most, adipocyte-specific genes (Devine et al., 1999; Dreyer et al., 1992; Schoonjans et al., 1996; Tontonoz et al., 1994a, c; Tontonoz et al., 1995b). It is expressed in the time frame in which the Hh pathway acts. By epistasis studies, we confirmed that Hh signaling function upstream of PPARγ. To identify the potential target controlled through Hh signaling during adipogenesis, we next examined the expression of Gata2, Gata3 and GILZ, three mammalian genes that fits in the following two criteria: 1) they are reported to regulate PPARγ expression and 2) they can be regulated by Hh signaling (Tong et al., 2000; Ingram et al., 2002; Shi et al., 2003). We found that expression levels of all three genes were elevated by Hh signaling under conditions in which the pathway inhibits adipogenesis. Gata2 also showed detectable expression change at the early time points, both 24h and 48h, in which Hh signaling alters adipogenesis. These time points are consistent with the previous microarray data by Ingram et al., in which the majority of the valid Shh targets showed a detectable expression change at 48h. We also found that Hh signaling required GATA function to suppress adipogenesis. So it is possible that Hh signaling inhibits adipogenesis at least in part by regulating GATA expression. Combined with previous description on Hh signaling pathway, our work that explored its underlying mechanism in adipogenesis leads to the following model described in Fig 30.

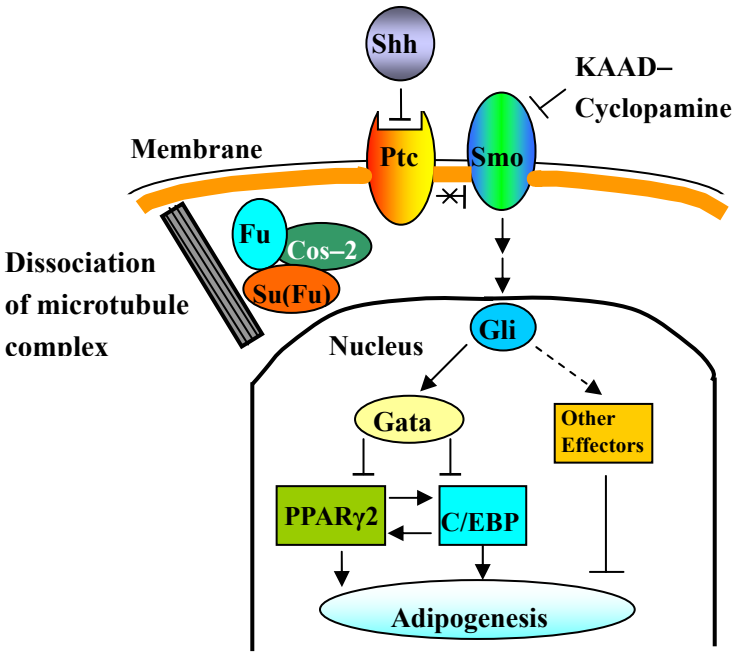

Figure 30 A model for Hh signaling in mammalian adipogenesis.

In mammals, binding of Shh releases Ptc inhibition on Smo and subsequently activates the Gli family of transcription factors. The process can be blocked by pharmacological agent, KAAD-cyclopamine. Gli activation, either directly or indirectly, induces antiadipogenic GATA factors, probably through transcriptional regulation. Gata2 and 3 repress the PPARγ2 promoter and inhibit the function of C/EBP proteins through a direct interaction. This does not exclude other possible factors that exist downstream of Shh in blocking adipogenesis.

4.2.3 Hh signaling stimulates osteogenesis

One of the most intriguing observations of our study is that Hh signaling altered the preadipocyte cell line 3T3-L1 from an adipocytic lineage to an osteoblastic lineage. For a cell to convert from one cell type to another, there must be suppression of differentiation of the original cell type and promotion of differentiation of the new one. Specifically, activation of the defined osteogenic transcription factors and repression of adipogenic ones are essential to the 3T3-L1's commitment to osteoblastic lineage. Osteoblastic differentiation is characterized by expression of the early marker alkaline phosphatase and the later marker Runx2 (Wagner and Karsenty, 2001). Adiopocytic differentiation is characterized by expression of early marker PPARγ and the later marker aP2 (Clarke et al., 1997; Tontonoz et al., 1994c). Therefore, elevated expression of alkaline phosphatase and Runx2 and down-regulated expression of PPARγ and aP2 in Shh treated cells suggested that 3T3-L1 cells differentiated from preadipocytes into obstetoblasts in response to Hh signaling. Although the potential function of Hh signaling on skeletal cells has been recently suggested by the induction of ectopic cartilage and bone formation by N-Shh (Kinto et al., 1997) and further characterized in 10T1/2 cells (Ahrens et al., 1993; Spinella-Jaegle et al., 2001), to our knowledge, this is the first time to show that Hh signaling could convert already committed cells to osteoblasts. In addition, it is surprising to find that 3T3-L1 cells are able to take on a second cell fate other than adipocyte fate, since it is generally believed to be a committed preadipocyte cell line which only

progresses along the adipogenic pathway. Hh signaling has been previously shown to control fate determination of many cell lineages, including neuronal cells, osteoblasts, skin cells, and hematopoetic cells (Goodrich and Scott 1998; Hammerschmidt et al., 1997). Our data further emphasized the role of Hh signals as critical morphogens in the induction of distinct cell fates. Further characterization of the role of Hh signaling was carried out in 10T1/2 cells. As in 3T3-L1 cells, Hh was anti-adipogenic and pro-osteogenic. Since GATA appeared to have similar functions as Hh, which also induced expression of osteogenic transcription factors, it may mediate the effect of Hh signaling to promote osteogenesis at the expense of adipogenesis. If so, the increase in fat and decrease in bone mass observed with aging might be a potential in vivo relevance of our results, in which Hh signaling might be reduced.

Hh signaling plays an integral role in differentiation and development of variety of cells and tissues. In addition, Hhs play critical roles in regulation of cell growth. Our results demonstrate that Hh signaling, possible mediated by GATA transcription factors, can inhibit fat formation in vitro and likely in vivo, based upon reduced expression of its signaling components in WAT sample of mouse models of obesity. In addition, it may function as a molecular switch to direct cells away from adipogenesis and towards an osteogenic fate. Further definition of the roles of Hh signaling in development of adipose tissues, and potentially as a modulator of obesity, await development and analyses of appropriate animal models.

4.3 Potential therapeutical application

Type 2 diabetes is characterized by hyperglycemia resulting from a failure of the pancreatic β-cells to compensate for insulin resistance. Obesity, which leads to insulin resistance, is a strong risk factor for the development of type 2 diabetes. Obesity and diabetes are now a major cause of morbidity and mortality throughout the world (discussed in Chapter One). Although the detailed molecular mechanism by which Hh signaling inhibits adipogenesis remain to be elucidated, our data suggest that since the Hh pathway has an inhibitory role in fat accumulation, it may provide novel targets for therapies designed to prevent obesity and diabetes.

With more advanced in age, an individual has a greater risk of developing osteoporosis, which is caused by a relative increase of bone resorption over bone formation. Disorder relating to bone loss is a major cause of morbidity and mortality in the elderly (Borgstrom et al., 2006; Koay and Brown, 2005). The increase in marrow adipogenesis associated with osteoporosis and age-related osteopenia is well known clinically (Bucay et al., 1998; Pacifici, 1998).

Both osteoblasts and adipocytes share a common multipotent progenitor cells, the mesenchymal stem cells. Our work demonstrated that Hh signal controls the balance between osteoblasts and adipocytes by inhibiting adipogenesis and promoting osteogenesis, which suggested that a large degree of plasticity exists between osteoblasts and adipocytes. In addition, the altered fate of 3T3-L1 preadipocytes from an adipogenic lineage to an

osteogenic lineage by Hh signaling further suggested that transdifferentiation is possible. It is very likely that inhibition of marrow adipogenesis with a concomitant increase in osteogenesis by Hh signaling could provide a therapeutic opportunity to either prevent further increases in adipocyte formation or divert existing adipocytes to become more osteoblasts with a resulting increase in functional bone cells. Therefore, studies on Hh pathway may provide exciting future pharmacological targets for the treatment of osteoporosis.

However, there are always issues with side-effect of a drug or therapy from inhibiting adipogenesis (Nadler and Attie, 2001; Buhman et al., 2004; Rosen, 2006). Two challenges have to be confronted: first, the drugs must direct function on the focus while can not influence tissue development elsewhere in the patient; second, if adipogenesis is inhibited, extra calories end up as ectopic lipid deposits in places like muscle and liver, i.e. there is a shift in the "lipogenic burden" from adipose tissue to other organs (Nadler and Attie, 2001). In order to develop proper drugs, Hh signaling has to be modulated in a way that it is not detrimental to normal tissues but will result in effective treatments for diseases. Therefore, the way to new drugs is far and further work is needed in order to translate the studies on Hh signaling pathways into therapeutics for obesity and osteoporosis.

4.4 Wnt and Hh signaling pathways in osteogenesis and adipogenesis

4.4.1 Similarities between the mechanism of Wnt and Hh signaling pathways

A large body of literature has shown that there are fundamental similarities between

Wnt and Hh signaling pathways. Both Wnt and Hh are lipid-modified signals that are destined for secretion. They are activated through a cell-surface receptor Frizzled (Fz) or Smo which are related to each other. In both pathways, there is phosphorylation-dependent proteolysis of a key effector: β-catenin for Wnt and Ci/Gli for Hh (Amit et al., 2002; Jia et al., 2002; Liu et al., 2002; Price et al., 2002; Yanagawa et al., 2002; Kalderon, 2002).

4.4.2 Similarities between the function of Wnt and Hh signaling in osteogenesis and adipogenesis

Developmental signaling pathways play crucial roles in the development of many tissues. Rather than each signaling pathway being specific for each tissue, it has been shown that the same signaling pathway is used repeatedly throughout development. Since both Wnt and Hh signaling pathways are evolutionarily related (Huelsken and Birchmeier, 2001; Ingham and McMahon, 2001), they often have related developmental roles (Baron, 2003; Hu et al., 2005). Therefore, our more extensive knowledge of Wnt pathways could be of predictive value for investigating Hedgehog signaling.

4.4.2.1 Both Wnt and Hh signaling pathways stimulate osteogenesis

Wnts have been shown to be critical for chondrocyte differentiation in vitro (Daumer et al., 2004), chondrogenesis during limb-formation at the earliest stages of development (Church et al., 2002; Tuan, 2003), maturation of chondrocytes and the onset of bone collar formation (Hartmann and Tabin, 2000), and chondrogenic differentiation during long bone

formation (Kawakami et al., 1999). In addition, Wnt signaling can also stimulates osteogenesis in a variety of osteogenic cell lines and induce ectopic bone formation (Bennett, et al., 2005; Hu et al., 2005; Jackson et al., 2005). Hh signaling, like Wnt, also stimulates osteogenesis. Both Shh and Ihh have unique sets of functions in regulation of osteogenesis, as discussed in detail in Chapter one.

4.4.2.2 Both Wnt and Hh signaling pathways inhibits adipogenesis

Although Wnt signaling stimulates bone formation, the pathway appears to negatively regulate adipogenesis. Wnt signaling maintains preadipocytes, 3T3-L1, in an undifferentiated state to block adipogenesis. Activation of Wnt signaling inhibits preadipocyte differentiation by blocking expression of C/EBPα and PPARγ (Ross et al., 2000; Bennett et al., 2002). Inhibition of adipogenesis by Wnt signaling is also likely in part through dysregulation of the cell cycle. The endogenous inhibitory Wnt signal may be initiated by Wnt10b, which is expressed in preadipocytes and stromal vascular cells but not in adipocytes (Ross et al., 2000; Bennett et al., 2002). In vivo function of Wnt10b was analyzed in transgenic mice, whose expression under the FABP4 promoter decreases accumulation of white adipose tissue by ~50% and completely blocks the development of brown fat (Longo et al., 2004; Kang et al., 2004).

As for the function of Hh signaling in adipogenesis, we as well as others have examined multipotent stem cells, such as C3H10T1/2, and found Shh blocked adipogenesis (Spinella-Jaegle et al., 2001). In 3T3-L1 preadipocytes, our data from both

sufficiency and necessity tests demonstrate that Hh signaling played an inhibitory role in adipogenesis. Activating the Hh signaling in a variety of ways (by direct exposure to Shh itself, a constitutively active form of its receptor Smo) inhibited adipocytes development and expression of the adipogenic marker genes. Conversely, blocking Hh signaling pharmacologically or genetically by a dominant-negative form of Gli promoted adipogenesis. In addition, Hh signaling, possibly mediated by GATA transcription factors, diverted 3T3-L1 preadipocytes to an alternative osteogenic fate and promoted osteogenesis at the expense of adipogenesis in the multipotent stem cells. In summary, Hh signaling, like Wnt, stimulates osteogenesis while inhibits adipogenesis.

4.4.3 Interaction and cooperation between Wnt and Hh signaling pathways in osteogenesis and adipogenesis

It has been reported that there are interactions between Hh and Wnt pathways in the patterning of different tissue types in vertebrate and invertebrate embryos (Roelink, 1996). Many development processes might be controlled together by several signaling pathways (Sarkar et al., 2000; Ryves and Harwood, 2003; Cayuso and Marti, 2005). Recently, a model that integrates Hh and Wnt signaling in osteoblast development has been proposed (Hu, 2005). In this model, Hh and Wnt signaling sequentially regulate osteoblast development and Hh acts upstream of Wnt. Hh induces expression of Wnt ligands that signal through β-catenin, which is in turn required for *Osx* expression and further osteoblast differentiation. This model suggests that there is complex interaction and

cooperation between Hh and Wnt signaling (Fig 31).

Both osteoblasts and adipocytes originated from the same multipotent progenitor cells, the mesenchymal stem cells (Pittenger et al., 1999). Consistently, an increased lipid accumulation in the bone marrow has been reported in association with age-related bone loss implying an inverse relationship between osteoblastogenesis and adipogenesis (Meunier, et al., 1971; Burkhhardt et al., 1987). Since both Hh and Wnt signaling function in a similar fashion during osteogenesis and adipogenesis, and they interact and cooperate with each other in the process of osteogenesis, it is plausible that there is also interaction and cooperation between Hh and Wnt signaling in inhibiting adipocyte formation. Further investigation of the interaction between Hh and Wnt may be of important value for future study on adipogenesis.

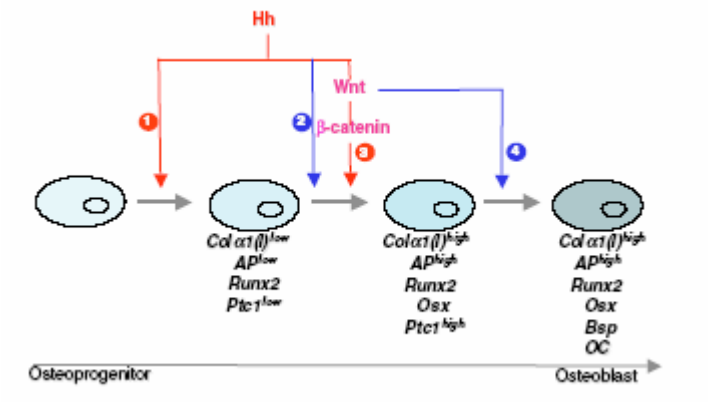

Figure 31 A model that integrates Hh and Wnt signaling in osteoblast development. Red arrows represent events supported by genetic evidence. Blue arrows indicate events revealed by studies in C3H10T1/2 cells. (Adopted from (Hu, 2005))

CHAPTER FIVE

CONCLUSIONS AND FUTURE DIRECTIONS

The previous chapters described my research on the role of Hh signaling in adipogenesis of both vertebrates and invertebrates. I will next outline the main conclusions of the results presented and some future directions of research.

The main conclusions:

-Activating Hh signaling inhibits adipocyte development

-Blocking Hh signaling promotes adipocyte development

-It is evolutionarily conserved from invertebrates to vertebrates

-Hh signaling diverts cells from an adipogenic lineage to an osteogenic lineage, partly through activation of GATA transcription factors

Future work includes:

-Further characterization of transdifferention in 3T3-L1 preadipocytes. Osteogenic differentiation was characterized by early expression of alkaline phospotase, later induction of cbfa1, and final extracellular matrix calcification. Our data have demonstrated Hh signals elevate expression of alkaline phosphotase and cbfa1 in 3T3-L1. Although *Cbfa1* regulates the expression of almost all known marker genes expressed in osteoblasts, whether 3T3-L1 can develop into mature mineralizing osteoblasts is not

known. Therefore, we need to address this issue and examine whether Hh signal itself or together with other signals leads to full osteogenic differentiation of 3T3-L1.

-Analysis of possible interaction between Hh and Wnt signaling in adipogenesis. As discussed in Chapter Four, there is great possibility that Hh and Wnt signaling interact with each other and function together in regulating adipogenesis. Our data suggest that GATA transcription factors are likely to mediate Hh signaling in blocking adipogenesis. However, it does not exclude the possibility that there are other mechanisms downstream of Hh exist. Therefore, examining possible interaction and cooperation between of Hh and Wnt could provide us further mechanistic insighs.

-Analysis of in vivo function of Hh signaling in adipogenesis. So far, most of our mammalian data about the function of Hh signaling are derived from in vitro analysis of cell culture model. Since our goal is to elucidate the in vivo mechanism of fat formation, the roles of Hh signaling in adipogenesis have to be ultimately tested in appropriate mouse models. Both sufficiency and necessity tests could be carry out using transgenic and knockout mouse models. For example, we can generate SmoA1 transgenic mice in which Hh signaling is constitutively activated in mouse adipose tissue using fat-specific promoter, such as aP2 promoter, or conditionally knockout Hh signaling in adipose tissue with appropriate fat-specific Cre driver, such as aP2-Cre.

-Clinical applications. A detailed understanding of molecular mechanisms of Hh signaling in controlling adipogenesis and osteogenesis will facilitate its use in developing therapies for obesity, diabetes and osteoporosis (discussed in Chapter Four).

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