IDENTIFICATION OF IMPORTANT CELL CYCLE REGULATORS AND NOVEL GENES IN SPECIFIC TISSUES USING MICROARRAY ANALYSIS, BIOINFORMATICS AND MOLECULAR TOOLS

DISSERTATION

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Most biological activities in animals are complicated processes regulated by expression of a large number of genes. In addition, most functions of one particular tissue are regulated not only by gene expression but also by signals delivered by other tissues, comprising an extremely complex network in an organism that is still a mystery in many aspects. Fortunately, microarray technology provides an efficient method to select the important genes involved in a complex physiological activity, as well as the novel genes belonging to a large category. Meta-analysis of various microarray studies dealing with similar problems can further provide a precise and economical way to identify critical or novel genes and predict gene functions based on comparison of gene expression. If we can further combine the meta-analysis of microarrays with various molecular technologies, it will be a powerful strategy in biological studies. Because the bioinformatic study of microarray data can reveal explicit targets and outline clear direction for molecular biological studies, whereas the molecular biological studies can in turn provide further confirmation of the target genes, reliable evidence for the predicted functions, as well as extended applications for the target genes, can be obtained from bioinformatic study of microarray data. These two strategies support each other to achieve a reliable conclusion in a cost-effective manner. The studies described in this dissertation provide good examples of utilization of this strategy in biological studies and can be extended to many other studies with similar objectives.
Study of cell cycle activity is critical to understand development of adipose tissue, because the switch on and off of cell cycle activity is tightly related to hyperplasia and hypotrophy of the fat cells. However, the cell cycle is a process that is regulated by many genes. The complexity of this process makes data mining necessary before focusing on any specific regulator. Fortunately, the Gene Expression Omnibus (GEO) DataSets (GDSs) provided on the website of the National Center for Biotechnology Information (NCBI) provides us an effective way to explore key cell cycle regulators during fat tissue development.

In the initial analysis, we tested the aforementioned hypothesis through meta-analysis of GDS2743 and GDS2366. These two datasets were obtained from microarray studies comparing gene expression between differentiating/differentiated and undifferentiated preadipocytes, respectively, in mouse and human. Microarray records of 129 cell cycle regulatory genes were then analyzed in the two datasets. The results of statistical analysis support our hypothesis. Among 82 positive regulators, the expressions of 30 genes in GDS2743 and 34 genes in GDS2366 were higher (P<0.05) in undifferentiated preadipocytes than in differentiating and differentiated preadipocytes, respectively. Among 42 negative regulators, 16 genes had higher expressions (P<0.05) in differentiated preadipocytes than in undifferentiated preadipocytes in GDS2743 and GDS2366, respectively.

After confirming the hypothesis, we set GDS2743 and GDS2366 as a standard for selection of key cell cycle regulators. Only the cell cycle activators highly expressed in undifferentiated preadipocytes and cell cycle inhibitors highly expressed in differentiating/differentiated preadipocytes were chosen for further analysis. In addition, GDS2659, which records gene expression at different time points during differentiation of 3T3-L1 cells, was used as another criterion to narrow the selection
of cell cycle genes that may be important in adipose development. The 3T3-L1 cell line is a commonly used fat cell line. Cell cycle activity generally becomes inhibited during its differentiation. Therefore, only cell cycle inhibitors showing increasing expressions and cell cycle activators showing decreasing expressions were selected for further study. However, redundant candidate genes still existed even with three datasets as selection criteria. In order to further narrow the focus of our study, we assumed that key cell cycle inhibitors should be highly expressed, whereas key cell cycle activators should be lowly expressed in fat tissue when compared to the other well-differentiated tissues. According to this hypothesis, GDS596 and GDS3142, which respectively compare gene expression among different tissues in adult human and mouse, were utilized for datamining. Finally, three cell cycle inhibitors – CCNG2, CDKN2C and PMP22 - and three cell cycle activators – CCND3, CCNA1 and ANAPC5 - were selected for further study.

In the first experiment, the three cell cycle inhibitors selected by datamining with GEO DataSets in human and mouse were verified in pigs by detecting gene expression in different tissues (fat, muscle, heart, lung, liver, kidney, spleen), and in stromal vascular cell and fat cell fractions during primary cell culture using quantitative real-time PCR (qPCR). Results were consistent with the GEO datasets except for tissue distribution, which may have been due to specie differences. In addition, expressions of the three cell cycle inhibitors were also detected in subcutaneous adipose tissue in different age groups (105-day embryos, 6-day postnatal Landrace and 120-day Landrace) using qPCR or western blotting. The results suggest that expression of these cell cycle inhibitors generally increases with the growth of pigs, which is consistent with our hypothesis.

In the second experiment, expression of the three cell cycle inhibitors was
further investigated in broiler chickens by conducting a similar study. For study of tissue distribution, subcutaneous and abdominal fat, thigh and pectoral muscle, heart, lung, liver and kidney tissues were collected. Results showed that all three cell cycle inhibitors are high in fat tissues, whereas all three cell cycle activators are low in fat tissues, which agrees with the GEO datasets and is different from results found in pigs. In addition, mRNA expression during primary cell culture and development of fat tissue also shows different patterns from pigs: the results suggest that PMP22 is not a good marker for the cell cycle in broiler fat tissue and CCNA1 is regulated differently from the other two cell cycle activators. Expression of the other four cell cycle regulators reaches a peak at post-hatch day 5 and decreases in the later stages, suggesting that day 5 may be an important time with active proliferation in one population of preadipocytes and active differentiation in another population of preadipocytes. To further investigate the development of fat tissue, hematoxylin and eosin (H&E) staining was used to measure the cell size in subcutaneous fat tissue of each age group including embryonic day 15 and 17 and post-hatch day 0, 5, 11 and 33. In addition, immunostaining of proliferating cell nuclear antigen (PCNA) was also utilized to detect proliferation activity with nuclear counterstain of 4',6-diamidino-2-phenyindole (DAPI). The results suggest that proliferation activity of fat cells keeps decreasing from embryo day 15 to post-hatch day 33, whereas fat cell size keeps increasing until post-hatch day 11. Combining the results of immunostaining and qPCR, we delineated the development of broiler subcutaneous adipose tissue: both hypertrophic and hyperplastic growth is active before post-hatch day 5. From post-hatch day 5 to day 11, as proliferation activity decreases, most preadipocytes become differentiated; therefore, hypertrophic growth gradually becomes dominant.

In the third experiment, the tissue specific secretory factors in mouse fat,
muscle, heart, lung, liver and kidney were selected based on analysis of GDS3142 and subsequent search of cellular locations of gene products. The tissue specific expressions of the selected genes were further confirmed using semi-quantitative PCR. The novel genes with unreported secretory activity were then validated through expression vector construction, subsequent transfection to HEK293 cells and protein detection using western blotting with cell lysate and medium. Finally, CTLA2A (cytotoxic T lymphocyte-associated protein 2 alpha) was identified as a novel secretory factor in lung; MUP19 was identified as a novel secretory factor in liver; and DEFB29 (defensin beta 29), WFDC15B (WAP four-disulfide core domain 15B) and SERPINA1F (serpin peptidase inhibitor, Clade A, member 1F) were identified as secretory factors in kidney. Their functions in the specific tissues were further predicted by integrating microarray data from different GEO profiles. In addition, alternative splicing inhibiting secretion was also identified in CTLA2A and SERPINA1F.

In the fourth study, a novel adipose specific gene HPS4 was revealed by DNA microarray analysis. Its higher expression in adipose tissue than in other tissues and in fat cells than in stromal vascular cells was detected by qPCR or western blotting. In addition, the increasing expression of HPS4 during primary cell culture in vitro and adipose development in vivo also suggests its important function in adipocytes. Finally, alternative splicing was identified and two different proteins were also detected in embryos and chickens in early ages in western blotting. However, the associations of the transcripts and proteins still need to be clarified and their functions still need to be studied.

In general, our studies not only developed a strategy to identify genes with important effects and novel genes in adipose and other tissues, but also increased our
understanding about adipose tissue and provided some guidance to studies in other tissues.
DEDICATION

To my family who are always doing their best to support me and motivate me to persistently pursue my dream. Especially, I dedicate this to my beloved wife and my dear son, who stand with me and render me infinite power to overcome the hard times.
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TABLE OF CONTENTS

ABSTRACT .................................................................................................................. ii
DEDICATION .............................................................................................................. viii
ACKNOWLEDGEMENTS .............................................................................................. ix
VITA .............................................................................................................................. xi
TABLE OF CONTENTS ............................................................................................... xiii
LIST OF TABLES ......................................................................................................... xiii
LIST OF FIGURES ........................................................................................................ xvi

CHAPTERS

1. INTRODUCTION ........................................................................................................... 1
2. REVIEW OF LITERATURE ............................................................................................. 5

   DEVELOPMENT OF WHITE ADIPOSE TISSUE IN ANIMALS ................................... 5
   REGULATION OF ADIPOGENESIS ............................................................................ 7
   TECHNOLOGIES FOR STUDYING DEVELOPMENT OF ADIPOSE TISSUE .............. 9
   MECHAMISMS OF CELL CYCLE REGULATION ...................................................... 15
   FUNCTION OF CELL CYCLE REGULATORS IN ADIPOGENESIS ......................... 19
   DETECTION METHODOLOGIES OF ADIPOCYTE CELL CYCLE ......................... 20
   METHODS TO STUDY GENE EXPRESSION AT DIFFERENT LEVELS ..................... 22
   DNA MICROARRAY AND GENE EXPRESSION OMNIBUS DATABASE .................... 27
   SUMMARY ..................................................................................................................... 28

3. DIFFERENTIAL EXPRESSION OF CYCLIN G2, CYCLIN DEPENDENT KINASE INHIBITOR 2C AND PERIPHERAL MYELIN PROTEIN 22 GENES DURING ADIPOGENESIS .................................................. 30

   ABSTRACT .................................................................................................................. 30
   IMPLICATIONS .......................................................................................................... 32
   INTRODUCTION ......................................................................................................... 32
MATERIALS AND METHODS ................................................................. 35
RESULTS ............................................................................................ 40
DISCUSSION ...................................................................................... 44
ACKNOWLEDGEMENTS ................................................................... 48
AUTHOR CONTRIBUTIONS ............................................................... 48

4. DIFFERENTIAL EXPRESSION OF CELL CYCLE REGULATORS DURING HYPERPLASTIC AND HYPERTROPHIC GROWTH OF BROILER SUBCUTANEOUS ADIPOSE TISSUE ............................................. 55
ABSTRACT ......................................................................................... 55
IMPLICATIONS ................................................................................... 56
INTRODUCTION .................................................................................. 57
MATERIALS AND METHODS .............................................................. 58
RESULTS ............................................................................................. 65
DISCUSSION ....................................................................................... 69
ACKNOWLEDGEMENTS ..................................................................... 76
AUTHOR CONTRIBUTIONS ............................................................... 76

5. IDENTIFICATION OF CTLA2A, DEFB29, WFDC15B, SERPINA1F AND MUP19 AS NOVEL TISSUE-SPECIFIC SECRETORY FACTORS IN MOUSE ........................................................................... 87
ABSTRACT ......................................................................................... 87
INTRODUCTION .................................................................................. 88
MATERIALS AND METHODS .............................................................. 91
RESULTS ............................................................................................. 95
DISCUSSION ....................................................................................... 104
ACKNOWLEDGEMENTS ..................................................................... 115
AUTHOR CONTRIBUTIONS ............................................................... 115

6. IDENTIFICATION OF HPS4 AS A NOVEL ADIPOSE-SPECIFIC GENE IN CHICKEN ADIPOSE TISSUE ......................................................................................... 127
ABSTRACT ......................................................................................... 127
INTRODUCTION .................................................................................. 128
MATERIALS AND METHODS .............................................................. 129
RESULTS ............................................................................................. 135
DISCUSSION ....................................................................................... 138
AUTHOR CONTRIBUTIONS ............................................................... 141
7. CONCLUSIONS ........................................................................................................146
LIST OF REFERENCES .............................................................................................150
LIST OF TABLES

Table 3.1 The cell cycle inhibitors expressed higher in adipose in both human and mouse .................................................................49

Table 4.1 Primer sequences of quantitative real-time PCR for selected cell cycle regulators .............................................................77

Table 4.2 Cell cycle activators with lower expression in adipose tissue of human and mouse ................................................................78

Table 4.3 Development of subcutaneous adipose tissue of broiler chickens at different ages ...................................................................79

Table 5.1 Primer sequences for semi-quantitative PCR .........................................................116

Table 5.2 Primer sequences for amplification of insertions into expression vectors .................................................................117

Table 5.3 Gene expression values of selected genes in GDS3142 ..................................118
<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1</td>
<td>Gene expression analysis of PMP22, CDKN2C and CCNG2 based on microarray datasets obtained from NCBI website</td>
</tr>
<tr>
<td>3.2</td>
<td>Expressions of CCNG2, CDKN2C and PMP22 in fat, muscle, heart, lung, liver, kidney, spleen and intestine of pigs</td>
</tr>
<tr>
<td>3.3</td>
<td>Relative gene expressions of DLK1, PPARγ, CCNG2, CDKN2C and PMP22 in the stromal vascular and fat cell fractions from pig adipose tissue</td>
</tr>
<tr>
<td>3.4</td>
<td>Expressions of DLK1, PPARγ, CCNG2, CDKN2C and PMP22 during pig primary adipocytes differentiation in vitro</td>
</tr>
<tr>
<td>3.5</td>
<td>Gene expression in subcutaneous adipose tissues of 105-day fetal, 6-day neonatal and 120-day pigs</td>
</tr>
<tr>
<td>4.1</td>
<td>Gene expression analysis of selected cell cycle regulators based on microarray datasets obtained from NCBI website</td>
</tr>
<tr>
<td>4.2</td>
<td>Change of fat cell size during development of chicken adipose tissues</td>
</tr>
<tr>
<td>4.3</td>
<td>Quantification of proliferating cells during development of chicken adipose tissues</td>
</tr>
<tr>
<td>4.4</td>
<td>mRNA expression of six selected cell cycle regulators in 21-day broiler chickens</td>
</tr>
<tr>
<td>4.5</td>
<td>Relative gene expression of DLK1, FABP4, ATGL and six cell cycle regulators in stromal vascular and fat cell fractions from chicken adipose tissue</td>
</tr>
<tr>
<td>4.6</td>
<td>Expression of DLK1, FABP4, ATGL and six cell cycle regulators during chicken primary adipocyte differentiation in vitro</td>
</tr>
<tr>
<td>4.7</td>
<td>Relative expression of DLK1, FABP4, ATGL and six cell cycle regulators during in vivo adipose development of pigs</td>
</tr>
</tbody>
</table>

xvii
broiler chickens

Figure 5.1 Expression of adult mouse gene transcripts detected by semi-quantitative PCR

Figure 5.2 Different isoforms of CTLA2A and their expression in HEK293 cell culture

Figure 5.3 Different isoforms of SERPINA1F and their expression in HEK293 cell culture

Figure 5.4 Detection of protein expression of MUP19, WFDC15B and DEFB29 in HEK293 cell culture

Figure 5.5 Expression profile in lung for CTLA2A in microarray DataSets obtained from NCBI website

Figure 5.6 Expression profile in liver for MUP19 in microarray DataSets obtained from NCBI website

Figure 5.7 Expression profile in kidney for kidney specific genes in microarray DataSets obtained from NCBI website

Figure 6.1 Expression of HPS4 in different tissues in 21-day broiler chickens

Figure 6.2 Comparison of expression of DLK1, PPARg and HPS4 between preadipocytes and fat cells in chicken adipose tissue

Figure 6.3 Expression of HPS4 during development of adipose tissue

Figure 6.4 Alternative splicing of chicken HPS4
CHAPTER 1
INTRODUCTION

The prevalence of obesity is increasing worldwide with the improvement of living quality and change of dietary and behavioral patterns (Popkin and Doak, 1998). As shown by the data of the National Health and Nutrition Examination Survey, the prevalence of obesity in the US increased nearly 8 percentage points between the 1976-1980 survey and the 1988-1994 survey and also between the 1988-1994 survey and the 1999-2000 survey. The prevalence of obesity was 35.5% among adult men and 35.8% among adult women in 2009-2010 (Flegal et al., 2012). Moreover, the prevalence of obesity in children and adolescents also increased in the 1980s and 1990s. Although it seemed to plateau afterwards, the prevalence of obesity reached 16.9% in children and adolescents in 2009-2010 (Ogden et al., 2012). The degree of obesity is closely related to obesity-related disorders including dyslipidemia, diabetes and hypertension (Janssen et al., 2002). In addition, excess body weight also increases the risk of cardiovascular diseases including stroke (Kurth et al., 2002), coronary disease and congestive heart failure in human (Hubert et al., 1983), as well as various cancers in multiple sites such as kidney, breast and colon (Calle et al., 2003). Obesity also increases the risk of death from cardiovascular disease and cancer (Calle et al., 1999). Finally, because adipose tissue secrets many regulatory factors, excess adipose tissue usually causes imbalance in endocrine and metabolic systems. It has been suggested that obese persons tend to have more inflammatory response including increases in macrophage and proinflammatory factors such as tumor necrosis.
factor α (TNF-α), Transforming growth factor β1 (TGF-β1) and monocyte chemotactic protein-1 (MCP-1) (Weisberg et al., 2003).

Due to the obesity epidemic and its risk to human health, various treatments for obesity, ranging from exercise and diet to pharmacotherapy and surgery, have been developed. Among these modalities, surgical therapy is most effective with the greatest extent and longest duration of weight reduction (Bult et al., 2008). However, it commonly leads to nutritional deficiencies such as carotenoid and other fat-soluble vitamin deficiency (Granado-Lorencio et al., 2011) and also carries considerable risk of morbidity or even mortality (Thomas and Agrawal, 2012). For example, obesity surgery is reported to increase the long-term risk of colorectal cancer (Derogar et al., 2013). For obesity pharmacotherapy, no anti-obesity drugs have been approved by the Food and Drug Administration since Orlistat was approved in 1999, because of the complexity of the obese population, which contains both healthy and sick people, and the lifelong nature of the treatments (Colon-Gonzalez et al., 2013). Therefore, research involving adipose tissue is of great interest in medical science.

Due to the critical relationship between the amount of fat in meat and meat quality, the fat quantity and quality in livestock is also being optimized by scientists (Webb and O’Neill, 2008). With the persistent selection of animal breeders and innovation of animal nutritionists, there has been significant improvement in feed efficiency and meat quality in domestic animals in the last few decades (Gao et al., 2007). However, due to the endless search for commercial profit in the animal industries and changing requirements from consumers, the pursuit of further understanding and more precise regulation of fat deposition never ends.

Adipose tissue in animals develops in two ways – increase of fat cell number (hyperplastic growth) and increase of fat cell size (hypertrophic growth) (Jo et al.,
Hyperplastic growth occurs through proliferation and differentiation of preadipocytes, whereas hypertrophic growth is due to accumulation of lipids in mature fat cells (Hausman et al., 2001). Therefore, terminal differentiation is an important watershed for development of adipose tissue. One significant change that marks the differentiation is inhibition of the cell cycle (Ntambi and Young-Cheul, 2000). Although some studies indicate that immature differentiated fat cells can still proliferate (Hanamoto et al., 2013), there is no doubt that the cell cycle is ultimately arrested in mature fat cells.

Although the cell cycle is important for study of adipose development, it is a complex process that involves a large number of regulators. The cell cycle can be divided into four stages: DNA synthesis (S) stage, mitosis (M) and two gaps between the two events (G1 and G2). When cells become differentiated, they can enter a resting stage called G0. These stages are coordinated in a fixed order, G1 followed by S after which cells enter G2 and finally M. After cell division, the cells can reenter another cycle or G0. If there is error in any step, the cell cycle will stop until it is fixed (Vermeulen et al., 2003). Such accurate regulation requires numerous proteins. Except for the core regulators, such as cyclins, cyclin-dependent kinases (CDK) and cyclin dependent kinase inhibitors (CKI), there are also many other proteins such as CDK activating enzymes and checkpoint proteins (Vermeulen et al., 2003).

The complex regulation of the cell cycle makes it hard to identify important cell cycle factors in adipose tissue. Fortunately, microarray technology, which enables detection of expression of thousands of genes simultaneously, makes it feasible to screen key factors in adipose tissue development by comparison across different tissues and between normal and pathological or treatment groups (Schena et al., 1995). As this technology became routinely used for high-throughput gene expression
detection, a huge amount of data was generated in the last decade by the scientific community. The Gene Expression Omnibus (GEO) repository was established in the National Center for Biotechnology Information (NCBI) archives (Barrett et al., 2007) to make most existing gene expression data accessible to the scientific community. The availability of microarray data facilitates meta-analysis, which allows more reliable and general conclusions to be made by combining information from multiple existing studies (Ramasamy et al., 2008).

One objective of the following studies is to identify important cell cycle regulators by utilizing the GEO database as a primary source for meta-analysis; to detect the expression of these cell cycle inhibitors and activators in adipose tissue and primary cell cultures in pigs and chickens using quantitative real-time PCR and western blotting; and to investigate development of broiler adipose tissue by combining the expression of these cell cycle regulators and proliferating status and change in size of fat cells in different age groups.

Another objective is to extend the application of the same strategy utilizing microarray data analysis, as well as bioinformatic and molecular technologies, to identification of tissue-specific secretory genes and novel adipose specific genes.
CHAPTER 2
REVIEW OF LITERATURE

DEVELOPMENT OF WHITE ADIPOSE TISSUE IN ANIMALS

Animal adipose tissues grow by two mechanisms: hyperplasia (increase of cell number) and hypertrophy (increase of cell size). As indicated by in vitro culture of preadipocyte cell lines, hyperplasia is achieved by proliferation of preadipocytes and their subsequent differentiation (Smyth et al., 1993), but a recent report claimed that differentiated adipocytes with little lipid accumulation, called small proliferative adipocytes (SPA), still have limited proliferation in vivo, indicating that the cell cycle and mitosis may be terminated gradually, rather than suddenly, during adipocyte differentiation in vivo (Hanamoto et al., 2013).

Hypertrophy usually occurs before hyperplasia cyclically during adipogenesis (Hausman et al., 2001). It seems that hyperplasia only occurs at early developmental stages in nonobese animals, whereas in obese animals, hyperplasia can also be triggered in the adults when the cell size and lipid content reach a certain level (Faust et al., 1978). Therefore, hyperplasia is highly correlated with severity of obesity (Hirsch and Batchelor, 1976). In human, fat cell number in the buttocks appears to increase dramatically after the body fat exceeds 25%. Although the fat cell number did not increase significantly before 1 year of age, it showed a significant increase between 1 and 2 years of age in the study of Knittle et al. (1979). After 2 years of age, the obese-prone children continued to exhibit increased numbers of adipocytes, whereas nonobese children only had increased adipocyte numbers from 10 to 18 years
of age to reach the final adult number (Knittle et al., 1979). In chicken, a genetic fat line showed significant increase of adipocyte numbers in the abdominal fat pad since 10 days of age compared to the lean line fed the same diet; the difference was intensified especially from 5 wk to 7 wk of age. The nonobese line stopped hyperplasia at 5 wk of age, whereas the obese line seemed to have no limit to the increases in fat cell numbers (Guo et al., 2011). In rats, the fat cell numbers in inguinal, testicular and perirenal-retroperitoneal adipose tissues increased for both obesity resistant and obesity susceptible lines from 24 to 105 days of age. This increase was further intensified by a high-fat diet in the obesity susceptible line and may be extended beyond 20 wk of age (Obst et al., 1981).

Although the cellularity of fat cells in vivo has been studied extensively, the detailed study of in vivo adipocyte development is difficult due to the infeasibility of isolating preadipocytes at a specific developmental stage (Ntambi and Young-Cheul, 2000). Therefore, most of our understanding about the mechanisms of adipocyte development is obtained from the in vitro study of various cell lines. During differentiation of several preadipocyte cell lines such as 3T3-F442A and 3T3-L1, the preadipocytes first proliferate through mitosis until they are arrested after confluence in cell culture. The arrested preadipocytes will differentiate into adipocytes after induction by an adipogenic cocktail (Tang and Lane, 2012). Before the initial differentiation, there is one important step characterized by several rounds of the cell cycle between confluence and initial differentiation. This proliferative step is called mitotic clonal expansion (MCE) (Gregoire et al., 1998). Although MCE was proven to be vital in in vitro cultures of some preadipocyte cell lines (Cornelius et al., 1994), it was still not proven to be vital in the in vivo adipocyte development. As reported by Entenmann and Hauner (1996), cell culture of human preadipocytes does not require
proliferation before differentiation since prevention of mitosis does not affect adipocyte differentiation, but it is still possible that the preadipocytes go through MCE \textit{in vivo} before isolation.

**REGULATION OF ADIPOGENESIS**

Although regulation of adipogenesis \textit{in vivo} is still not fully understood, the mechanisms are being gradually revealed by the \textit{in vitro} culture of various cell lines, such as pluripotent C3H10T1/2 cells, which serve as a faithful MSC model (Tang and Lane, 2012), and 3T3-F442A and 3T3-L1 cells, which are mostly used in preadipocyte cell lines (Gregoire \textit{et al.}, 1998). Cell culture of C3H10T1/2 stem cells has demonstrated that two bone morphogenetic protein (BMP) family members, BMP4 and BMP2, are important activators during commitment of pluripotent stem cells to preadipocytes. These proteins can bind to their receptor BMPr1, which forms a complex with BMPr2, and thereby phosphorylate and activate the BMPr1 kinase. The BMP receptors then phosphorylate Smad-1, -5 and -8, which form a complex with Smad-4 and regulate downstream gene expression (Huang \textit{et al.}, 2009). In addition, the wnt signaling pathway is also involved in activation of the commitment. Activation of the wnt signaling pathway prevents degradation of β-catenin, and, therefore, allows accumulation of β-catenin in the nucleus where it activates lymphoid factors and triggers the transcription of downstream genes like c-myc (Davis and Zur Nieden, 2008). However, unlike the BMP pathway, the wnt pathway inhibits adipogenesis during late stage differentiation, since it can inhibit the adipogenic transcription factors CCAAT/enhancer binding protein α (C/EBPα) and peroxisome proliferator-activated receptor γ (PPARγ) (Ross \textit{et al.}, 2000). Certainly, there are also other regulatory pathways during commitment of stem cells into preadipocytes, such as the hedgehog (Hh) signaling pathway (James \textit{et al.}, 2010). However, the
underlying mechanisms need further exploration.

Compared to the mechanisms of commitment, the mechanisms of differentiation of preadipocytes to adipocytes have been clarified to a much greater degree using cell cultures of 3T3-L1 and 3T3-F442A. For example, growth of the 3T3-L1 cell lines is normally arrested after cell confluence, at which the expression of lipoprotein lipase (LPL) occurs (Amri et al., 1986). After induction of differentiation by an inducer combination including insulin, dexamethasone (DEX) and an agent to elevate cellular cAMP such as methylisobutylxanthine (MIX), the cyclic AMP response element-binding protein (CREB) increases and becomes phosphorylated (Zhang et al., 2004). The phosphorylated CREB can then activate expression of CCAAT/enhancer binding protein β (C/EBPβ), which is then phosphorylated by mitogen-activated protein kinase (MAPK) and glycogen synthase kinase-3 β (GDK-3β) (Tang et al., 2005). The phosphorylated C/EBPβ can then dimerize with another phosphorylated C/EBPβ to create a DNA-binding pocket (Vinson et al., 1989), which facilitates the transcriptional activation of C/EBPα and PPARγ by C/EBPβ (Christy et al., 1991). The activated C/EBPα and PPARγ then cross activate each other via their respective C/EBP regulatory elements (Rosen et al., 2002) and activate a large number of downstream adipocyte genes. PPARγ is both necessary and sufficient for fat formulation, and, thus, is a master regulator of adipogenesis (Farmer, 2006). Therefore, PPARγ is also regulated by many other regulators, such as sterol regulatory element binding transcription factor 1c (SREBP1c), which can induce expression of PPARγ (Kim and Spiegelman, 1996). Moreover, not all of the C/EBPs are pro-adipogenic. For example, one member named CHOP10 in the C/EBPs family acts as a dominant-negative isoform, since it lacks the DNA-binding region, but can still
heterodimerize with C/EBP (Ron and Habener, 1992). Certainly, there are many other regulators involved in adipogenesis in addition to PPARγ and C/EBPs. The finely-tuned process of adipogenesis is thus the result of a balance between the pro- and anti-adipogenic transcription factors.

TECHNOLOGIES FOR STUDYING DEVELOPMENT OF ADIPOSE TISSUE

One important technology to study adipogenesis is cell culture because it provides a simple way to investigate cell proliferation and differentiation and interpret the outcome of different treatments. During the last 40 years, many adipocyte cell lines were developed for in vitro study of adipogenesis, including multipotent stem cell lines such as 10T1/2 (Reznikoff et al., 1973), CHEF/18 (Sager and Kovac, 1982), Balb/c 3T3 (Sparks et al., 1986) and RCJ3.1 (Grigoriadis et al., 1990) and preadipocyte cell lines such as 3T3-F442A, 3T3-L1, TA1 (Chapmen et al., 1984), 30A5 (Konieczny and Emerson, 1984) and Ob17 cells (Negrel et al., 1978).

The 10T1/2 cells, which are cloned from C3H mouse embryos, represent stem cells blocked at the mesodermal pathway and can be converted to adipocyte, myocyte and chondrocyte linages after treatment with a demethylating agent such as 5-azacytidine (Taylor and Jones, 1979). Other multipotent stem cell lines can also undergo determination upon treatment with 5-azacytidine. These multipotent fibroblasts are good models for understanding the events in cellular determination. The 3T3-F442A and 3T3-L1 cells were originally isolated from disaggregated 17- to 19-day Swiss 3T3 mouse embryo cells for their ability to accumulate cytoplasmic triacylglycerol (Green and Kehinde, 1974), but 3T3-F442A are more committed in the adipocyte differentiation process. They are widely used cell lines for in vitro study of adipogenesis, because they provide reliable models of preadipocytes as verified by successful in vivo cell transplantation (Green and Kehinde, 1979) and the same
ultrastructure features as adipocytes in situ (Novikoff et al., 1980). The TA1 and 30A5 cell lines are both obtained by treating 10T1/2 cells with 5-azacytidine. Ob17 cells, which were generated from adipose precursors in epididymal fat pads of genetically obese (ob/ob) mice, represent a later preadipocyte stage (Negrel et al., 1978). All preadipocyte cell lines are morphologically similar to fibroblasts before induction of differentiation, but they are already committed solely to adipocyte lineage. However, these cell lines represent different stages of fat cell growth as indicated by variation in differentiation requirements (Cornelius et al., 1994).

Due to the homogeneous cellular population of these cell lines that allow definitive response to treatments and their indefinite passage that provides a consistent source of preadipocytes, the growth of adipocyte cells from commitment to differentiation was revealed in detail through in vitro culture (Ntambi and Young-Cheul, 2000). However, we still cannot generalize these discoveries to in vivo conditions, because these cell lines exhibit aneuploidy, and, therefore, often possess different characteristics from the primary preadipocyte cells isolated from adipose tissue (Cornelius et al., 1994). Although primary cell cultures also have drawbacks, such as a heterogeneous cellular population and limited life span in culture (Ntambi and Young-Cheul, 2000), they can be used to verify the findings obtained from immortalized cell lines, because they better reflect the context of adipose tissue in vivo.

In addition to the clonal cell lines and primary preadipocytes, dedifferentiated fat cells derived from mature fat cells through ceiling culture technology can also proliferate and differentiate again to mature fat cells both in vitro and in vivo (Yagi et al., 2004). Because they are more homogeneous than primary preadipocyte cultures, they can potentially replace the latter (Matsumoto et al., 2008). The dedifferentiated
preadipocytes can differentiate to multiple lineages and share characteristics of adult stem cells. Ullah et al. (2013) have claimed that transdifferentiation between adipocytes and osteocytes or chondrocytes is via dedifferentiation rather than the cell-to-cell conversion.

Even though mesenchymal stem cells, dedifferentiated fat cells, and primary preadipocytes provide tools to study adipogenesis, they all suffer from the same disadvantages, such as limited proliferative potential and decreased differentiation. This obstacle was overcome by Ahfeldt et al. (2012) who generated mesenchymal progenitor cells from several pluripotent stem cell lines and then programmed them to white and brown adipocytes through gene transfection. The programmed cells later gave rise to an ectopic fat pad with morphological and functional characteristics of white and brown adipose tissue after transplantation.

The availability of various adipose clonal cell lines and primary preadipocyte cell cultures has facilitated investigation of the process of adipogenesis and the effects of various hormones, gene transfections and knockdowns, growth factors and pharmacological compounds on adipogenesis. Among the treatments for cell lines, gene transfection and knockdown are advanced technologies that are currently widely used to study gene functions. For example, Ahfeldt et al. (2012) programmed mesenchymal progenitor cells to white adipocytes with a lentiviral construct containing a doxycycline-inducible promoter driving PPARG2, a construct controlling expression of PPARG2 and treatment of doxycycline, indicating the master role of PPARG2 in white adipose development. Sjölund et al. (2014) found that knockdown of Hipk2 through short hairpin RNAs (shRNAs) significantly reduced differentiation of 3T3-L1 cells into mature fat cells, indicating the essential function of Hipk2 in development of white adipose tissue.
In addition to the *in vitro* study of adipose development with various cell lines, scientists are also trying to investigate fat tissue development *in vivo* using various strategies. One of the important strategies to study adipogenesis *in vivo* is knockout of specific genes. One can apply global knockout of the gene of interest and collect embryonic fibroblasts, preadipocytes or primary adipocytes to study adipogenesis ex vivo. For example, Oh *et al.* (2005) generated Acc2⁻/⁻ mutant mice and compared fatty acid and glucose oxidation between wild type and Acc2⁻/⁻ knockout mice in primary cultures of isolated fat cells. However, the drawback of global knockout is that it may not only cause change in adipose tissue but also in other tissues, and sometimes the phenotypic observation may be just an indirect effect of gene knockout in other cell types. Fortunately, tissue specific knockout is facilitated by Cre-loxP-dependent strategies. This is done by introducing short sequences called loxP sites around the target gene and Cre recombinase driven by fat specific markers. aP2-Cre mice and Adipoq-Cre Mouse have been generated by Barlow *et al.* (1997) and Wang *et al.* (2010). Cre recombinase is driven by the Fabp4 flanking sequence in the former mouse model and control elements from the adiponectin locus in the latter line. When Cre recombinase is expressed in adipose tissue under the control of adipose specific regulatory sequence, it can recognize and recombine loxP sites and delete the gene flanked by these sites. The Retn-Cre line, in which expression of Cre recombinase is driven by resistin, was created by Mullican *et al.* (2013), but it did not exhibit adipose-specific expression as expected.

By crossing the adipose-specific Cre lines to reporter mice (R26R-lacZ), Cre-mediated recombination can be visualized and quantified by lacZ staining. All Cre lines can induce recombination by expressing Cre mRNA and protein. However, non-adipose recombination can also occur in some Cre lines. For example, significant
recombination was also detected in brain and skeletal muscle in aP2-Cre and Retn-Cre lines (Kang et al., 2014). In some aP2-Cre mice, the recombinase can also “leak” to germ lines and cause heterozygous whole-body knockout in the next generation (Dubois et al., 2006). In addition, variation in copy numbers and effects of the Cre transgene in different positions of the same model can also lead to different recombination efficiencies. For example, Adipoq-Cre mice show greater recombination efficiency in brown adipose tissue (BAT) than in white adipose tissue (WAT) (Lee et al., 2013), whereas Retn-Cre mice show higher recombination efficiency in WAT and a mosaic recombination pattern in BAT (Mullican et al., 2013).

If gene knockout is one loss-of-function strategy, gene overexpression is one gain-of-function strategy that is also widely used to study gene function in vivo. However, it also involves finding an adipose-specific marker to drive expression of a transgene such as that for Cre recombinase in knockout models. Therefore, the variation of transgene expression in different locations is also a concern as mentioned for Cre recombinase efficiency. To avoid such a problem, one can introduce the Cre transgene to the ROSA26 locus, where there are frequent gene-targeting events. ROSA26, which was identified by Friedrich and Soriano (1991), is ubiquitously expressed in embryonic and adult tissues. When the Cre transgene is introduced to the ROSA26 locus, which is downstream of a STOP cassette flanked by loxP sites, transcription of the transgene will be induced due to deletion of the STOP cassette upon induction of Cre (Soriano, 1999). Although Cre-loxP is able to control transgene expression in a time- and cell-type-specific fashion, it cannot silence gene expression after induction. To solve this problem, a tetracycline (Tet)-controlled system can be applied to make the ROSA26 transgenes inducible (Beard et al., 2006).

Recently, the Scherer lab developed a system called AdipoChaser Mouse using
all of the strategies mentioned above (Wang et al., 2013). The mouse model is generated by crossing three transgenic lines so that it contains three transgenic factors: transcriptional factor rtTA that can be activated by treatment of diet doxycycline and driven by the adiponectin promoter; cre recombinase gene that is activated by rtTA, and loxP-stop-loxP–β-galactosidase construction that is driven by the Rosa26 promoter (Rosa26-loxP-stop-loxP-lacZ). Therefore, under the treatment of doxycycline, activated rtTA can induce expression of cre recombinase, which in turn eliminates the floxed stop codon cassette and thus turns on expression of β-galactosidase. In this case, if the cell is stained with β-gal, it will turn to a blue color. With prolonged treatment of doxycycline, all of the existing mature fat cells can be labeled with lacZ. With this mouse model, scientists can easily determine whether hyperplastic growth is triggered by a high fat diet in a specific location (Wang et al., 2013).

Another commonly used approach is gene knockdown using antisense nucleotides. The short oligonucleotides can bind to mRNA through Watson-Crick base pairing so that translation to corresponding proteins can be blocked (Zamecnik and Stephenson, 1978). In addition, binding of some antisense nucleotides to RNA can recruit and activate RNase-H, which can in turn lead to degradation of target mRNA (Jepsen and Wengel, 2004). As are knockout models, the antisense treatment is also a powerful strategy to study gene function. For example, knockdown of Protein tyrosine phosphatase 1B (PTP1B) in vivo not only led to significant reduction in adiposity but also downregulated several genes involved in lipogenesis, indicating an important function of this gene in enlargement of adipose tissue and development of obesity (Rondinone et al., 2002). However, the effect is not limited to fat. Instead, the effects are usually seen in multiple cell types in the fat pad or other organs such as the
MECHANISMS OF CELL CYCLE REGULATION

Animal development from a single-cell zygote to a multi-cellular organism requires many rounds of the cell cycle. The cell cycle involves a series of events and various regulators in a cell that result in mitosis and cell proliferation. The whole process in somatic cells can be divided into four distinct phases in sequence: G1, S, G2 and M phases. G1 and G2 are two “gaps” in the cell cycle, whereas S and M stand for DNA synthesis and mitosis. In the G1 phase, the cell size increases and everything needed for DNA synthesis is prepared. In the S phase, the DNA is synthesized quickly to avoid damage of the base pairs by other external factors, and, therefore, the chromosomes become a double structure. In the G2 phase, the cell size continues to increase and everything is checked to be ready for the M phase and cell division. These three phases take the most time in the cell cycle and are referred to collectively as interphase, because there is no cell division. In the M phase, the cell stops increasing in size, but is divided into two daughter cells (William et al., 2011).

Although the M phase is short, there are so many activities taking place that it can be further subdivided into four sequential phases: prophase, prometaphase, metaphase, anaphase and telophase. During prophase, the two centrosomes migrate respectively to the opposite ends of the cell, and the nuclear envelope begins to break. Meanwhile, the chromosomes become condensed under the mediation of the condensin complex. During prometaphase, the nuclear membrane breaks apart and the chromosomes form kinetochores, which then bind to the microtubules in the spindle formed by the two centrosomes at the poles of the cell. During metaphase, the chromosomes are aligned at the equatorial plane in the midline region of the cell.
During anaphase, the two sister chromatids of each chromosome separate and are drawn to opposite ends of the cell by the gradually shortened spindle fibers caused by the removal of tubulin subunits. During telophase, the daughter chromosomes arrive at the poles of the cell and are surrounded by the reformed nuclear membrane. Meanwhile, the spindle fibers degenerate and the chromosomes become diffuse and invisible chromatins. In addition, cytokinesis occurs and the cell is finally divided into two daughter cells. The daughter cells can either reenter the cell cycle or enter the G0 phase and become quiescent or senescent (William et al., 2011).

Because the cell cycle is an elaborative process with sequential phases, there are some checkpoints that make sure each event occurs after another. The first major checkpoint is the G1/S checkpoint, where the cell ensures its adequate size and intact DNA before DNA synthesis. The second major checkpoint is the G2/M checkpoint, where DNA replication is checked before mitosis. The last major checkpoint is the M checkpoint, where the assembly of the spindle fiber system and the connections between the spindle fibers and the kinetochores are monitored before separation of sister chromatids (William et al., 2011). If a mistake is found at these checkpoints or a differentiation signal is received, the cell cycle will be arrested until the mistake is corrected or eliminated. If the mistake cannot be corrected, cell apoptosis will occur. If the checkpoint is overridden due to impairment of the regulatory system, the cell cycle will be uncontrollable and the cells may become cancerous (Pietenpol and Stewart, 2002).

The strict regulation of checkpoints and smooth progression of the cell cycle are dependent on proper communication among the cell cycle regulators. The two central families of cell cycle regulators are cyclins and cyclin dependent kinases (cdks). Cyclins are needed for activation of cdks, so they usually form complexes in
which the cyclins are regulatory subunits for cdks and targeting subunits for particular substrates, whereas the cdks are catalytic subunits for particular substrates (Schafer, 1998). Different cyclins can activate different cdks that play various roles in different phases of the cell cycle. The activity of specific complexes at appropriate times in the cell cycle is regulated by the cyclic synthesis and degradation of cyclins. In the G0 phase, the retinoblastoma protein (Rb) is hypophosphorylated and binds with E2F to inhibit its activity by recruiting histone deacetylases (HDAC) (Brehm et al., 1998) and lysine/arginine methyl-transferases (Fabbrizio et al., 2002). When the cell exits the G0 phase after receiving a mitogenic signal, cyclin D is induced and binds with cdk4 and cdk6. The cyclinD-cdk4/6 complex phosphorylates the retinoblastoma protein and thus releases E2F, which regulates many genes involved in DNA synthesis, as well as other cyclins downstream (Schafer, 1998). Cyclin E is the next cyclin activated by E2F during the G1 phase and associates with cdk2 (Koff et al., 1992). The cyclin E-cdk2 complex can then further phosphorylate Rb on additional sites and thus lead to a positive feedback cycle for the accumulation of E2F (Lundberg and Weinberg, 1998). When the accumulation of cdk2 activity is above a certain threshold, the cell will pass the restriction point and proliferate irreversibly in a mitogen-independent fashion (Darzynkiewicz et al., 1996). Meanwhile, E2F also promotes accumulation of cyclin A at the G1/S transition. Cyclin A associates with cdk2 in the S phase and later with cdk1 in the G2 phase. The cyclin A- cdk1/2 complex is required for entry into and completion of the S phase, as well as entry into the M phase (Malumbres and Barbacid, 2005). However, unlike cyclin E, cyclin A participates in a negative feedback cycle with E2F to prevent excessive DNA replication (Xu et al., 1994). Finally, entry into the M phase induces expression of cyclin B, which in turn activates cdk1 (Porter and Donoghue, 2003). The cyclin B-cdk1 complex not only
promotes mitosis and cell division, but also activates the cyclosome/anaphase-promoting complex (APC) with Polo-like kinase (PLK) (Golan et al., 2002). The cyclin B-cdk1 complex must be degraded by cyclosome/APC before anaphase to enable the cells to enter anaphase and to exit mitosis (Chang et al., 2003).

In addition to regulation by expression and destruction of their cyclin partners, cdks are regulated by phosphorylation and dephosphorylation at specific residues to facilitate tight control of the cell cycle. For example, during the G2 phase, cdk1 is phosphorylated at tyrosine 15 (Y15) and threonine 14 (T14) and inhibited by wee1 (Parker and Piwnica-Worms, 1992) and myelin transcription factor 1 (myt1) (Mueller et al., 1995) to prevent early onset of mitosis. However, as the cell cycle approaches the M phase, cdk1 is dephosphorylated and activated by cell division cycle 25C (cdc25C) (Boutros et al., 2006). In addition to wee1, there is one special complex termed CDK-Acting Kinase (CAK), which phosphorylates cdk1 at threonine 161 (T161) and other cdks at corresponding residues. However, phosphorylation at this site is required for activation rather than inhibition of cdks (Nigg, 1995). Another interesting fact is that the CAK complex is itself a cyclin-cdk complex that consists of cyclin H, cdk7 and menage A trois homolog1 (mat1) (Lolli and Johnson, 2005).

The last method of regulation of cdks is the expression and destruction of cdk inhibitors. There are two important families of inhibitors – p16 and p21 (Schafer, 1998). The p16 family includes p16, p15, p18 and p19 (Nigg, 1995). This family of inhibitors exclusively inhibits the cyclin D-dependent kinases (cdk4 and cdk6) to arrest the cell at the G1 phase. Therefore, they are also called the INK4 family (Sherr and Roberts, 1999). Moreover, the INK4-sequestered cdks cannot be rescued by increased cyclins (Nigg, 1995). The p21 family includes p21, p27 and p57. These proteins can bind most cdks and cyclins to inhibit activity of cdks, but the inhibition
can be reversed by increasing cyclins (Schafer, 1998). These inhibitors play crucial roles at the three checkpoints, especially when the cell cycle needs to be arrested. For example, when there is a genotoxic agent, p21 will be induced and then bind to cyclin D-cdk4/6 and cyclin E-cdk2 complexes, resulting in hypophosphorylation of Rb and cell cycle arrest at the G1/S checkpoint. At the G2/M checkpoint, p21 can also repress the cyclin B-cdk1 complex and lead to cell cycle arrest (Pietenpol and Stewart, 2002).

In addition to the important regulators and pathways mentioned above, other regulators and pathways are being discovered to expand our understanding of cell cycle regulation. For example, although cdc25C can antagonize wee1 and myt1, and dephosphorylate and activate cdk1, when the cell cycle is arrested at the G2/M checkpoint, it can also be phosphorylated by chek1 and chek2, which is activated by ATM, and then exported out of the nucleus by 14-3-3 proteins, so that the activity of cdk1 is inhibited (Pietenpol and Stewart, 2002).

**FUNCTION OF CELL CYCLE REGULATORS IN ADIPOGENESIS**

As stated in section 1.1, hyperplasia of fat cells requires proliferation of preadipocytes and immature adipocytes and their terminal differentiation, which are dependent on timely activation and inhibition of the cell cycle. In addition, the dedifferentiation of adipocytes into fibroblast-like cells also upregulated expression of some cell cycle promoters and downregulated that of some cell cycle inhibitors, indicating that cell cycle gene expression may contribute to the lineage restriction during adipogenesis (Ullah et al., 2013).

The Cdk-cyclin-E2F-Rb pathway, which has been extensively studied in the mitotic cellular expansion (MCE) of preadipocyte cell lines, is the main cell cycle regulatory mechanism during the transition from proliferation to differentiation. When the preadipocytes become arrested, the E2F1/Rb complex appears. When the arrested
preadipocytes go through MCE, Rb is hyperphosphorylated by cyclin D/CDK4/6, which is activated following downregulation of p27, so E2F is released to facilitate cell cycle activity (Farmer, 2006). However, once the cells become quiescent after terminal differentiation, Rb becomes hypophosphorylated, and thus can inhibit not only E2F but also C/EBPβ (Cole et al., 2004). In addition, it can also inhibit PPARγ activity by recruiting histone deacetylase 3 (HDAC3) to PPARγ target genes (Fajas et al., 2002). On the other hand, E2F1 not only promotes the cell cycle by activating the expression of cyclin D1 and cyclin E in the Cdk-cyclin-E2F-Rb signaling cascade, but also enhances adipocyte differentiation by positively modulating the expression of peroxisome proliferator-activated receptor PPARγ (Fajas et al., 2002). Therefore, these cell cycle regulators play an important role not only in cell cycle regulation during adipocyte differentiation, but also in adipogenesis regulation.

As more cell cycle regulators were studied, the complex network between them and adipogenic regulators was gradually unveiled. For example, the cyclin D3-cdk6 complex not only promotes cell cycle progression in the G1 phase, but also phosphorylates and activates PPARγ (Sarruf et al., 2005), which can be also activated by cdk4 (Abella et al., 2005) but repressed by cyclin D1 (Fu et al., 2005). The intimate interrelationship between the cell cycle regulators and adipogenic regulators also makes the adipogenic regulators cell-cycle dependent. For example, C/EBPβ is dephosphorylated when the preadipocytes are in the G1- growth arrested state. Phosphorylation of C/EBPβ occurs as preadipocytes traverse to the G1-S checkpoint at the onset of mitotic clonal expansion and the completion of dual phosphorylation of C/EBPβ coincides with entry into the S phase (Tang et al., 2005).

**DETECTION METHODOLOGIES OF ADIPOCYTE CELL CYCLE**

Since fine dissection of the cell cycle is imperative for profound understanding
of normal cell proliferation and differentiation, as well as carcinogenesis, various methods have been developed for cell cycle detection. Some methods detect the cell cycle through measurement of DNA synthesis. Among them, the simplest method is to extract DNA and measure the amount of DNA within the tissue (Chen et al., 2014). However, it is just a general method to measure cell cycle activity in a relatively wide time span, because change in amount of DNA cannot be detected within a short period of cell cycle activity. To detect DNA synthesis in a real-time manner, bromodeoxyuridine (BrdU) is usually utilized as a marker of DNA synthesis during the S phase of the cell cycle, because it can incorporate DNA of dividing cells as a thymidine analog and it can be easily detected using a monoclonal antibody developed by Gratzner (1982). Before BrdU labeling was developed, [3-H] thymidine autoradiography was utilized for decades to detect the cell cycle through the same mechanism as BrdU. However, compared to BrdU, [3-H] thymidine autoradiography requires much more time and labor. In addition, the result is not reliable due to poor resolution and low signal-to-noise ratio of the microscopic image (Duque and Rakic, 2011). Therefore, [3-H] thymidine autoradiography was replaced by BrdU. However, BrdU also has disadvantages (Salic and Mitchison, 2008). The first disadvantage is the harsh staining conditions needed to denature the DNA, which inevitably degrade the structure of the specimen. The second disadvantage is the limited size of tissue that can be stained due to limited penetration of the antibody. In 2008, a new method was developed by Salic and Mitchison to detect DNA synthesis in proliferating cells. Similar to BrdU, it is based on incorporation of 5-ethynyl-2’ deoxyuridine (EdU), which is also a thymidine analogue and can be detected through the reaction with the fluorescent azides in a Cu (I)-catalyzed [3 + 2] cycloaddition. In contrast to BrdU, this method does not require sample fixation or DNA denaturation. In addition, it can stain
large tissue and organ fragments, because its small size allows for rapid penetration and diffusion.

However, all of the above methods can only detect cells at the S phase of the cell cycle. In order to distinguish cells at different phases of the cell cycle, different markers with different colors must be used. There are a wide variety of markers available for phase determination during the cell cycle. For example, geminin is a well-known marker for the S/G2/M phase (McGarry and Kirschner, 1998) and Cdt1 is also a popular marker for the G1 phase (Nishitani et al., 2001). Phosphorylated histone gamma H2A.X is a recruiter of multiple DNA damage response proteins and thus is a marker for initiation of apoptosis (Yuan et al., 2010). Because the antibodies for the three markers show different colors, an immunochemistry-based cell cycle detection method that can simultaneously stain cells at the G1 and S/G2/M phases and cells undergoing apoptosis has been developed with the antibodies of the three markers (Yanagita et al., 2012).

Another popular marker for detection of cells with cell cycle activity is the proliferating cell nuclear antigen (PCNA). PCNA is a processivity factor for DNA polymerase delta and epsilon (Kelman, 1997) showing rapid increase during the S phase of the cell cycle (Celis et al., 1985), and, therefore, can be used as an index of cell cycle and cell proliferation in fixed tissues (Hall et al., 1990). On the other hand, Ki-67, a nuclear antigen present in S, G2 and M phases and G1 phase following the M phase, can also be utilized as a reliable marker for immunostaining of proliferating cells in frozen tissues (Gerdes et al., 1984).

METHODS TO STUDY GENE EXPRESSION AT DIFFERENT LEVELS

Gene expression analysis is becoming increasingly important in many fields of biological research. It is not only imperative for selection of novel biomarkers of
various diseases in humans and various production traits in domestic animals, but is also essential for understanding the functions of specific genes. Gene expression can be measured through mRNA quantification and protein quantification. At the mRNA level, one detection method is northern blot analysis, which is based on the ability of complementary single-stranded nucleic acids to form hybrid molecules with the target sequences (Denhardt, 1966). In brief, RNA is transferred to a nylon or nitrocellulose membrane after separation according to size on an agarose gel under denaturing conditions, and then hybridized with a $^{32}$P-labeled nucleic-acid probe. After washing off the unbound and non-specifically bound probe, the radiolabeled RNA can be detected by autoradiograph on an X-ray film (Brown, 1993). One disadvantage of northern blot analysis is that radioactive procedures make it time-consuming and potentially dangerous. However, as safer alternatives, nonradioactive approaches, such as digoxigenin- (DIG) (Trayhurn et al., 1994) and biotin (Löw and Rausch, 1994) labeled probes, were developed. Northern blot analysis is still the most-widely used method for validating small RNAs identified by high-throughput approaches (Kim et al., 2010). Despite possessing less sensitivity than other analytical methods, northern blot analysis can readily reveal irrelevant products and provide both size and expression level information for both small RNAs and their precursors (Várallyay et al., 2008).

Another widely used method for measuring mRNA abundance is real-time quantitative PCR. In this technique, cDNA template is first generated from mRNA through reverse transcription. The cDNA template is then amplified with fluorescence labeled hybridization probes or intercalating dye and a standard curve is constructed based on the change in emitted fluorescence during the DNA amplification process. Then the gene expression value can be calculated as the number of copies of original
mRNA in absolute quantification based on the standard curve or as change in expression compared to some reference group in relative quantification (Livak and Schmittgen, 2001). If the PCR product is just loaded for electrophoresis to compare the quantity based on the band strength, the process is called semi-quantitative PCR. Although quantitative PCR is much more sensitive than northern blot analysis, for correct quantification of the initial target sequences, it is essential to find a housekeeping gene with constant gene expression in all samples to normalize each sample to equal input amounts of nucleic acid. In case a single gene for normalization can lead to relatively large errors, the geometric mean of multiple housekeeping genes can be utilized for normalization (Vandesompele et al., 2002). In another method called quantitative competitive (QC) – PCR, a known amount of internal control competitor is included in each reaction, and a relative quantification of target PCR product can be obtained through comparison with the known competitor PCR product. In this case, an internal competitor that amplifies with the same efficiency as the target gene must be carefully designed (Heid et al., 1996).

Although quantitative PCR can measure mRNA expression relatively accurately, one of its disadvantages is that it cannot provide information about the location of the transcripts within cells or tissues. In order to detect spatial location of the transcripts, in situ hybridization (ISH) can be utilized. ISH is performed based on hybridization between the specifically labeled probe and its complementary RNA sequences in fixed cells or tissues. The target transcript can then be visualized through detection of the label on the probe. When ISH was initially developed, the probes were also radioisotope labeled. Although it was highly sensitive, the method was time consuming and also carried risk to the researchers and environment due to radioactivity (Qian and Lloyd, 2003). However, when immunological detection was
introduced, ISH underwent further development. In the immunological detection method, the probe is labeled by hapten, which can bind specific antibody-enzyme conjugates. Then the enzyme can react with the precipitating chromogens to make the RNA visible (Tautz and Pfeifle, 1989). In addition, introduction of fluorescence-labeled probes facilitates fluorescence in situ hybridization (FISH). After continuous optimization and numerous technical advancements, FISH became a foremost biological technology with improved sensitivity, specificity and simultaneous visualization of multiple RNAs (Levsky and Singer, 2003).

Although northern blotting, quantitative PCR and ISH have their unique merits, all of these methods can only detect RNA expression of a limited number of genes at one time. To quantify the complete set of transcripts in a cell or tissue, microarray analysis can be utilized. As is FISH, microarray analysis is a hybridization-based approach. In microarray analysis, a fluorescently labeled probe is applied to a custom-made microarray containing known genome sequences instead of a fixed tissue. However, microarray analysis also has limitations. First, it can be only conducted under the premise that the genome sequence is known. Second, the background is usually high due to cross-hybridization, which limits the dynamic range of detection (Okoniewski and Miller, 2006). Finally, it is often difficult and requires a complicated normalization method to compare gene expression across different experiments. With the development of next-generation sequencing, a more powerful technology - RNA-Seq - has been developed and is widely used. In this method, a population of RNA is first converted to a cDNA library with adaptors attached to one or two ends of each fragment. Each molecule is then sequenced from the adaptors in a high-throughput manner. The resulting reads after sequencing are then aligned to reference genome or transcripts or assembled to produce a genome-scale transcription
map. Finally, the density of reads covering RNA from each exon, splice event or new candidate gene is then obtained to calculate expression level of each gene (Mortazavi et al., 2008). Unlike microarray analysis, RNA-Seq does not require existing genomic sequence to detect transcripts. In addition, it not only can measure gene expression, but also can precisely locate the transcription boundaries, and provide information on how the exons are connected and transcripts are spliced (Wang et al., 2009).

Although amount of mRNA can provide much information concerning gene expression, the levels of transcripts are usually unable to reflect the corresponding protein levels, because not all transcripts will be translated to proteins. For protein expression analysis, one of the most widely used methods is western blotting. In this method, the proteins are first separated by electrophoretic migration in a discontinuous standard Tris/glycine SDS-polyacrylamide (SDS-PAGE) gel according to molecular weight. After electrophoretic transfer of the proteins to a two-dimensional membrane that can support and bind proteins, the target proteins are then visualized by specific antibodies or ligands that can bind the proteins. The quantity of the target proteins can then be compared based on the strength of the fluorescence (Brunner, 2005). As is the case for the ISH method used for mRNA, immunohistochemical (IHC) staining is a method for in situ detection of protein expression in different sections of a fixed tissue. The principle of this method is the same as in western blotting. It is also based on binding of antibodies to antigens and detection of the protein through labeling of the antibodies (Ramos-Vara and Miller, 2014). For the measurement of expression of specific proteins, mass spectrometry can also be utilized. In this method, the proteins to be analyzed are first isolated through SDS-PAGE and then excised and digested to peptides with C-terminally protonated amino acids. The peptides are then separated in very fine capillaries through high-
pressure liquid chromatography and nebulized in small highly charged droplets in an
electrospray ion source. Finally, the mass spectrum of the peptide is taken in the mass
spectrometer and the intensity of the mass spectrum provides a measurement of
protein expression (Aebersold and Mann, 2003). The matrix-assisted laser desorption
ionization (MALDI) mass spectrometry can also be applied to a thin tissue section to
map the distribution of peptides and proteins. In this method, the frozen sections are
cut, thawed and mounted on a metal plate. The UV-absorbing matrix is then deposited
uniformly over the section after being completely dried. Finally, a raster, which is a
dot matrix data structure of the tissue, is performed over a predetermined array or grid,
generating a full mass spectrum at each grid in a mass spectrometer. A density map
can then be generated based on the intensity of a given mass-to-charge value in each
spectrum (Chaurand et al., 2002). The density maps for different sections can then be
combined to construct a three-dimensional expression map of the whole tissue
(Andersson et al., 2008).

DNA MICROARRAY AND GENE EXPRESSION OMNIBUS DATABASE

A DNA microarray is a high-throughput technology that allows monitoring of
expression of thousands of genes simultaneously with a collection of probes
immobilized on different DNA spots. The probes are cDNA fragments or
oligonucleotides with known sequence. When the mRNA samples labeled with
fluorescence are applied to the microarray chips, the target mRNAs will hybridize to
the probes by base paring (adenine pairs with uracil and cytosine pairs with guanine).
After the unbound mRNA or nonspecific bindings are washed out, only the target
mRNA stays on the probes. Then the abundance of target mRNAs is measured by the
intensity of fluorescent images (Bunney et al., 2003). This technology facilitates
selection of genes differentially expressed between different tissues, time points and
conditions, and thus became widely used in recent decades as the cost became less (Pan, 2002).

However, as utilization of the high-throughput technology became popular, there was increasing demand for open access to the high-throughput datasets in the published literature. As demand for a public database of high-throughput expression data grew, the Gene Expression Omnibus (GEO) was established by the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) (Edgar et al., 2002). By now, thousands of microarray datasets have been submitted to the GEO database, providing a useful tool to compare gene expression under various physiological, developmental or nutritional conditions. Therefore, we can often find useful data supporting our research conclusions by exploring these datasets. In addition, these datasets also provide free tests of our hypotheses, and, thus, guide our study in the right direction without wasting time, money and effort in the initial exploration of the hypotheses. With these advantages, the GEO database has been successfully used in the investigation of novel tissue-specific genes (Song et al., 2013).

There are two main sections in the database: GEO DataSets and GEO Profiles. By searching GEO DataSets, microarray datasets, as well as original series and platform records that are related to a particular research interest, can be found. By searching GEO Profiles, the spatial and temporal expression of single genes under different conditions can be investigated. In this study, we first searched the GEO DataSets for useful microarray datasets for our study of cell cycle regulators in adipocyte differentiation, and then started the exploration of target cell cycle regulators in the GEO Profiles.
SUMMARY

The microarray technology is a powerful strategy for comparison of expression of a large number of genes, providing a tool to select master genes involved in various biological processes, marker genes for various diseases and phenotypes, and important genes regulated by various treatments, as well as novel genes with potential physiological functions. Therefore, the GEO database, as a huge repository of microarray data from thousands of studies, provides an economical and efficient way to identify critical or novel genes and predict their potential functions through comparison of gene expression among different conditions, locations and time points. With the broad application of microarrays and increasing deposition of microarray data in the GEO database, bioinformatic study of gene expression can provide more strength and more distinct direction for molecular research. With the strategy integrating bioinformatic and molecular technologies, more important genes related to function and development of adipose tissue, as well as more novel genes with potential significance, can be revealed to deepen our understanding of complicated biological activities.
CHAPTER 3
DIFFERENTIAL EXPRESSION OF CYCLIN G2, CYCLIN DEPENDENT KINASE INHIBITOR 2C AND PERIPHERAL MYELIN PROTEIN 22 GENES DURING ADIPOGENESIS

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ABSTRACT

Increase of fat cells (FCs) in adipose tissue is attributed to proliferation of preadipocytes or immature adipocytes in the early stages, as well as adipogenic differentiation in the later stages of adipose development. Although both events are involved in the FC increase, they are contrary to each other, because the former requires cell cycle activity, whereas the latter requires cell cycle withdrawal. Therefore, appropriate regulation of cell cycle inhibition is critical to adipogenesis. In order to explore the important cell cycle inhibitors and study their expression in adipogenesis, we adopted a strategy combining the Gene Expression Omnibus (GEO) database available on the NCBI website and the results of quantitative real-time PCR.
(qPCR) data in porcine adipose tissue. Three cell cycle inhibitors - cyclin G2 (CCNG2), cyclin-dependent kinase inhibitor 2C (CDKN2C) and peripheral myelin protein (PMP22) - were selected for study because they are relatively highly expressed in adipose tissue compared with muscle, heart, lung, liver and kidney in humans and mice based on two GEO DataSets (GDS596 and GDS3142). In the latter analysis, they were found to be more highly expressed in differentiating/ed preadipocytes than in undifferentiated preadipocytes in human and mice as shown respectively by GDS2366 and GDS2743. In addition, GDS2659 also suggested increasing expression of the three cell cycle inhibitors during differentiation of 3T3-L1 cells. Further study with qPCR in Landrace pigs did not confirm the high expression of these genes in adipose tissue compared with other tissues in market-age pigs, but confirmed higher expression of these genes in FCs than in the stromal vascular fraction, as well as increasing expression of these genes during in vitro adipogenic differentiation and in vivo development of adipose tissue. Moreover, the relatively high expression of CCNG2 in adipose tissue of market-age pigs and increasing expression during development of adipose tissue was also confirmed at the protein level by western blot analysis. Based on the analysis of the GEO DataSets and results of qPCR and western blotting we conclude that all three cell cycle inhibitors may inhibit adipocyte proliferation, but promote adipocyte differentiation and hold a differentiated state by inducing and maintaining cell cycle inhibition. Therefore, their expression in adipose tissue is positively correlated with age and mature FC number. By regulating the expression of these genes, we may be able to control FC number, and, thus, reduce excessive fat tissue in animals and humans.
IMPLICATIONS

The cell cycle is necessary for preadipocyte proliferation, whereas adipocyte differentiation requires its inhibition. In this study, three cell cycle inhibitors were found to be low in preadipocytes and increasingly expressed during adipogenic differentiation and adipose tissue development, serving as potential indicators for rates of proliferation and adipocyte differentiation. Moreover, modulation of their expression or activities of their encoding proteins during adipose growth and development may provide a prospective method for controlling fat deposition in animals and humans by reducing the fat cell number and size. Finally, this study provides an efficient research strategy combining an online database with experimental data.

INTRODUCTION

As the most flexible tissue in size and weight, adipose tissue can range from 2 to 3% of body weight in the fittest athletes to 60 to 70% of body weight in extremely obese individuals (Hausman et al., 2001). Growth of adipose tissue is achieved through increases of cell size (hypertrophy) or cell number (hyperplasia). Although it seems that hyperplasia only occurs at early developmental stages in non-obese animals, it was also found to be triggered in adult obese animals when the cell size and lipid content reached a certain level (Faust et al., 1978; Guo et al., 2011). The different patterns of adipose development are closely related to timely cell cycle inhibition, because proliferation of preadipocytes and immature adipocytes, which is dependent on cell cycle activity, precedes differentiation of preadipocytes into mature fat cells (FCs), which is characterized by cell cycle withdrawal (Ntambi and Young-
Cheul, 2000), whereas promotion of cell proliferation can inhibit adipogenesis (Hou et al., 2013).

Therefore, we postulated a hypothesis that there may be important cell cycle inhibitors that inhibit cell proliferation in the early stage of development of adipose tissue, but allow FC differentiation in the later stage through cell cycle inhibition. Therefore, they should be highly expressed in mature FCs and well-differentiated adipose tissue and increasingly expressed during adipose development. If such cell cycle inhibitors can be found, they may become potential markers for selection of reduced fat tissue in the animal industry. Meanwhile, since increase of FC number is one of the contributors to severe obesity (Hirsch and Knittle, 1970; Faust et al., 1978), increased expression of these cell cycle inhibitors in the early stage of adipose development and decreased expression in the later stage may provide avenues for curing severe obesity.

Although some cell cycle inhibitors such as retinoblastoma protein (Chen et al., 1996; Richon et al., 1997) have been well studied for their function coupling cell cycle inhibition and adipocyte differentiation, the functions of most other cell cycle inhibitors in adipogenesis are still unclear. In addition, to the best of our knowledge, there have been no comprehensive studies of cell cycle inhibitors in adipose development. Fortunately, the availability of hundreds of microarray data in the Gene Expression Omnibus (GEO) database on the NCBI website makes it feasible to initiate a comprehensive study of cell cycle inhibitors by comparing gene expression in various physiological, developmental or nutritional conditions.

In this study, three important cell cycle inhibitors - Cyclin G2 (CCNG2), Cyclin-dependent kinase inhibitor 2C (CDKN2C) and Peripheral Myelin Protein 22 (PMP22) - were found to be expressed to a greater extent in adipose tissue by
analyzing microarray data from two GEO datasets, GDS596 and GDS3142, which compare gene expression in various tissues of humans and mice. An unconventional cyclin, CCNG2, not only inhibits cell proliferation by blocking transition from the G1 to the S phase in the cell cycle, but also promotes transcriptional activity of PPARγ by forming a transcriptional complex (Aguilar et al., 2010). CDKN2C, which is also called p18INK4C, is also well-known as a key inhibitor of the cell cycle and can block cell cycle G1 progression by preventing activation of cyclin dependent kinase 4 or 6 (CDK4/6) (Hirai et al., 1995). CDKN2C has been identified as a protein that is indirectly regulated by PPARγ in the differentiation-dependent cascade (Morrison and Farmer, 1999). Therefore, CCNG2 and CDKN2C are both important regulators coupling cell cycle arrest and adipogenic differentiation. PMP22 is involved in G0 growth arrest as a homologue of the murine growth arrest-specific 3 (gas3) (Manfioletti et al., 1990; Welcher et al., 1991) and has been demonstrated to negatively regulate DNA synthesis and retard the transition from G0/G1 to the S + G2/M phases in Schwann cells (Zoidl et al., 1995). In addition, gas3/PMP22 is upregulated in human neonatal fibroblasts arrested by serum starvation or confluence, but is downregulated when these cells re-enter the cell cycle (Karlsson et al., 1999), indicating that it is important for maintaining a growth arrest state.

Although there are some reports regarding the three genes in the in vitro preadipocyte cell culture, there are no reports about the in vivo expression patterns of these three genes during adipose development. In this study, the expression of the three genes in adipose tissue was investigated by combining GEO DataSets for the human and mouse with qPCR and western blotting results for the pig. The spatial expression was studied for distribution in different tissues and comparison between stromal vascular (SV) cells and FCs. Meanwhile, the temporal expression was
examined during in vitro culture of porcine primary preadipocytes and in vivo development of porcine subcutaneous adipose tissue.

MATERIALS AND METHODS

Data Sources and Processing

The microarray expression profiles from six tissues (heart, liver, lung, muscle, kidney and adipose) were derived from two GEO datasets (GDS) available on the NCBI website: GDS596 for adult human (more than 20 years old) and GDS3142 for adult mouse (10 to 12 weeks old). The microarray in GDS596 contains 22,215 spots other than the housekeeping spots, whereas the microarray in GDS3142 contains 45,037 spots other than the housekeeping spots. There are two samples for each tissue in GDS596 and three to four samples for each tissue in GDS3142.

First, all of the genes in the two microarray datasets were ranked in descending order based on the ratio between mean expression value of the genes in adipose tissue and mean expression value of the genes in the other five tissues as previously described (Song et al., 2013). The 108 cell cycle regulatory genes published by Cell Cycle RT² Profiler™ PCR array in the QIAGEN company (http://www.sabiosciences.com/rt_pcr_product/HTML/PAHS-020Z.html) and 21 other important cell cycle regulators mentioned in published paper (Schafer, 1998) were then selected and analyzed in the two datasets. Finally, three inhibitors – CCNG2, CDKN2C and PMP22, were selected as they are directly involved in the cell cycle and showed high ranks in both datasets.

The expression levels of the three inhibitors were then further compared among different differentiation stages of preadipocytes by analyzing GDS2743 and
GDS2366. GDS2743 compares the gene expression between primary white preadipocytes from epididymal white adipose tissue of mouse cultured for 4 and 7 days, which corresponds to the undifferentiated stage and differentiating stage (n=8 for undifferentiated preadipocytes and n=6 for differentiating preadipocytes) of adipocytes. GDS2366 compares the gene expression between undifferentiated and differentiated preadipocytes from subcutaneous adipose tissues in human (n=3 for each of the two groups). In addition, GDS2659, which records gene expression at several time points (preconfluence, confluence and 1, 3, 7 and 28 days after addition of differentiation cocktail) during differentiation of 3T3-L1 preadipocytes, was also used to analyze gene expression of the three inhibitors during differentiation of adipocytes in vitro.

**Experimental Animals**

All animal care and use procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at The Ohio State University. Pigs were reared at the Ohio Agricultural Research and Development Center (OARDC) Western Agricultural Research Station in South Charleston, OH. Neonatal pigs were nursed by sows. Market-age pigs were fed with three different diets during the three phases of their growth. When the pigs weighed 32 to 64 kg, the diet contained 18% crude protein (CP) and 0.95% lysine with metabolizable energy (ME) of 3234 kcal/kg. When the pigs weighed 64 to 91 kg, the diet contained 16.3% CP and 0.78% lysine with 3243 kcal ME/kg. When the pigs weighed more than 91 kg, the diet contained 14.3% CP and 0.68% lysine with 3247 kcal ME/kg. Rearing of the pigs used to detect the tissue distribution of gene expression was the same as that for the pigs used to detect gene expression during adipose tissue development (Crawford, *et al.*, 2010).

To detect the tissue distribution of the three inhibitors - CCNG2, CDKN2C
and PMP22 - in pigs, adipose tissue, muscle, heart, lung, liver, kidney, spleen and intestine were collected from four Landrace pigs at 120 days of age. In addition, for western blot analysis of tissue distribution of CCNG2, the same tissues except intestine were also collected from one Duroc pig at 200 days of age. To detect expression of the three genes during development of adipose tissue in vivo, subcutaneous adipose tissues were collected from the middle of the back of 105-day fetal pigs, 6-day post-natal pigs and 120-day market-age Landrace pigs with three individuals in each group. All of the collected samples were kept at -80℃ after being snap frozen in dry ice for total RNA isolation and qPCR (Deiuliis et al., 2008; Ahn et al., 2013).

Separation of Stromal Vascular and Fat Cell Fraction

To compare gene expression between the SV and FC fractions, the two fractions were separated from three 5 g subcutaneous adipose tissues that were collected from each of the three 120-day market-age Landrace pigs mentioned above. The subcutaneous adipose tissues were first minced using razor blades before incubation with 3.2 mg/mL of collagenase II (Sigma-Aldrich, St. Louis, MO) at 37℃. After incubation for 1 h in a shaking water bath, the suspension was passed through a 100 µm nylon cell strainer to remove the large pieces. Finally, the floating FC fraction was separated from the SV fraction in the pellet after centrifugation of the filtrate at 500×g for 5 min (Deiuliis et al., 2006).

In Vitro Culture of SV Cells

After the SV fraction, which consists mostly of preadipocytes, was isolated, the cells were diluted in DMEM culture medium containing 10% fetal bovine serum (Invitrogen Inc., Grand Island, NY, USA) and a mixture of penicillin and streptomycin (Invitrogen). The diluted cells were then maintained and grown to
confluence (day 0) at 37°C in 5% CO₂. After induction of differentiation of dexamethasone (80 nM) for 3 days post confluence, the differentiation mixture containing 0.5 mM of isobutylmethylxanthine, 5 µg/mL of transferrin and 5 µg/mL of insulin was added for a 6-day culture to promote differentiation into adipocytes. The cells were collected on day 0, 3, 6 and 9 after induction of differentiation for extraction of total RNA samples, which were stored at -80°C for subsequent RT-PCR.

**cDNA Synthesis and qPCR**

The total RNA samples from different tissues and cell cultures were isolated using Trizol reagent (Invitrogen) according to the manufacturer’s instructions and the quality was assessed by electrophoresis on 1% gels. Then the cDNA was synthesized using 1 µg of total RNA, oligo dT and moloney murine leukemia virus (M-MLV) reverse transcriptase (Invitrogen) according to the manufacturer’s instructions. The synthesized cDNA was then used for qPCR to measure expression of CCNG2 (Accession number: XM_003129099; forward 5’-CAGCTGAAAGCTTGCAACTGC -3’ and reverse 5’-TGAAAAATAGGCCAGATCTGATCTGA-3’), CDKN2C (Accession number: NM_001262; forward 5’-GGGACCTAGAGCAACTTACTAGTTGT-3’ and reverse 5’- GTGTCAGGAAACCTGCTCTG-3’) and PMP22 (Accession number: NM_000304; forward 5’-CTCCACGATCGTCAGCCA AT-3’ and reverse 5’-GTGAAGAGCTGGCCAGAAGACAG-3’) with primers shown in the parentheses. qPCR was performed using AmpliTaq Gold polymerase (Applied Biosystems, Grand Island, NY, USA), SYBR green I as a detection dye and cyclophilin (cyc) as an internal control with cycling parameters as follows: 95°C for 10 min, followed by 40 cycles of 94°C for 30 s, 60°C for 1 min and 82°C for 30s. The relative gene expression was then calculated as the ratio of target gene to cyc expression. In addition, in order to verify separation of the SV and FC fractions,
differentiation of preadipocytes in vitro and development of adipose tissue in vivo, the expression of one preadipocyte marker, delta-like 1 homolog (DLK1), and one adipocyte marker, peroxisome proliferator-activated receptor γ (PPARγ), was also measured by qPCR. The sequences of primers for cyc, DLK1 and PPARγ in qPCR were described in previous reports (Li et al., 2007).

**Protein Isolation and Western Blot Analysis**

In order to detect protein expression among different tissues and different time points during adipose development, protein was isolated from different tissues of one 120-day Landrace pig and one 200-day Duroc pig, as well as adipose tissues of two individuals at each developmental stage. Due to lack of available peptide sequence and antibody, only expression of CCNG2 was detected. Western blot analysis was carried out following the procedure described in our previous report (Li et al., 2012). Protein was extracted from ~100mg of tissue after homogenization in 1 mL of lysis buffer followed by centrifugation at 12,000 rpm for 5 min at 4°C. Coomassie stain was used to determine an equal amount of protein loaded for each sample before western blotting. For western blotting, the proteins in each sample were transferred to polyvinylidene fluoride membrane (Amersham Biosciences, Piscataway, NJ, USA) after separation in SDS-PAGE by the mini-Protein system (Bio-Rad, Hercules, CA, USA). The membrane was then blocked for 30 min in 4% nonfat dry milk in 1×Tris-Buffered Saline Tween-20 (TBST; 0.1% Tween 20) and incubated overnight at 4°C with a primary antibody raised against the region 170-210aa of human CCNG2 protein (Bioss Inc., Woburn, MA, USA) in 4% nonfat dry milk. After being washed with 1×TBST for 4 min each time for seven times, the membrane was incubated with a horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology
Inc., Danvers, MA, USA) in 4% nonfat dry milk for 1 h at room temperature, and then washed again with 1×TBST for 4 min each time for seven times. Finally, bands were detected with Hyperfilm (Amersham Biosciences) in a dark room after the Amersham ECL Plus Western Blotting Detection Reagents were applied on the membrane. To ensure that the bands detected were the target bands (38.9KDa), the protein isolated from muscle of a postnatal 20-day mouse was used as a positive control.

**Statistical Analyses**

Statistical analysis for the tissue distribution of gene expression was performed using a mixed model (MIXED) procedure available in SAS software (version 9.3, SAS Institute Inc., Cary, NC, USA). The DIFF option was used to detect significant differences between pairs of least squares means. Comparison of gene expression between two groups was conducted in SAS using Student’s t-test. Multiple comparisons among the different time points during *in vitro* differentiation and *in vivo* development were carried out using one-way ANOVA followed by Fisher’s post hoc test. P-values lower than 0.05 were treated as significant. All of the results are presented as least squares means plus or minus standard errors of the least square means (SEM).

**RESULTS**

**Data Analysis Based on the GEO DataSets**

A total of 22,215 genes in the GDS596 (human) and 45,037 genes in the GDS3142 (mouse) datasets were ranked in descending order based on the ratio between average expression in adipose tissue and average expression in the other five tissues. By comparing high-ranking cell cycle regulators across the human and mouse,
three cell cycle inhibitors were selected that had a relatively high expression in the adipose tissue of both the human and mouse (Table 3.1). The ranks of CCNG2, CDKN2C and PMP22, respectively, were 2,102, 654 and 2,023 in GDS596 and 1,878, 55 and 2,007 in GDS3142. Although expression of the three genes was not necessarily highest in adipose tissue, the three genes generally had high expression in lung or adipose tissue and the lowest expression in liver.

In the cell culture of preadipocytes from mouse epididymal adipose tissue in GDS2743, all three cell cycle inhibitors showed significantly greater expression in differentiating preadipocytes (7 days in cell culture) than in undifferentiated preadipocytes (4 days in cell culture) (Figure 3.1A). In addition, the differentiated preadipocytes in human subcutaneous adipose tissues in GDS2366 also exhibited greater expression of the three cell cycle inhibitors compared with undifferentiated preadipocytes (Figure 3.1B). The high expression of these genes in differentiated preadipocytes was further confirmed by the 28-day timeline records of gene expression during differentiation of 3T3-L1 preadipocytes in GDS2659, because the three inhibitors were generally expressed to a greater degree at the end of differentiation than at the initiation of differentiation (Figure 3.1C). However, there was also a transient fluctuation in expression for PMP22 and CCNG2 from preconfluence to 3 days after differentiation before another strong increase, which may have been caused by the addition of differentiation cocktail.

**Expression of the Three Selected Genes in Various Tissues of Pigs**

mRNA expression of the three cell cycle inhibitors in the Landrace pigs showed a slightly different pattern from that in human (GDS596) and mouse (GDS3142). None of the three genes showed the highest expression in adipose tissue. For PMP22, the highest expression occurred in the lung, but without significant
differences from muscle and adipose tissue. For CCNG2 and CDKN2C, the highest expression was observed in muscle with the expression in adipose tissue close to the mean value of eight tissues. This result was especially obvious for CDKN2C, where expression in muscle was high with the expression in other tissues significantly low. Therefore, expression of CDKN2C seemed to be muscle-specific. Although the three genes showed different distributions of expression among the eight tissues, they exhibited consistency in that lowest expression occurred in the liver (Figure 3.2A). However, the protein expression of CCNG2 in different tissues showed some discrepancy from the mRNA expression. According to western blot analysis, although the CCNG2 protein is also the most abundant in muscle, it was only detected in fat, muscle and heart in both Landrace and Duroc (Figure 3.2B).

**Expression of the Selected Genes in SV and FC Fractions in Pigs**

In qPCR with pig subcutaneous adipose tissue, all three genes showed greater expression in the FC fraction. Expression of CCNG2 and CDKN2C was more than 18 and 2 times greater, respectively, in the FC fraction than in the SV fraction (P<0.01), whereas PMP22 was expressed less than two times as much, and, therefore, was expressed nonsignificantly more in the FC fraction than in the SV fraction (Figure 3.3). Meanwhile, expression of the preadipocyte marker DLK1 was nearly 50-fold greater in the SV fraction than in the FC fraction (P<0.05), whereas expression of the adipocyte marker PPARγ was more than 15 times greater in the FC fraction than in the SV fraction (P<0.001) (Figure 3.3), indicating that isolation of the two fractions was successful.
Expression of the Selected Genes during Adipogenic Differentiation

During the 9-day *in vitro* differentiation of preadipocytes in the SV fraction isolated from porcine subcutaneous adipose tissue, the expression of CCNG2 showed a continuous and significant increase from 3 to 9 day after induction of differentiation with expression doubling every three days (P<0.05). For CDKN2C, there were two significant increases in expression (P<0.05). The first increase occurred from day 0 to day 3, while the second one occurred from day 6 to day 9. For PMP22, although the increased expression also seemed continuous, it was only significant from day 6 to day 9 (P<0.05) with an increment of 83% (Figure 3.4). Meanwhile, DLK1 expression decreased 94.5% from day 0 to day 3 (P<0.05) and remained at a very low level from 3 days after induction of differentiation onward. In contrast, expression of PPARγ was low at day 0 and 3, but increased more than four times and two times, respectively, by day 6 and 9 (P<0.05) (Figure 3.4), indicating successful differentiation of preadipocytes into adipocytes.

Expression of the Selected Genes during Adipose Development

As the three cell cycle inhibitors all increased during adipogenic differentiation, all of them also increased significantly during adipose development *in vivo* from 6 days to 120 days after birth (P<0.05). The mRNA expression of CCNG2, CDKN2C and PMP22 increased more than 33, 4 and 9 times, respectively, during this period, coincident with a seven-fold increase of PPARγ, which is contrary to the 100-fold decrease of DLK1 from the fetal stage to the neonatal stage (P<0.05) (Figure 3.5A). However, all three genes maintained a low level of expression in 105-day fetuses and 6-day old piglets, as in the case of PPARγ. This trend differed from that of DLK1, which was highly expressed in 105-day fetuses (Figure 3.5A). Western blot
analysis of CCNG2 also showed results consistent with those of qPCR, since the protein expression at different time points of development showed the same trend as the mRNA expression (Figure 3.5B). In 105-day fetuses and 6-day old piglets, there was only a small amount of mRNA without detectable protein, while in the 120-day pigs, there was abundant mRNA and protein expression.

**DISCUSSION**

In this study, we applied a powerful strategy that combined microarray data deposited in NCBI's GEO public database with the data from our qPCR and western blotting experiment, and, therefore, conducted more comprehensive analysis and made stronger conclusions with less time and financial cost due to experimentation. There are nearly 100 GEO DataSets on FCs and adipose tissues; therefore, we can often find useful data supporting our research conclusions by exploring these datasets. In addition, these datasets also provide free tests of our hypothesis, and, thus, guide our study in the right direction without wasting time, money and effort in the initial exploration of the hypothesis. For instance, selection of the three important cell cycle inhibitors in this study would have been difficult and costly without the five GEO DataSets, because there are so many cell cycle inhibitors.

The differentiation of preadipocytes to FCs has been acknowledged to be tightly coordinated with changes in the cell cycle, because the preadipocytes undergo cell proliferation, which is arrested in the mature FCs (Fajas et al., 1998). Therefore, it is reasonable to hypothesize greater expression of some important cell cycle inhibitors in mature FCs than in the adipocyte precursors. This hypothesis was proven by examination of the GDS2743 and GDS2366 datasets, because the three cell cycle inhibitors that we studied exhibited much greater expression in differentiating or
differentiated preadipocytes than in undifferentiated preadipocytes in both humans and mice. This result is also supported by the higher expression of the three genes in the FC fraction compared with the SV fraction, which mainly contains preadipocytes, suggesting that the three cell cycle inhibitors may be related to cell cycle inhibition during adipogenic differentiation and in the maintenance of the quiescent state of mature FCs.

In addition, results of the 9-day porcine preadipocyte primary culture also agreed with results of the 3T3-L1 cell culture in the GDS2659 dataset in terms of the increasing expression of the three genes during differentiation. The highest level of DLK1 gene expression and the lowest level of PPARγ expression at day 0 indicated an undifferentiated preadipocyte stage, whereas the dramatic decrease in DLK1 expression after induction of differentiation, which coincided with the gradual increase in PPARγ expression, indicated that differentiation was promoted (Smas and Sul, 1993; Deiuliis et al., 2008; Li et al., 2009). The gradual increase in expression of the three cell cycle inhibitors may therefore indicate that inhibition of the cell cycle during the differentiation process is gradual rather than immediate. This conclusion is also supported by the discovery of small proliferative adipocytes (SPA), which have tiny or no lipid droplets, expression of adipocyte markers and limited proliferative ability, and, thus, may represent the middle stage of differentiation (Hanamoto et al., 2013). Moreover, it seems that a significant increase of PMP22/gas3 only occurred in the later stage of differentiation, suggesting that it may be related more to the growth arrest state in the mature adipocytes than in the differentiation process. Based on the above analysis, it seems that the three cell cycle inhibitors promote the differentiation process mainly by cell cycle inhibition, even though there are some reports of positive correlations of CCNG2 (Morrison and Farmer, 1999) and CDKN2C (Hirai et al., 1995).
with PPARγ, because PPARγ also induces cell cycle withdrawal during adipocyte differentiation (Altiok et al., 1997). Therefore, we speculate that CCNG2 and CDKN2C may form a positive feedback loop with PPARγ coupling cell cycle inhibition and the adipogenic differentiation process.

The PPARγ-dependent expression pattern of the three cell cycle inhibitors also seems to be age-dependent during in vivo development of adipose tissue, because all three genes and PPARγ exhibited much greater expression in market-age pigs than in the early age groups. For CCNG2, this is further confirmed at the protein level. In the 105-day fetal pigs, the development of adipose tissue is mainly characterized by an increase of FC number by proliferation of preadipocytes and immature FCs (Desnoyers et al., 1980). Therefore, the expression of DLK1 was high, while expression of the three cell cycle inhibitors and PPARγ was low. In the 6-day postnatal pigs, DLK1 and PPARγ as well as the three cell cycle regulators, had low expression levels, because this developmental stage is characterized by an increase of SPA cells, which express adipocyte markers, but still can proliferate (Anderson and Kauffman, 1973). In the 4-month old pigs, the dramatic increase in expression of the three cell cycle inhibitors and PPARγ may indicate an increasing number of mature FCs during this period, because the increase in FC size becomes dominant in the development of adipose tissue, whereas the increase in FC number gradually slows during this period (Anderson and Kauffman, 1973). However, even in this stage, cell cycle activity may not completely stop in adipose tissue, because there are still preadipocytes or SPA cells with the potential to differentiate into mature FCs. This is perhaps the reason why expression of the three genes in adipose tissue in the tissue distribution analysis for 4-month old pigs is not as high as that for adult humans (>20
years old) and mice (10-12 weeks old) in the GDS596 and GDS3142 datasets. The FC number seems minimally increase after 18 years of age in humans (Knittle et al., 1979) or after 3 weeks of age in mice (Johnson and Hirsch, 1972), but still increases in pigs before 7 months of age (Anderson and Kauffman, 1973).

In addition to the close relationship of these three genes in adipocyte proliferation and differentiation, the low expression of the three cell cycle regulators in the liver of the human, mouse and pig may also reflect a high potential of hepatocytes to proliferate compared with the differentiated cells in other tissues, because they can re-enter the cell cycle soon after injury to facilitate regeneration (Albrecht et al., 1998). Moreover, the high expression of PMP22 in the lung of the human, mouse and pig may also be important for normal function in the quiescent state of lung cells, because expression of PMP22 was reported to be down-regulated in lung tumors of mice (Re et al., 1992). Finally, the high mRNA expression and low protein expression of CCNG2 in lung and intestine may indicate that some post-transcriptional mechanisms are involved in the regulation of this gene and that CCNG2 may not be the major regulator of cell cycle inhibition in these tissues.

In conclusion, CCNG2, CDKN2C and PMP22 are three important genes in adipogenesis, because they may promote adipocyte differentiation through cell cycle inhibition. Hyperplasia, which requires cell cycle activity, precedes differentiation from preadipocytes into FCs. Therefore, overexpression of these cell cycle inhibitors in the early developmental stage and inhibition of their expression in the later stage of adipogenesis may limit the increase of FC number in animals and humans caused by FC proliferation or differentiation (Hirsch and Knittle, 1970; Faust et al., 1978), and, therefore, provide potential methods of reducing excessive fat tissue in dietary meat and curing obesity in humans.
In addition, since the cell cycle is activated in differentiated and dedifferentiated liposarcoma compared with normal fat tissue (Singer et al., 2007), induction of terminal differentiation and cell cycle inhibition of liposarcoma may provide a possible therapeutic method for the treatment of liposarcoma (Tontonoz et al., 1997). For example, CDK4 is the main target for amplification of chromosome 12 in well-differentiated or dedifferentiated liposarcomas (Louis-Brennetot et al., 2011), whereas CDKN2C is one of the main inhibitors of CDK4, and, thus, may provide a prospective remedy for this disease. However, more in-depth studies still need to be done before application of these cell cycle inhibitors in these areas.

ACKNOWLEDGEMENTS

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AUTHOR CONTRIBUTIONS

**Table 3.1.** The cell cycle inhibitors expressed higher in adipose in both human and mouse

<table>
<thead>
<tr>
<th>Species</th>
<th>Gene (Rank)</th>
<th>Adipose</th>
<th>Muscle</th>
<th>Heart</th>
<th>Lung</th>
<th>Liver</th>
<th>Kidney</th>
<th>A/O</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>CCNG2(2102)</td>
<td>238±55</td>
<td>221±54</td>
<td>102±27</td>
<td>138±54</td>
<td>45±26</td>
<td>342±26</td>
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<td></td>
<td>CDKN2C(654)</td>
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<td>58±29</td>
<td>310±198</td>
<td>92±3</td>
<td>42±29</td>
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<td>PMP22 (2023)</td>
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<td>2674±248</td>
<td>1242±175</td>
<td>2764±48</td>
<td>140±13</td>
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<td>Mouse</td>
<td>CCNG2(1878)</td>
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<td>492±13</td>
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<td>3711±68</td>
<td>123±18</td>
<td>248±14</td>
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</table>

*a-e* Different superscript indicate significant difference (P<0.05).

Abbreviations: A/O = ratio between average expression in adipose tissue and that in the other tissues; PMP22 = Peripheral Myelin Protein 22; CDKN2C = Cyclin dependent kinase inhibitor 2C; CCNG2 = Cyclin G2.
Figure 3.1. Gene expression analysis of PMP22, CDKN2C and CCNG2 based on microarray datasets obtained from NCBI website. A, Expression comparison of the three genes between undifferentiated (EU, cultured for 4 days, n=8) and differentiating (ED, cultured for 7 days, n=6) preadipocytes from mouse epididymal adipose tissue based on GDS2743. B, Expression comparison of the three genes between undifferentiated (SU, n=3) and differentiated (SD, n=3) preadipocytes isolated from subcutaneous adipose tissue of human based on GDS2366. C, Expression of the three genes at various time points during differentiation of 3T3-L1 preadipocytes in GDS2659. In the histograms, each bar represents mean±SEM and statistical significance of Student’s t test is indicated by * (P<0.05), ** (P<0.01) and *** (P<0.001).
Figure 3.2. Expressions of CCNG2, CDKN2C and PMP22 in fat (F), muscle (M), heart (H), lung (Lu), liver (Li), kidney (K), spleen (S) and intestine (I) of pigs. A, mRNA expressions of the three genes were measured by qPCR with porcine cyclophilin (cyc) as a control for normalization. Bars represent means ± SEM. A mixed model was used with DIFF option to compare expressions in different tissues (n=4 for each tissue). Tissues with different letters above the bars are significantly different (P<0.05). B, CCNG2 protein levels in different tissues of one 120-day Landrace pig and one 200-day Duroc pig. Protein from muscle of 20-d postnatal mouse (M+) was used as positive control.
Figure 3.3. Relative gene expressions of DLK1, PPARγ, CCNG2, CDKN2C and PMP22 in the stromal vascular (SV) and fat cell (FC) fractions (n=3 for each group) from pig adipose tissue. The bars represent means±SEM. Statistical significance is indicated by * (P<0.05) and *** (P<0.001). The expression values are normalized by porcine cyclophilin (cyc) gene.
Figure 3.4. Expressions of DLK1, PPARγ, CCNG2, CDKN2C and PMP22 during pig primary adipocytes differentiation in vitro. Gene expressions were detected at initiation of differentiation (day 0) and after differentiation (day 3, 6 and 9) and normalized by porcine cyclophilin (cyc) gene. ANOVA followed by Fisher’s post hoc test was performed to examine the differences of gene expressions among the four time points (n=3 for each time point) which are indicated by different letters (a - c) if significant (P<0.05).
Figure 3.5. Gene expression in subcutaneous adipose tissues of 105-day fetal (Fetus), 6-day neonatal (6 d old) and 120-day pigs (Adult). A, mRNA expression of DLK1, PPARγ, CCNG2, CDKN2C and PMP22 during pig adipose development in vivo. Gene expressions were normalized by porcine cyc gene. ANOVA followed by Fisher’s post hoc test was performed to examine the differences in gene expressions among the three groups (n=3 for each group) which are indicated by different letters (a - b) if significant (P<0.05). B, Protein expression of CCNG2 in subcutaneous adipose tissues of 105-day fetal (F1, F2), 6-day neonatal (N1, N2) and 120-day pigs (A1, A2). Coomassie staining results are provided to show equal amounts of proteins loaded in different wells in the same gel. Protein from muscle of 20-d postnatal mouse (M+) was used as a positive control.
CHAPTER 4
DIFFERENTIAL EXPRESSION OF CELL CYCLE REGULATORS DURING HYPERPLASTIC AND HYPERTROPHIC GROWTH OF BROILER SUBCUTANEOUS ADIPOSE TISSUE

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ABSTRACT

Hyperplastic growth and hypertrophic growth within adipose tissue is tightly associated with cell cycle activity. In this study, CCNG2 and CDKN2C were found to be correlated with cell cycle inhibition during fat cell differentiation, whereas CCND3, CCNA1 and ANAPC5 were positively associated with cell cycle activity during fat cell proliferation after selection based on GEO datasets available on the NCBI website. The findings were validated through comparison of expressions of these genes among different tissues/fractions in broiler chickens and time points during primary cell culture using quantitative real-time PCR. Development of broiler subcutaneous
adipose tissue was investigated on embryonic days 15 and 17 and on post-hatch days 0, 5, 11 and 33 using H&E staining and PCNA immunostaining with DAPI counter stain. In addition, mRNA expressions of five cell cycle regulators, as well as precursor cell and adipocyte markers, were detected at those time points. The results suggest that cellular proliferation activity decreased as the fat pad grew, but a population of precursor cells seemed to be maintained until post-hatch day 5 despite increasing differentiation activity. Hypertrophic growth gradually intensified despite a slight cessation on post-hatch day 0 due to increased energy expenditure during hatching and delayed food access. From post-hatch day 5 to day 11, most of the precursor cells may have become differentiated. After post-hatch day 11, hyperplastic growth seemed to slow, while hypertrophic growth may have become dominant. This study provides further understanding about broiler fat tissue development, which is imperative for effective control of fat deposition.

**IMPLICATIONS**

This study provides a novel method of investigating the development of adipose tissue by combining conventional cellularity studies and cell cycle regulator expression. Using this method, we acquired more precise and comprehensive understanding of the development of broiler subcutaneous adipose tissue, which is essential for effective control of fat deposition in animals and production of meat with better quality. In addition, the study also presents an efficient strategy for combining bioinformatic tools such as GEO DataSets with molecular and cellular studies. Using this strategy, much time and effort can be saved to derive reliable conclusions.
INTRODUCTION

Understanding development of adipose tissue is crucial for curing obesity and controlling fat deposition in meat-producing animals to yield higher-quality meat with improved feed efficiency. Two processes of adipose tissue development – hypertrophy (enlargement of fat cell size) and hyperplasia (increase of fat cell number) – are generally acknowledged. These two developmental mechanisms are associated with fat cell proliferation and differentiation, ultimately controlling the cell cycle (Hausman et al., 2001). Most studies concerning cellularity of fat cells have suggested that hyperplastic growth occurs before hypertrophy to establish immature fat cells for lipid deposition (Jo et al., 2009). However, it is not yet clear the ways in which the two mechanisms are orchestrated at various time points.

Studies concerning fat cell size and number at specific ages or weights have been conducted in Athens-Canadian and commercial Cobb broiler chickens (Cartwright et al., 1986; Cartwright, 1988). Proliferation status was also examined by DNA mass and proliferating cell nuclear antigen (PCNA) immunostaining during the first 7 weeks following hatch (Guo et al., 2011). A previous study in our lab also compared the hypertrophic and hyperplastic growth from embryonic day 12 to post-hatch day 1 between Leghorn and broiler chickens (Chen et al., 2014). These studies indicated increasing fat cell size and fat cell number as the body weight increased, as well as decreasing rate of fat cell proliferation during post-hatch development. However, none of the studies examined both embryonic and post-hatch development of adipose tissue or investigated expression of cell cycle regulators during the development of adipose tissue.

In our previous study, we found three important cell cycle inhibitors – cyclin
G2 (CCNG2), cyclin-dependent-kinase inhibitor 2C (CDKN2C) and peripheral myelin protein 22 (PMP22) - in pig adipose tissue by utilizing the Gene Expression Omnibus (GEO) database and data from quantitative real-time PCR (qPCR) and western blotting (Zhang, et al., 2014). All three genes showed higher expression in mature fat cells than in stromal vascular cells, as well as increasing expression during adipocyte differentiation and development of adipose tissue, indicating a role in cell cycle inhibition during adipocyte differentiation. Therefore, the same study was conducted for these inhibitors in broiler chickens to help us characterize hyperplastic growth of broiler chicken adipose tissue. In response to the previous confirmation of high expression of cell cycle inhibitors in differentiated fat cells and increasing expression of cell cycle inhibitors during fat cell differentiation and adipose tissue development (Zhang et al., 2014), we hypothesized that there should be some important cell cycle activators that are highly expressed in preadipocytes and that have decreased expression during fat cell differentiation and adipose tissue development. In order to test this hypothesis, we explored the GEO datasets (GDS) and conducted qPCR in broiler chickens. To the best of our knowledge, this is the first study that combines cellularity study and cell cycle regulator expression during the development of broiler chicken adipose tissue.

MATERIALS AND METHODS

Data Sources and Processing

The data mining process using two microarray datasets (GEO DataSets: GDS596 and GDS 3142) was described in our previous report (Zhang, et al., 2014). Briefly, mean expression values from six tissues (heart, liver, lung, muscle, kidney and
adipose) were calculated, and genes were ranked in descending order based on the ratio between expression value in adipose tissue and mean expression value in the other five tissues. Finally, three cell cycle inhibitors with high ranks (CCNG2, CDKN2C and PMP22) and three cell cycle activators (CCND3, CCNA1 and ANAPC5) with low ranks in both datasets were selected.

The expression levels of the six genes were then further compared among different differentiation stages of preadipocytes in culture by analyzing GDS2743 and GDS2366. GDS2743 was generated to compare gene expression between undifferentiated and differentiating primary white preadipocytes from epididymal white adipose tissue of mouse. GDS2366 was generated to compare gene expression between undifferentiated and differentiated preadipocytes from subcutaneous adipose tissues in human. To determine gene expression pattern of the six genes during differentiation of adipocytes in vitro, GDS2659 was also analyzed, as it records mRNA expression at preconfluence, confluence and 1 day, 3 days, 7 days and 28 days after initiation of differentiation cocktail during differentiation of 3T3-L1 preadipocytes. The gene expression values were calculated as percentages compared to the maximum values in the GEO profiles.

**Experimental Animals**

Broiler eggs (Ross 708) were incubated and chickens were raised after hatching with free access to a broiler start diet (crude protein = 21.0%, metabolizable energy = 3,121 kcal/kg) provided by the Ohio Agricultural Research and Development Center (OARDC, Wooster, OH). The diet meets the NRC nutrient recommendations for chickens (NRC, 1994). During this process, total subcutaneous adipose tissues on the two thighs were collected using forceps after removing the skin and feathers with scissors at embryonic days (E) 15 and 17 and post-hatch days (P) 1,
5, 11 and 33 (n=5 for each age group) to study adipogenesis at different developmental stages. The fat pad location is shown in our previous paper (Chen et al., 2014). At P21, subcutaneous and visceral adipose tissue at the abdomen, thigh and pectoral muscles, heart, lung, liver and kidney were collected (n=4 for each tissue) to investigate tissue distribution of gene expression. For post-hatch chicks, euthanasia was conducted through CO₂ inhalation before tissue collection. All of the procedures were approved by The Ohio State University Institutional Animal Care and Use Committee. After tissue collection, all of the tissues were snap-frozen immediately in liquid nitrogen and stored at -80 °C for RNA isolation and quantitative real-time PCR (qPCR) (Lee et al., 2009; Lee et al., 2012).

**Histological Processing and Immunostaining**

In order to quantify the change in fat cell size and number of proliferating cells in the adipose tissue, Hematoxylin and Eosin (H&E) staining, as well as immunostaining of proliferating cell nuclear antigen (PCNA) with counterstain of 4’, 6-diamidino-2-phenylindole (DAPI), were conducted. Fat tissues from chickens of different age groups (n=5 for each group) were embedded in paraffin and H&E slides were made by the Goss Histology Lab. (Histology and Immunohistochemistry Core, OSU College of Veterinary Medicine, Columbus, OH) after being fixed with 10% neutral-buffered formalin for 24 to 48 h and dehydrated in 70% ethanol. Sections (6 μm) were cut with a microtome and mounted on glass slides for PCNA and DAPI staining and were rehydrated with a series of ethanol treatments (100%, 2×3 min; 95%, 2×3 min; 70%, 3 min and deionized water, 2×2 min) after paraffin was removed with xylene (3×5 min). After being equilibrated in Tris-buffered saline (TBS, pH 7.6; 3×10 min) and incubated in TBS containing 0.025% Triton X-100 and 1% nonfat dry milk for 30 min, tissue sections were then incubated overnight at 4 °C with a monoclonal PCNA
antibody (PC10, Santa Cruz Biotechnology, Santa Cruz, CA) diluted in a milk solution at 1:500 ratio and rinsed 10 times by TBS with 0.025% Triton X-100. Finally, the tissue sections were incubated for 30 min with Rhodamine conjugated goat anti-mouse secondary antibody at 1:500 dilution at room temperature and rinsed 10 times with TBS containing 0.025% Triton X-100. To visualize cell nuclei, sections were counterstained with 300nM DAPI (Invitrogen) in TBS for 10 min, and then rinsed 10 times with TBS. After being dehydrated in a series of increasing alcohol concentrations (70%, 2 min; 95%, 2×2 min; 100%, 2×3 min), sections were washed with xylene (2×3 min) and temporarily mounted with phosphate buffered saline (PBS). Stained slides were observed and imaged with a fluorescence microscope equipped with AxioCam MRc5 camera (Zeiss, Thornwood, NY). Average size of fat cells and percentage of PCNA positive nuclei were determined using ImageJ software (NIH ImageJ 1.47; http://imagej.nih.gov/ij/) from H&E stained sections and PCNA&DAPI stained sections, respectively. For H&E staining, 15 areas with 1000-2000 cells in total were randomly selected for each individual. The average cross sectional area (CSA) for each cell was calculated by dividing the total area by the total number of cells. For PCNA&DAPI staining, five sections were imaged for each individual, and the percentage of PCNA positive cells was obtained by dividing the number of cells stained by both DAPI and PCNA by the total number of cells stained by DAPI after the DAPI and PCNA images of the same section were merged.

Separation of Stromal-Vascular and Fat Cell Fraction

The stromal-vascular (SV) and adipocyte cell (FC) fractions were isolated from visceral adipose tissue at the abdomen of four 21-d-old chickens following the procedures described in our previous report (Deiuliis et al., 2006 and 2008). Briefly, the adipose tissues were minced with a razor blade after being washed with PBS and
were incubated at 37 °C with 5 mL of digestion buffer per gram of tissue containing 3.2 mg/mL of collagenase II (Sigma-Aldrich, St. Louis, MO) for 1 h in a shaking water bath (180 rpm). The suspension was then passed through a nylon cell strainer (BD Falcon, Franklin Lakes, NJ) with 100-μm mesh pores to remove undigested tissue debris. The filtered cells were further separated into FC (top layer) and SV pellets after centrifugation at 200×g for 5 min. Finally, the two fractions were collected for primary cell culture, as well as RNA isolation and subsequent qPCR analysis.

**Primary Stromal Vascular Cell Culture**

After collection of SV cells from four chickens, they were cultured according to a method described in our previous report (Oh et al., 2011). The cells were first washed with Dulbecco’s modified Eagle’s medium (DMEM) before centrifugation at 500×g for 10 min at room temperature. After centrifugation, the pellet was re-suspended in DMEM medium containing 10% fetal bovine serum (FBS) to a concentration of 6×10⁶ cells/mL. Cells were seeded in 12-well plates at a density of 3×10⁵ cells/mL in culture medium and maintained in a humidified incubator with 5% CO₂ at 37 °C until 50% confluence. Then, the cell medium was changed to contain 10% FBS, 50mM linoleic and oleic acids and 10 μg/mL of insulin in DMEM to induce adipocyte differentiation for 3 days. Cells were collected at day -1 (before differentiation), day 0 (differentiation initiation), and day 1, 2 and 3 (after differentiation) for RNA isolation and qPCR analysis.

**cDNA Synthesis and Quantitative Real-time PCR**

Tissue samples were homogenized in Trizol reagent (Invitrogen, Carlsbad, CA) with a Tissuemiser homogenizer (Fisher Scientific, Pittsburgh, PA) to isolate total RNA according to the manufacturer’s protocol. For primary cell culture, cells were
removed from plates using Trizol (Invitrogen) and vortexed to isolate total RNA according to the manufacturer’s instructions (Hassan et al., 2014). After being assessed via gel electrophoresis and a Nanodrop machine (NanoDrop Technologies, Wilmington, DE, USA), 1 μg of total RNA was used in reverse transcription (RT) to generate cDNA with M-MLV reverse transcriptase (Moloney murine leukemia virus RT, Invitrogen). RT conditions for cDNA amplification were 65 °C for 5 min, 37 °C for 52 min and 70 °C for 15 min. Gene expression of the six cell cycle regulators was quantified by SYBR Green real-time PCR with primers listed in Table 4.1. The primers with Tm around 61°C were designed using Primer Express Software (Applied Biosystems) to span genomic introns (>1Kb) to avoid amplification of possible genomic DNA contamination. The qPCR was performed on an ABI 7300 real-time PCR Instrument (Applied Biosystems, Foster City, CA). The PCR reaction consisted of 1 μL of cDNA, 0.5 μL of 10mM deoxynucleoside triphosphate mix (dNTP), 2.5 μL of GeneAmp 10×PCR buffer II containing 100mM Tris-HCl, PH 8.3 and 500mM KCl, 2 μL of 25mM MgCl₂, 0.5 nM of each of the forward and reverse primers, 0.385 μL of 0.2% Rox as reference dye, 0.034 μL of 0.2% SYBR green as detection dye, 0.125 μL of AmpliTaq Gold polymerase (Applied Biosystems) and UltraPure DNase/RNase-free distilled water (Invitrogen) up to 25 μL. Efficient amplification of real time PCR was confirmed by slope calculation of a standard curve and gel electrophoresis. The standard curve slope was determined using serial dilutions (1:1, 1:2, 1:4 and 1:8) of each template cDNA sample. The optimized thermal cycling procedure was confirmed by a normal amplification curve, one major peak in melting point analysis, and a single target band in gel electrophoresis. Correct PCR product and product size were assessed through a melting curve provided by qPCR software (Applied Biosystems) and gel electrophoresis. The conditions of qPCR were 95 °C for
10 min, 40 cycles of 94 °C for 15 s, 60 °C for 40 s, 72 °C for 30 s and 82 °C for 32 s. The relative level of expression of a target gene was calculated from the expression value determined by the ABI software using the comparative \(2^{-\Delta\Delta Ct}\) method for relative quantification (Livak and Schmittgen, 2001). The geNorm program of qbasePLUS software (Biogazelle, Zwijnaarde) was used to evaluate stability of four reference genes including β-actin, RPS13, OAZ1 and RPL27. Reference genes with average expression stability values lower than 1.5 were then selected for qPCR normalization. Finally, β-actin (gACTB) was selected as an internal control (housekeeping gene) for mRNA expression in different tissues and cell fractions and at different primary cell culture time points. Ribosomal protein S13 (gRPS13) was selected as an internal control for mRNA expression in different age groups (Serr et al., 2011). For the tissue comparison, one single relative quantity (ΔCt) was used to reflect variation in multiple tissues. However, for comparisons within the same tissue, we selected the one with lowest expression value as a calibrator. Gene expressions in different groups after normalization of the housekeeping gene were then displayed as fold increases compared to the calibrator. In order to examine separation of the stromal-vascular and fat cell fractions, differentiation of primary cells in vitro and development of adipose tissue in vivo, expressions of two adipocyte markers, adipose triglyceride lipase (ATGL) and fatty acid binding protein (FABP4), and one precursor cell marker, delta-like 1 homolog (DLK1), were also measured by qPCR. Primer sequences of chicken RPS13 (GenBank: NM_001001783), ACTB (GenBank: NM_205518), ATGL (GenBank: EU852334), FABP4 (NM_204290) and DLK1 (EU288039) have been described in our previous reports (Yang et al., 2013; Lee et al., 2009).
Statistical Analyses

For tissue distribution of gene expression, comparisons were performed by a mixed model (MIXED) procedure using SAS software (version 9.3, SAS Institute Inc., USA) with the DIFF option to detect significant differences between pairs of least squares means. Gene expressions between two groups were compared in SAS using the Student’s t-test. Multiple comparisons of gene expression at different time points during in vitro cell culture and in vivo development were carried out as one-way ANOVA followed by Fisher’s post-hoc test. P-values lower than 0.05 were treated as significant. All of the results are presented as least squares means with standard errors of the least squares means (SEM).

RESULTS

Data Analysis based on the Gene Expression Omnibus (GEO) Datasets

Using the same methodology and datasets as in our previous paper (Zhang et al., 2014), three cell cycle activators were selected that had relatively low expression in the adipose tissue of both human and mouse (Table 4.2). The ranks of CCND3, CCNA1 and ANAPC5, were 14,570, 13,622 and 9,835, respectively, in GDS596 (22,215 genes in total) and 32,066, 27,701 and 40,067, respectively, in GDS3142 (45,037 genes in total). Because the genes were ranked according to the ratio between gene expression in adipose tissue and that in the other five tissues, the low ranks of these genes are due to their low expression in adipose tissue. Although there is no significant difference in expression of CCNA1 among different tissues in mouse (Table 4.2), we still chose CCNA1 as one target gene because of its well-known involvement in the cell cycle and its decreased expression during fat cell differentiation in other microarray studies (Figure 4.1).
As indicated by analysis of GDS2743 and GDS2366, contrary to the lower expression of the three cell cycle inhibitors – CCNG2, CDKN2C and PMP22 – in undifferentiated preadipocytes in these datasets (Zhang et al., 2014), ANAPC5 showed higher expression in undifferentiated preadipocytes than in differentiating or differentiated preadipocytes in mouse (Figure 4.1A) and human adipose tissue (Figure 4.1B). The other two cell cycle activators also appeared to have higher expression in undifferentiated preadipocytes (Figure 4.1A and 4.1B).

Consistent with the findings in the previous two datasets, expression of the three cell cycle activators also showed an increasing trend, whereas that of the three cell cycle inhibitors showed a decreasing trend during differentiation of 3T3-L1 preadipocytes in GDS2659 (Figure 4.1C).

**Cellularity of Fat Cells during Development of Chicken Adipose Tissue**

Tissue collection data indicated that, as the broiler chickens grew, both body weight and leg fat pad weight expanded at an increasing rate. The percentage of fat pad weight relative to the body weight increased from E15 to D0 but kept decreasing from D0 to D33 (Table 4.3).

H&E staining of tissue sections of adipose tissue in different age groups of chickens showed a gradual increase in average cross-sectional area (CSA) of adipocytes from the embryonic stages to P11 with the greatest increase from E15 to E17 (121 μm²/cell/day, P<0.001) and the smallest increase from P0 to P5 (20 μm²/cell/day). However, from P11 to P33, the average fat cell size did not change (Figure 4.2).

PCNA immunostaining with counterstain of DAPI suggested that the proliferating cells gradually decreased during the growth of broiler chickens (Figure 4.3A). The percentage of PCNA positive cells at E15, E17, P0, P5 and P11 were 70%,
56%, 40%, 30% and 18%, respectively. The decreases in the percentage of PCNA positive cells between E15 and E17, P0 and P5, and P11 and P33 were significant (P<0.05) (Figure 4.3B).

Expression of Selected Genes in Various Tissues of Broiler Chickens

Although the qPCR data did not show statistical differences between fat and all the other tissues, all three cell cycle inhibitors tend to be highly expressed and all three cell cycle activators tend to be expressed at low levels, in both subcutaneous and visceral fat tissue at the abdomen. The three inhibitors showed lowest mRNA expression in the liver. However, highest expression of the three activators was not observed in the same tissues. CCND3 demonstrated the highest expression in thigh muscle and lung, and CCNA1 showed the highest expression in both thigh and pectoralis major muscle, whereas ANAPC5 exhibited the highest expression in the liver and kidney (Figure 4.4).

Expression of Selected Genes in Stromal Vascular (SV) and Fat Cell (FC) Fraction in 21-day Broiler Chickens

The DLK-1 gene, a marker for adipose-derived precursor cells, showed more than 5-fold greater expression in the SV fraction than in the FC fraction (P<0.01). On the other hand, FABP4 and ATGL, which are involved in free fatty acid transportation and mobilization, were expressed 16-fold (P=0.056) and 8-fold (P<0.05) higher, respectively, in the FC fraction than in the SV fraction. Because the SV fraction should consist mainly of precursor cells that can differentiate to fat cells under adipogenic conditions, whereas the FC fraction contains mature fat cells, the expression of the three marker genes indicates that separation of the two fractions was successful. Expression of the three cell cycle activators – CCND3, CCNA1 and ANAPC5 – was 3.6 (P<0.05), 6 (P<0.001) and 3-fold (P<0.0001) higher, respectively,
in the SV fraction than in the FC fraction, whereas expression of the two cell cycle inhibitors – CCNG2 and CDKN2C – was 2.7 (P=0.085) and 6 times (P<0.01) higher, respectively, in the FC fraction than in the SV fraction, indicating that cell cycle activity is high in the SV fraction and low in the FC fraction. However, unlike the other two cell cycle inhibitors, PMP22 showed an inverse pattern – more than 4-fold greater (P<0.01) expression in the SV fraction than in the FC fraction, indicating that PMP22 may possibly be involved with processes other than the cell cycle (Figure 4.5).

Expression of Selected Genes during Primary Cell Culture

During primary cell culture, the induction of differentiation brought about a 34% decrease (P<0.05) in expression of DLK1, but a two-fold increase (P<0.05) in expression of ATGL. From day 1 to day 3 after differentiation, DLK1 expression was at the lowest level, whereas ATGL remained at a continuously high level. Unlike ATGL, another fat cell marker – FABP4 – exhibited a constant increase after 1 day of differentiation. After 1 day of differentiation, there was a rapid increase in expression with more than a 9-fold increase between day 1 and day 2 (P<0.05), as well as a 2-fold increase between day 2 and day 3 (P<0.05) (Figure 4.6).

As was the case for FABP4, expression of the cell cycle inhibitors CCNG2 and CDKN2C also increased as the cells matured. Expression of CCNG2 was maintained at a low level before day 3 but displayed more than a 2-fold increase from day 2 to day 3 (P<0.05), whereas expression of CDKN2C increased every day (P<0.05) during the cell culture. However, PMP22 exhibited a unique pattern because it showed a decreasing trend from day 0 to day 2. Expression of both CCND3 and ANAPC5 increased from day 0 to day 1 (P<0.05). However, CCNA1 seemed to be more sensitive to differentiation induction, as it decreased immediately after the
induction of differentiation, and stayed at the low level until day 3 (P<0.05) (Figure 4.6).

Expression of Selected Genes during Development of Chicken Adipose Tissue

Expression of DLK1 was steady from E15 to P11 but decreased on P33 relative to P5. Expression of FABP4 and ATGL was low in the embryos and increased 9-fold (P<0.01) and 70-fold (P<0.0001) respectively from E17 to the time of hatch (P0). From P0 to P5, expression of FABP4 decreased by 38% (P<0.05) and expression of ATGL decreased by 75% (P<0.01). From P5 to P33, expression of all three genes seemed to exhibit a gradually decreasing trend as the chickens grew (Figure 4.7).

The five cell cycle regulator genes, other than CCNA1, were expressed at a low level in embryonic stages, but increased significantly on P0 or P5. For CCND3 and CDKN2C, the expression decreased significantly on P11 compared to P5 and remained at the same low level as in embryos until P33. For CCNG2 and PMP22, there was no significant change of expression from P5 to P33. Expression of ANAPC5 demonstrated a decreasing trend from P5 to P33. The expression on P33 was lower than on P5 but higher than on E15 and E17. Unlike the other genes, expression of CCNA1 was at a low level from E15 to P5 and increased to a high level on P11 and P33 (P<0.05) (Figure 4.7).

DISCUSSION

As indicated by Table 4.3, age is a significant factor for increases in fat cell size and decreases in the quantity of proliferating cells. Therefore, hyperplastic growth activity is attenuated, while hypertrophic growth is promoted during
development of adipose tissue. On the other hand, the close relationship between growth mechanism of fat tissues and adipocyte proliferation or differentiation makes study of fat cell proliferation and differentiation a potential route for better understanding and regulation of adipose tissue development. Due to the fact that broiler chickens have greater adipose weight than layers (Emmerson, 1997), minimizing their fat accumulation is advantageous to improve feed conversion and maximize productive efficiency of the industry. In this study, subcutaneous fat tissue from the broiler legs showed continuous accumulation with increasing speed as body weights increased; the same as for abdominal fat tissue in a previous report (Guo et al., 2011). Moreover, the proliferation rate on P11 and P33 indicated by PCNA immunostaining was also similar to that of abdominal fat tissue on P10 and P28 (Guo et al., 2011). However, unlike the continuous increase of fat cell size in abdominal adipose tissue, the increase of fat cell size in subcutaneous adipose seems to stop after P11, indicating adipose development and function varies depending upon the specific location. Therefore, investigation of the subcutaneous fat tissue growth in this study is useful for a comprehensive understanding of adipose tissue development.

Results of PCNA & DAPI staining and H&E staining indicate that the proliferation of fat cells decreased as fat cell size increased from E15 to P11, indicating that hypertrophic growth gradually became dominant, whereas hyperplastic growth slowed. Although the proliferation activity of fat cells showed a decreasing trend from E15 to P33, the average fat cell became smaller. This result seems to contradict the previous reports that fat cell size in abdominal fat tissue keeps increasing during this period (Hood, 1982; Guo et al., 2011), indicating developmental differences between subcutaneous and abdominal fat tissues.
Unlike previous reports that only studied cellularity in fat tissue (Hood, 1982; Guo et al., 2011; Chen et al., 2014), we also detected expression of some precursor cell and adipocyte markers, as well as selected cell cycle markers, for a better understanding of the developmental mechanisms of broiler subcutaneous fat tissue. Higher expression of the DLK1 gene in the SV fraction agrees with our previous paper (Shin et al., 2008), and supports previous reports that claim DLK1 as a precursor cell marker (Lee et al., 2003) and as an inhibitory factor in adipose development (Smas and Sul, 1993; Lee et al., 2003; Wang et al., 2006). Greater expression of FABP4 and ATGL in the FC fraction also agrees with our previous reports (Shin et al., 2008; Lee et al., 2009), validating their reliability as adipocyte markers.

Compared to the three cell cycle inhibitors selected in the previous paper (Zhang et al., 2014), all three activators showed contrary expression patterns in GDS596, GDS3142, GDS2743, GDS2366 and GDS2659, suggesting their different association with fat cell differentiation compared to the three cell cycle inhibitors. The different association is possibly related to their different functions during the cell cycle. The function of these genes within the cell cycle is well known. Cyclin A associates with cyclin-dependent kinase (cdk) 2 in the S phase and later with cdk1 in the G2 phase. The cyclin A-cdk1/2 complex is required for entry into and completion of the S phase, as well as entry into the M phase of the cell cycle (Malumbres et al., 2005). CCNA1, as one member of A-type cyclins, is reported to contribute to G1/S cell cycle progression in somatic cells (Ji et al., 2005). D-type cyclins are essential for G1 progression in the cell cycle. As a member of D-type cyclins, CCND3 is a subunit of the cyclinD-cdk4/6 complex. CyclinD-cdk4/6 phosphorylates the retinoblastoma protein and thus releases E2F transcriptional factors, which in turn regulate many
genes involved in DNA synthesis, as well as other cyclins downstream (Schafer, 1998). ANAPC5 is a subunit of anaphase-promoting complex/cyclosome (APC/C), which mediates timely degradation of cell cycle regulators in mitosis and in the G1 phase to promote cell cycle progression (Harper et al., 2002). In general, high expression of CCNA1, CCND3 and ANAPC5 may promote DNA synthesis and completion of mitosis during cell proliferation.

On the other hand, the three cell cycle inhibitors exhibit adverse functions during the cell cycle. CCNG2 is an unconventional cyclin that blocks G1/S transition (Aguilar et al., 2010); CDKN2C is a member of the INK4 family of cyclin-dependent kinase inhibitors and can block G1 progression by inhibiting cyclin-dependent kinase 4 or 6 (CDK4/6) (Hirai et al., 1995); PMP22 is a homologue of murine growth arrest-specific 3 (gas3), which is involved in G0 growth arrest (Welcher et al., 1991). Therefore, high expression of CCNG2, CDKN2C and PMP22 may promote cell cycle inhibition during cell differentiation.

Because the cell cycle becomes inhibited at the G0 phase after terminal differentiation of fat cells from preadipocytes (Tang & Lane, 2012), CCNA1, CCND3 and ANAPC5 showed higher expression in the SV section which mainly contains precursor cells, whereas CCNG2 and CDKN2C showed higher expression in the FC section. In primary cell culture, CCNA1 and ANAPC5 exhibited decreased expression after initiation of differentiation, whereas CCNG2 and CDKN2C exhibited increasing expression during adipocyte differentiation. Moreover, high expression of ATGL and FABP4 after 3 days of differentiation suggests that many stromal vascular cells have differentiated into fat cells, because ATGL and FABP4 are respectively involved in
triglyceride hydrolysis and fatty acid trafficking in maturing fat cells (Zimmermann et al., 2004; Serr et al., 2009).

The validation of involvement of CCNA1, CCND3, ANAPC5, CCNG2 and CDKN2C in adipocyte differentiation based on the GEO database and comparison of mRNA expression between different tissues and fractions, as well as different time points in primary cell culture, together with the marker genes for precursor cells and adipocytes, provide a possible way to analyze the early-stage development of broiler adipose tissue at the molecular level. Although all three cell cycle inhibitors showed lowest expression in liver as in pigs (Zhang et al., 2014), PMP22 exhibited a reverse pattern of expression from CCNG2 and CDKN2C. Therefore, its regulation in broilers may be different from that in pigs (Zhang et al., 2014). Due to limitations of the current studies regarding mRNA expression and the lack of literature concerning PMP22, we cannot make inferences about the regulation of PMP22 in broiler adipose tissue. Further study of the function of PMP22 is needed.

Although PCNA&DAPI staining indicated that the proliferation rate continued declining from the embryonic stage onward, expression of precursor cell marker, DLK1, reached its highest point at P5, which may indicate that a large proportion of precursor cells were maintained until P5. This conclusion is also supported by the increasing expression of two of the cell cycle activators – CCND3 and ANAPC5 – during this period. However, the increasing expression of the three cell cycle inhibitors also suggests that increasing differentiation activity may also occur in a group of preadipocytes to promote increase of adipocyte number when the other group is proliferating. Meanwhile, change in fat cell size shown by H&E staining also indicates a dramatic increase in cell size of the differentiated fat cells. Therefore, the
period before P5 is an important stage for both hypertrophic and hyperplastic growth with hyperplastic growth occurring before hypertrophic growth. In addition, P0 also seems to be an important time point because hypertrophic growth is undermined by dramatic hydrolysis of triglycerides as an energy supply during hatching (Lee et al., 2009; Chen et al., 2014). In addition, the starved condition immediately after hatch but before access to food may also aggravate the loss of fat (Lee et al., 2009; Chen et al., 2014). Therefore, the expression of ATGL dramatically increased in terms of both mRNA and at the protein level (Lee et al., 2009). Meanwhile, FABP4 also reached peak expression in both mRNA and protein (Chen et al., 2014), perhaps due to its function in transportation of cellular fatty acids during lipolysis as reported by Coe et al. (1999) and Verhoef et al. (2013).

After P5, the precursor cells were fewer in number as indicated by DLK1 expression, leading to less proliferation activity demonstrated by PCNA staining. Instead, many precursor cells may have become differentiated. However, as indicated by the decreased expression of CCNG2 and CDKN2C at P11, the differentiation activity of preadipocytes seems less intensive at this time because both genes have been shown to be promoted by fat cell differentiation (Zhang et al., 2014). Therefore, dramatic differentiation activity may occur sometime between P5 and P11. On the other hand, H&E staining showed that the size of fat cells did not increase from P11 to P33, whereas fat pad weight increased more than four times, indicating dramatic increase of mature fat cell number. With fewer precursor cells and consequently reduced potential for differentiation in the whole adipose tissue, one possible reason for the increase of mature fat cell number is that a population of immature differentiated cells becomes filled with lipid during this period. Therefore, hypertrophic growth should still be dominant during this period. Moreover, these
immature fat cells may be able to proliferate as reported by other researchers who defined these cells as small proliferative adipocytes (SPA) (Kajita et al., 2012; Hanamoto et al., 2013). Upregulation of CCNA1 may be related to this process. However, due to the complicated conditions in vivo, such as communication between different types of cells and interaction between different groups of regulators, we can only speculate tentatively from a cellular study and cell cycle point of view. To verify this speculation, further research needs to be done.

In summary, our research suggests that the period from embryonic stages to P5 may be an important period for development of broiler leg adipose tissue with both dramatic proliferation and differentiation of fat cell precursors as indicated by peak expression of both cell cycle activators and inhibitors. However, the proliferation activity declines, whereas the differentiation activity increases from E15 to P5 as evidenced by the fact that the number of precursor cells decreased. Therefore, by the period between P5 and P11, a large proportion of preadipocytes may have become differentiated. Meanwhile, the average cell size also exhibits a continuous increase. Therefore, during the period before P11, both hypertrophic and hyperplastic growth is active. However, during the period from P11 to P33, hypertrophic growth seems to contribute more to the development of subcutaneous fat tissue, because both proliferation and differentiation activities decline as indicated by a decrease of PCNA positive cells and expression of cell cycle regulators. Therefore, the conclusion of our study is consistent with most reports involving animal adipose tissue in that hyperplastic growth mainly occurs in the early developmental stage. In general, this study provides further understanding of fat development in broiler chickens and thus will be helpful for regulation of fat deposition in the broiler industry.
ACKNOWLEDGEMENTS

This work was supported by an Ohio Agricultural Research and Development Center (K. Lee) SEEDS grant and Agriculture and Food Research Initiative Competitive Grant no. 2010-65206-20716 from the USDA National Institute of Food and Agriculture.

AUTHOR CONTRIBUTIONS

### Table 4.1. Primer sequences of quantitative real-time PCR for selected cell cycle regulators

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession number</th>
<th>Forward (5’-3’)</th>
<th>Reverse (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCNG2</td>
<td>XM_420475.4</td>
<td>AGCTGTGGTGGTTCTCTGTGT</td>
<td>GATCGCTAGGAGGAGAACCTGTAAG</td>
</tr>
<tr>
<td>CDKN2C</td>
<td>XM_004936780.1</td>
<td>ACCATGCTGCAACTCTCAATTTG</td>
<td>TTAACGTCGCGTGGAAC</td>
</tr>
<tr>
<td>PMP22</td>
<td>NM_001277071.1</td>
<td>TCTTCGTCCTCACCACGTCA</td>
<td>GTGAAGAGCTGGCAGAAGACAG</td>
</tr>
<tr>
<td>CCND3</td>
<td>NM_001008453.1</td>
<td>CTGGATGCTGGAGGGTGTGT</td>
<td>TGATGGAGAATGTGAGCCCAAGA</td>
</tr>
<tr>
<td>CCNA1</td>
<td>XM_417097.4</td>
<td>AGAGGGAGCTGCAAGTTGTA</td>
<td>TCAGCAGGACCAATCTCA</td>
</tr>
<tr>
<td>ANAPC5</td>
<td>NM_00106187.1</td>
<td>GTTGATCCAGTGGAGGAAATC</td>
<td>CACTGGCCACCAAGACATG</td>
</tr>
<tr>
<td>Species</td>
<td>Gene (Rank)</td>
<td>Adipose</td>
<td>Muscle</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
<td>---------</td>
<td>--------</td>
</tr>
<tr>
<td>Human</td>
<td>CCND3(14570)</td>
<td>352±16 b</td>
<td>487±168 b</td>
</tr>
<tr>
<td></td>
<td>CCNA1(13622)</td>
<td>117±3 b</td>
<td>529±82 a</td>
</tr>
<tr>
<td></td>
<td>ANAPC5 (9835)</td>
<td>252±31 b</td>
<td>1109±328 a</td>
</tr>
<tr>
<td>Mouse</td>
<td>CCND3(32066)</td>
<td>118±8 b</td>
<td>128±7 b</td>
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<td></td>
<td>CCNA1 (27701)</td>
<td>75±1</td>
<td>87±7</td>
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<tr>
<td></td>
<td>ANAPC5(40067)</td>
<td>478±12 b</td>
<td>1398±89 a</td>
</tr>
</tbody>
</table>

Different superscripts indicate significant differences (P<0.05). A/O: ratio between average expression in adipose tissue and that in other tissues.
Table 4.3. Development of subcutaneous adipose tissue of broiler chickens at different ages

<table>
<thead>
<tr>
<th>Trait</th>
<th>Embryo 15</th>
<th>Embryo 17</th>
<th>Post-hatch 0</th>
<th>Post-hatch 5</th>
<th>Post-hatch 11</th>
<th>Post-hatch 33</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW (g)</td>
<td>17.5±0.6</td>
<td>24.4±1.2</td>
<td>47.8±1.3</td>
<td>92.6±2.5</td>
<td>248.0±5.9</td>
<td>1801.6±72.8</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>FPW (g)</td>
<td>0.08±0.01</td>
<td>0.14±0.01</td>
<td>0.27±0.01</td>
<td>0.41±0.03</td>
<td>0.85±0.08</td>
<td>3.70±0.48</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>FPP (%)</td>
<td>0.48±0.03</td>
<td>0.56±0.02</td>
<td>0.57±0.01</td>
<td>0.44±0.04</td>
<td>0.34±0.03</td>
<td>0.21±0.02</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

* Different superscripts indicate significant difference (*P*<0.05). BW: Body weight; FPW: Fat pad weight; FPP: Fat pad weight percentage.
Figure 4.1. Gene expression analysis of selected cell cycle regulators based on microarray datasets obtained from NCBI website.  

A, Expression comparison of the three cell cycle activators between undifferentiated (EU, cultured for 4 days, n=8) and differentiating (ED, cultured for 7 days, n=6) preadipocytes from mouse epididymal adipose tissue based on GDS2743.  

B, Expression comparison of the three cell cycle activators between undifferentiated (SU, n=3) and differentiated (SD, n=3) preadipocytes isolated from subcutaneous adipose tissue of human based on GDS2366.  

C, Expression of the selected cell cycle activators and inhibitors at preconfluence (PC), confluence (CF), and day (D) 1, 2, 3, 4 and 7 after induction of differentiation of 3T3-L1 preadipocytes in GDS2659. The Y axis is the percentage of gene expression compared to a maximum value in the GEO profile. Each bar in the histograms represents mean ± SEM and statistical significance of Student’s t test is indicated by * (P<0.05) and ** (P<0.01).
Figure 4.2. Change of fat cell size during development of chicken adipose tissues. A, Histological images of broiler leg subcutaneous adipose tissue on embryonic day 15 (E15) and day 17 (E17), post-hatch day 0 (P0), 5 (P5), 11 (P11) and 33 (P33) in 50 × objective lens. Scale bar = 100 μm. B, Cross sectional area (CSA) of adipocytes in subcutaneous fat of broiler chickens on E15 and E17, P0, P5, P11 and P33 (n=5 for each group). Each bar indicates mean±SEM for one time point with 15 sections for each individual.
Figure 4.3. Quantification of proliferating cells during development of chicken adipose tissues. **A**, Representative pictures of proliferating cell nuclear antigen (PCNA, red) immunostaining with nuclear counterstain of 4',6-diamidino-2-phenylindole (DAPI, blue) on embryonic day 15 (E15) and day 17 (E17), post-hatch day 0 (P0), 5 (P5), 11 (P11) and 33 (P33) in 200× objective lens. Scale bar = 50 μm. PCNA positive cells were counted as overlap of two color channels. **B**, Percentage of PCNA positive cells in subcutaneous fat of broiler chickens on E15 and E17, P0, P5, P11 and P33 (n=5 for each group). Each bar indicates mean±SEM for one time point with at least 5 sections for each individual.
Figure 4.4. mRNA expression of six selected cell cycle regulators in 21-day broiler chickens. Total RNA was isolated from subcutaneous (SQ) and visceral fat tissue at the abdomen (AF), thigh (TM) and pectoral (PM) muscle, heart (H), lung (Lu), liver (Li) and kidney (K). Expression of the three genes was measured by quantitative real-time PCR with Chicken β-actin (gACTB) as a control for normalization. Bars represent means ± SEM. A mixed model with DIFF option in SAS 9.3 was used to compare expression in different tissues (n=4 for each tissue). Different letters above the bars indicate significant differences (P<0.05).
Figure 4.5. Relative gene expression of DLK1, FABP4, ATGL and six cell cycle regulators in stromal vascular (SV) and fat cell (FC) fractions (n=4 for each group) from chicken adipose tissue. Each bar represents mean ± SEM. Statistical significance is indicated by * (P<0.05), ** (P<0.01) and *** (P<0.001). The expression values are normalized by gACTB gene.
Figure 4.6. Expression of DLK1, FABP4, ATGL and six cell cycle regulators during chicken primary adipocyte differentiation in vitro. mRNA expression was detected at 1 day before differentiation induction (day -1), initiation of differentiation (day 0), and 1, 2 and 3 days after differentiation induction and normalized by gACTB gene. Differences in gene expression among the four time points (n=4 for each time point) were examined using ANOVA followed by Fisher’s post-hoc test. Significant differences (P<0.05) between different time points are indicated by different letters (a - e).
Figure 4.7. Relative expression of DLK1, FABP4, ATGL and six cell cycle regulators during *in vivo* adipose development of broiler chickens. mRNA expression was detected in subcutaneous adipose tissue on embryonic day 15 (E15) and day 17 (E17), post-hatch day 0 (P0), 5 (P5), 11 (P11) and 33 (P33) and normalized by chicken ribosomal protein S13 (gRPS13) gene. ANOVA followed by Fisher’s post-hoc test was performed to examine the differences in gene expression among the six groups (n=4 for each group), which are indicated by different letters (a - d), if significant (P<0.05).
CHAPTER 5

IDENTIFICATION OF CTLA2A, DEFB29, WFDC15B, SERPINA1F AND MUP19 AS NOVEL TISSUE-SPECIFIC SECRETORY FACTORS IN MOUSE

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ABSTRACT

Secretory factors in animals play an important role in communication between different cells, tissues and organs. Especially, the secretory factors with specific expression in one tissue may reflect important functions and unique status of that tissue in an organism. In this study, we identified potential tissue-specific secretory factors in the fat, muscle, heart, lung, kidney and liver in the mouse by analyzing microarray data from NCBI’s Gene Expression Omnibus (GEO) public repository and searching and predicting their subcellular location in GeneCards and WoLF PSORT, and then confirmed tissue-specific expression of the genes using semi-quantitative PCR reactions. With this approach, we confirmed 11 lung, 7 liver, 2 heart, 1 heart and muscle, 7 kidney and 2 adipose and liver-specific secretory factors. Among these...
genes, 1 lung-specific gene - CTLA2A (cytotoxic T lymphocyte-associated protein 2 alpha), 3 kidney-specific genes - SERPINA1F (serpin peptidase inhibitor, Clade A, member 1F), WFDC15B (WAP four-disulfide core domain 15B) and DEFB29 (defensin beta 29) and 1 liver-specific gene - MUP19 (major urinary protein 19) have not been reported as secretory factors. These genes were tagged with hemagglutinin at the 3’end and then transiently transfected to HEK293 cells. Through protein detection in cell lysate and media using western blotting, we verified secretion of the 5 genes and predicted the potential pathways in which they may participate in a specific tissue through data analysis of GEO profiles. In addition, alternative splicing was detected in transcripts of CTLA2A and SERPINA1F and the corresponding proteins were found not to be secreted in cell culture media. Identification of novel secretory factors through the current study provides a new platform to explore novel secretory factors and a general direction for further study of these genes.

INTRODUCTION

The secretory factors are a large group of proteins synthesized by ribosomes bound to rough endoplasmic reticulum (ER). During the process of protein synthesis, the proteins are directed from the cytosolic face of the ER membrane to the ER lumen by the ER signal sequences at the N-terminus of the proteins (Corsi and Schekman, 1996). The proteins in the ER lumen are subsequently packaged into transport vesicles that fuse with the cis-Golgi vesicles. Cis-Golgi vesicles then move toward the plasma membrane and change to trans-Golgi cisternae (Glick and Malhotra, 1988). Some secretory factors such as hormones are stored in secretory vesicles and are only released upon triggers of hormonal or neural signals. Other secretory factors such as those found in the extracellular matrix are continuously secreted and exist in all cell
types (Lodish et al., 2000).

Due to the ubiquitous property of secretory factors, they play an important role in various cells, organs and systems in animals. As increasing numbers of secretory factors are explored, functions of one cell type and tissue and connections between different cell types and tissues become further understood and complex networks in living organisms become extensively revealed. For example, fat tissue was once regarded only as a lipid reservoir for excess energy. However, since the discovery of leptin by Zhang et al. (1994), perceptions about fat tissue have gradually changed. Leptin is a protein secreted by mature adipocytes, and can regulate food intake and body fat mass by binding to its receptor in the hypothalamus. Since the identification of leptin, more than 100 secretory factors have been identified that are produced and released by adipose tissue (Hauner, 2005). Among these factors, some adipose-specific secretory proteins such as adiponectin, resistin and visfatin are involved in the immune system (Lago et al., 2007), while some proteins such as angiotensinogen (Massiéra et al., 2001) and plasminogen activator inhibitor type I (Alessi et al., 1997) are related to vascular function. Therefore, adipose tissue is now thought of, not only as a lipid-storing organ, but also as an endocrine organ that maintains intensive cross talk with other organs.

Since tissue-specific expression of one novel secretory factor usually indicates novel function of that tissue, it is necessary to explore novel tissue-specific secretory factors. Tissue specific expression of various genes can be detected by microarrays with measurements of transcript abundance in various tissues. There are thousands of microarray data records from various studies with open access in the Gene Expression Omnibus (GEO) database on the NCBI website, providing a useful tool to explore tissue-specific genes and predict their functions (Barrett et al., 2005). In our previous
study we successfully selected some novel tissue-specific genes in the human and mouse and predicted their potential function by taking advantage of this database (Song et al., 2013). The subcellular location of various proteins can also be found in some databases such as GeneCards (Rebhan et al., 1998) and UniProt (Wu et al., 2006) or predicted by WoLF PSORT (Horton et al., 2007). These powerful bioinformatic tools greatly facilitate the selection of novel secretory factors with tissue specific expression.

The objective of this study is to identify and evaluate novel tissue-specific secretory genes in the fat, muscle, heart, lung, liver and kidney in the mouse by performing microarray data analysis, literature search, protein location information search and prediction, semi-quantitative PCR analysis and western blotting after transfection of expression vectors in cell culture. With the bioinformatics approach developed by Song et al. (2013), we detected novel tissue-specific genes in the 6 tissues and filtered potential secretory genes by searching and predicting protein location. After confirmation of tissue-specific expression through semi-quantitative PCR analysis, 30 genes were identified in 6 tissues in adult mice. Among them, 11 genes are lung-specific; 7 genes are liver-specific; 2 genes are heart-specific; 1 gene is muscle and heart-specific; 7 genes are kidney-specific and 2 genes are adipose and liver-specific. After the literature study, 5 novel tissue-specific secretory genes were discovered: CTLA2A (cytotoxic T lymphocyte-associated protein 2 alpha) in the lung, SERPINA1F (serpin peptidase inhibitor, Clade A, member 1F), WFDC15B (WAP four-disulfide core domain 15B) and DEFB29 (defensin beta 29) in the kidney, and MUP19 (major urinary protein 19) in the liver tissue. The secretory properties of these proteins were detected by western blot analysis using cell lysate and medium after transient transfection of expression vectors to HEK293 cells. The biological activities
in which these genes may participate in the specific tissues were predicted according to GEO profiles under different physiological and pathological conditions. This study provides a novel strategy combining bioinformatics tools and molecular technologies for exploration of novel tissue-specific secretory genes.

**MATERIALS AND METHODS**

**Experimental Animals**

Mice raised in a mouse housing facility at The Ohio State University with free access to feed were euthanized by CO₂ inhalation and subsequent cervical dislocation at 3 months of age. Then white adipose tissue, muscle, heart, lung, liver and kidney were harvested (n=3). All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of The Ohio State University. After tissue collection, all of the tissues were snap-frozen immediately in liquid nitrogen and stored at -80°C for total RNA isolation.

**Data Mining and Literature Search**

In order to find the secretory factors that are highly expressed in the adipose tissue, muscle, heart, lung, liver and kidney in the adult mouse, the microarray expression records in GDS3142, which is one Gene Expression Omnibus DataSet (GDS) available on the NCBI web site, were analyzed following the methods proposed by Song et al. (2013). In brief, expression records for each gene in the 6 different tissues were derived and ratios were calculated between expression in each tissue and average expression in the other 5 tissues; so there was one list of ratios for each tissue. After ranking the genes in descending order according to each list of ratios, the top 200-300 genes were selected for each tissue. For genes with several
spots on the microarray, only one record was kept. Then, each of these genes was checked in Genecards (http://www.genecards.org/) and UniProt (http://www.uniprot.org/) and predicted by WoLF PSORT (http://www.genscript.com/psort/wolf_psort.html) to see whether the corresponding protein was secreted or not. Finally, only the secretory genes were kept and a literature search was conducted in Pubmed (http://www.ncbi.nlm.nih.gov/pubmed) and Google Scholar (http://scholar.google.com/) to check whether there are reports concerning high expression of each gene and secretion of the protein in certain tissues. After the 5 novel secretory factors were selected, their functions in specific tissues were further predicted based on analysis of various GEO profiles. Function of CTLA2A was predicted based on GDS3950, GDS4582, GDS4914 and GDS2709; function of MUP19 was predicted based on GDS1261, GDS1053, GDS279, GDS1517 and GDS1374; function of SERPINA1F was predicted based on GDS2031, GDS4316 and GDS3675; function of WFDC15B was predicted based on GDS2030, GDS4316, GDS3675, GDS2817, GDS1583 and GDS4449; and function of DEFB29 was predicted based on GDS2031, GDS4316, GDS1583, GDS4839 and GDS3612.

**cDNA Synthesis and Semi-quantitative PCR Analysis**

For the genes whose tissue-specific expressions are not reported in existing publications, we conducted a semi-quantitative PCR to detect their expression in different tissues. Total RNA was isolated from the adipose tissue, muscle, heart, lung, liver and kidney of the adult mice using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and then reverse-transcribed to cDNA using moloney murine leukemia virus (M-MLV) reverse transcriptase (Invitrogen) and oligo dT. The conditions for reverse transcription were 65°C for 5 min, 37°C for 50 min and 70°C for 15 min. After the RNA was reverse-transcribed to cDNA, PCR was conducted with 1 μL of cDNA, 0.2
μL of 10mM deoxynucleotide triphosphate mix (dNTP), 1 μL of 10× ThermopoIII (Mg-free) reaction buffer, 0.2 μL of 100 mM MgSO₄, 0.2 nM of each of the forward and reverse primers, 0.05 μL of Taq DNA polymerase (New England BioLabs, Ipswich, MA, USA) and nuclease-free water up to 10 μL. The cycling parameters for PCR were 95°C for 1 min, followed by 32 to 40 cycles of 94°C for 30 s, 58°C for 30 s and 72°C for 40 s with a final elongation for 10 min. To ensure that equal amounts of cDNA were added to the PCR reaction for different tissues, expression of the cyclophilin (CYC) gene was used as a control and cDNA was diluted until the PCR products of CYC showed similar brightness for different tissues in agarose gel electrophoresis. After the equalization of cDNA, PCR was conducted for the other genes. The PCR primers for all of the genes are listed in Table 5.1.

**Vector Construction and Cell Transfection**

The subcellular locations of 5 of the selected genes were not found in the literature. To verify secretion of their encoding proteins from cells, we constructed plasmids containing target genes to transfect cells to detect the location of expressed protein. First, coding sequences of these genes were amplified with primers shown in Table 5.2. For CTLA2A, 2 forward primers were used to amplify 2 transcripts with alternative start codons. Hemagglutinin (HA) tag sequence, which is underlined in the table, was linked to the 5’ end of all of the reverse primers to add the HA-tag to the C terminal of the target proteins. After gel extraction of the PCR product, the amplified sequences were ligated to the pCR2.1 vector (Invitrogen) using T4 DNA ligase (New England BioLabs). Then, the plasmid with an insertion of each PCR product was transformed to *E.coli* cells, which were then spread over plates containing 5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside (X-gal). Because the lacZ gene in the plasmid is disrupted by inserted sequences, the colonies were white, whereas, the
plasmids with no insertion had intact lacZ genes expressing β-galactosidase, and its reaction with X-gal stained the colony in a blue color. After selection of positive colonies and incubation in Luria-Bertani (LB) broth, plasmids were extracted using a miniprep Kit (QIAGEN, Valencia, CA, USA) and the direction of inserted genes was verified through restriction enzyme digestion and PCR with M13 primers and the primers of inserted genes. The plasmids with correct orientation of insertion were then digested with HindIII and XhoI (New England BioLabs) and ligated to a pcDNA 3.1 vector, which was also digested with the same enzymes. The constructed vectors were then transformed to E. coli and insertion was verified by digestion with HindIII and XhoI and subsequent electrophoresis in 0.6% agarose gels. After confirmation of the correct ligation, the constructed vectors were extracted using a plasmid midi kit (QIAGEN).

**Transient Transfection of Plasmids in HEK 293 Cells**

One day before transfection, human embryonic kidney 293 (HEK293) cells were centrifuged and resuspended in Dulbecco’s modified Eagle’s medium (DMEM, Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS, Gibco). On the day of transfection, the cells were transfected with pcDNA3.1 vector containing HA-tagged putative secretory proteins or the same vector without an insert using lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. The medium was changed to fresh complete growth medium after 6 h. Then, the cells were incubated for 48 h prior to protein extraction for western blot analysis.

**Western Blot Analysis**

After collecting the cell medium, proteins expressed in HEK293 cells were extracted using ice-cold 1× lysis buffer (125 mM Tris-HCl pH 6.8, 0.5% SDS). Proteins in the medium were precipitated using Tricholoroacetic acid (TCA). In brief,
the medium was spun down at 1,200 × g to pellet the cell debris and the supernatant was centrifuged at 16,000 × g after adding TCA (25% v/v). The protein pellet was washed 2 times with ice-cold acetone, and then resolved in 2X loading buffer. Equal amounts of proteins were loaded and separated by 10-15% SDS-PAGE and wet-transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, CA). After blocking for 30 min in Tris-buffered saline with Tween-20 (TBST) containing 4% nonfat dry milk, the membranes were incubated overnight at 4°C with mouse anti-HA-tag (1:3000; Cell Signaling, Danvers, MA) antibody as a primary antibody. Horseradish peroxidase-linked anti-mouse IgG (1:5000; Cell Signaling) was used as a secondary antibody for an 1-h incubation at room temperature. After detecting signals with ECL plus reagents (GE Healthcare Biosciences, Pittsburgh, PA), proteins were visualized by exposure of the membranes to X-ray films (GE Healthcare Biosciences).

Statistical Analysis

A mixed model (MIXED) procedure was performed for comparison among more than two groups using SAS software (version 9.3, SAS Institute Inc., USA). The DIFF command was used to detect significant differences between pairs of least squares means. Student’s t-test was performed in JMP 10 to compare gene expression between two groups. Differences with p-values lower than 0.05 were treated as significant. Statistical values are presented as least squares means with standard errors of the least squares means (SEM).

RESULTS

Discovery of Tissue-Specific Secretory Genes

Six Excel spreadsheet files were generated with the top 200-300 genes in terms of highest expression in the fat, muscle, heart, lung, liver and kidney in the
adult mice based on GDS3142. After conducting searches of the Genecards and Uniprot websites, protein subcellular localization prediction in WoLF PSORT and literature searches in Pubmed and Google Scholar, non-secretory genes and known secretory genes with reported tissue-specific expression consistent with our findings in GDS3142 were deleted and 31 genes were kept with their average expression values in 6 different tissues from GDS3142 listed in Table 5.3. Among these genes, 11 are more highly expressed in the lung, 7 genes are more highly expressed in the liver; 2 are more highly expressed in the heart, and one – (GPC1) shows nearly equal expression in heart and muscle; one gene (DHRS7C) shows the highest expression in the muscle without much difference in expression in the heart; 7 genes are expressed more highly in the kidney; 2 genes show the highest expression in adipose with little difference in expression in the liver. The expression patterns of these genes were confirmed by semi-quantitative PCR and gel electrophoresis. As shown in Figure 5.1, both adipose-specific genes showed similar expression in adipose tissue and liver; DHRS7C and GPC1 showed similar expression in muscle and heart; whereas, all other genes only showed detectable expression in one specific tissue.

As shown in Table 5.3, 25 of the selected genes have been reported to produce extracellular proteins. In the lung-specific genes, CHIL4 encodes Chitinase-like protein 4, which is also called secreted Ym2 protein. It has been identified in respiratory secretions and reported to be increasingly expressed in the allergic lung (Webb et al., 2001). Neuron-Derived Neurotrophic Factor (NDNF) was reported to be a secreted glycosylated protein by Kuang et al. (2010), but its secretion was reported in the brain and neuron cell culture rather than in the lung. NPNT encodes nephronectin, which is an extracellular matrix protein and has been reported to be secreted from the embryonic kidney (Brandenberger et al., 2001) and the MC3T3-E1
cell line (Kahai et al., 2009). PCOLCE2 encodes procollagen C-endopeptidase enhancer 2, a collagen-binding protein that was reported to be secreted in transfected human 293 EBNA-1 cells (Steiglitz et al., 2002). Leucine-rich glioma inactivated 3 (LGI3) is a secreted leucine-rich repeat protein. It was reported to be highly expressed in the brain and, thus, has been extensively studied in the nervous system (Park et al., 2010), but there is no report in lung. EGFL6 encodes an epidermal growth factor–like protein, which has been reported to be secreted in an osteoblastic-like cell culture (Chim et al., 2011). LYZ1 encodes lysozyme, which has been reported to be primarily secreted by serous cells of submucosal glands in the lung (Dajani et al., 2005).

Hypoxia Inducible Lipid Droplet-Associated (HILPDA) was reported to be secreted in COS7 cell culture, but its expression was only reported in fetal kidney and renal cell carcinoma (Togashi et al., 2005). SEMA3G encodes semaphorin 3G - a member of murine class 3 semaphorin. It is expressed in the lung and kidney and secreted in HEK293-T cells (Taniguchi et al., 2005). SPON1 encodes F-spondin, which is an extracellular matrix protein secreted in the floor plate in the embryonic neural tube (Klar et al., 1992). Its secretion and function have been extensively studied in the nervous system. However, there are few reports about this gene in the lung.

Among the liver-specific genes, SERPINA1C encodes one isoform of murine a-1-antitrypsin, which is produced in the liver, secreted in serum and circulated to the lung (Song et al., 1998). Secretion of SERPINA1C has also been reported by Paterson and Moore (1996). CPB2 encodes plasma Carboxypeptidase B2, which is secreted from the liver (Eaton et al., 1991). Both ITIH1 and ITIH3 encode one heavy chain of inter-alpha-trypsin inhibitor (ITI), which is one plasma protease inhibitor, and is synthesized and secreted in a hepatoma HepG2 cell culture (Bourguignon et al., 1989) and COS7 cell culture (Martin-Vandelet et al., 1999). C8B
encodes the β subunit of complement component C8, which is a component of the membrane attack complex (MAC) and has been reported to be secreted by hepatocytes into serum. The a, b and g subunits normally associate with each other before secretion (Ng et al., 1987). KLKB1 encodes plasma kallikrein, which is synthesized in the liver (Borges et al., 1981).

Among heart-specific genes, FNDC5 encodes fibronectin type III domain-containing protein 5, which is a membrane protein proteolytically cleaved and secreted as a hormone peptide – Irisin. Irisin is mainly secreted by cardiac and skeletal muscle, with the former producing more than the latter (Kuloglu et al., 2014). IL15 encodes interleukin 15, whose production has been detected in various tissues. This gene produces 2 different isoforms through alternative splicing. The 21-aa signal isoform mRNA is expressed in the heart, thymus, appendix and testis, translated efficiently but not secreted. The 48-aa signal isoform mRNA is expressed in skeletal muscle, placenta, heart, lung, liver, thymus and kidney, translated less efficiently and secreted at a low level (Fehniger and Caligiuri, 2001). GPC1 encodes glypican-1, which is a cell membrane heparan sulfate proteoglycan. It is attached to the cell membrane by a glycosylphosphatidylinositol (GPI) linkage. The GPI anchor can be cleaved and thus lead to secretion of glypican-1 or shedding into the extracellular matrix (Velleman et al., 2013).

Among the kidney-specific genes, SPINK3 encodes pancreatic secretory trypsin inhibitor in the mouse. It is produced in acinar cells in the pancreas and secreted with digestive enzymes into secretory granules (Wang et al., 2008). However, its secretion in the kidney has not been reported. C1QTNF3 encodes C1q/TNF-related Protein-3, which is an adipokine and, thus has been studied extensively in adipose tissue and cell lines (Peterson et al., 2010). However, it is rarely reported in the kidney.
CGREF1, which is also known as CGR11, encodes hydrophobestin. This protein has been reported to be secreted in MtT/S cell culture (Devnath et al., 2009). SECTM1B encodes secreted and transmembrane protein 1. This protein shows a perinuclear Golgi-like pattern; its N-terminal domain was reported to be secreted in peripheral blood leukocytes and breast cancer cell lines (Slentz-Kesler et al., 1998). However, there are no reports about this gene in the kidney.

As shown by semi-quantitative PCR, both LRG1 and SERPINA3N are highly expressed in both liver and adipose tissue (Figure 5.1). However, they are only reported to be secreted in liver. Leucine-Rich Alpha-2-Glycoprotein (LRG1) is a plasma glycoprotein primarily produced in the liver (Shirai et al., 2009). It is also synthesized and secreted by ovarian cancer cells (Andersen et al., 2010). SERPINA3N is one isoform of murine α-1 antichymotrypsin (ACT), which has also been reported to be highly expressed in the liver. ACT is primarily synthesized and secreted by hepatocytes and released in plasma. In addition, SERPINA3N is reported to be secreted in Sertoli cell cultures (Sipione et al., 2006).

Among the remaining 6 genes, DHRS7C is located in the sarcoplasmic reticulum of the heart and skeletal muscle (Lu et al., 2012), although it is predicted to be secreted by WoLF PSORT. The other 5 genes - CTLA2A in the lung, MUP19 in the liver and WFDC15B, SERPINA1F and DEFB29 in the kidney have not been reported to be secreted in currently available publications.

**Identification of Alternative Splicing of CTLA2A and SERPINA1F**

To detect whether proteins translated by the 5 genes are secreted or not, the open reading frame (ORF) of each gene was amplified by a forward primer at the start codon and a reverse primer linked with HA-tag sequence. Alternative splicing of CTLA2A (NM_007796 and NM_001145799) and SERPINA1F (NM_026687 and
NM_001164742) were identified in GenBank. As shown in Figure 5.2A, there are alternative start codons near the end of exon 1 and beginning of exon 2, whereas the alternative start codon in exon 2 only occurs together with the alternative donor site in intron 1, resulting in a loss of 24 N-terminal amino acids. However, the amount of this short isoform is too small to be amplified by PCR. Unlike CTLA2A, the alternative splicing of SERPINA1F occurs within an exon rather than an intron. The ORF of SERPINA1F starts from the second base pair in the second exon and ends at 186 bp in exon 5. As shown in Figure 5.3A, in-frame alternative splicing occurs in the region transcribed from exon 3, which contains an alternative acceptor site, resulting in deletion of 165 nt in mRNA and 55 amino acids in protein.

The alternative splicing in both genes fits the classical splicing model outlined by Keller and Noon (1984) and Padgett et al. (1986). In the classical model, the consensus sequence at the donor site should usually be AG at the 3’ end of an exon followed by GT at the beginning of the adjacent intron. This feature is located not only at the 5’ end of intron 1, but also 21 bp from the 5’ end of intron 1 of CTLA2A, making alternative splicing feasible. On the other hand, the consensus sequence at the acceptor site is usually AG at the 3’ end of an intron followed by G at the beginning of adjacent exon. The sequence CTA/GAC/T at the branching point should be 20-50 base pairs from the 3’ splice site. These features are located not only in the 3’ end but also in the middle area of exon 3 in Serpina1f, which facilitates the alternative splicing.

**Detection of Novel Secretory Genes in HEK293 cell culture**

Western blotting demonstrates that CTLA2A with full-length amino acids is secreted as it was detected in both cell lysate and conditioned media. However, the short isoform with deletion of N-terminal amino acids is not secreted as predicted by
WoLF PSORT (Figure 5.2B), because it lacks signal peptide. For SERPINA1F, the long isoform is detected in both cell lysate and conditioned media, indicating that it is secreted outside the cells. However, the short isoform, which lacks 55 amino acids in the middle region of the protein, is not secreted, because it was only detected in cell lysate (Figure 5.3B). WFDC15B, DEFB29 and MUP19, which do not have alternative splicing, are also detected in both cell media and lysate (Figure 5.4), indicating their secretion out of cells. Finally, for the cells transfected with empty vectors, no protein was detected. This validates the successful insertion of target genes into the vector and expression of target genes in HEK293 cells.

**GEO Data Analysis of Novel Secretory Genes**

For the 5 novel secretory genes, there are few reports about their temporal and spatial expression, as well as function, in the tissues where they are highly expressed. In order to understand potential functions of these genes, gene expressions were compared between different age groups, different tissue sections and different treatment groups or genetic lines by analyzing GEO profiles.

Expression of CTLA2A in the lung seems to be affected by bacterial infection and other injuries. As can be seen from Figure 5.5A, GDS3950 shows that CTLA2A expression exhibits an increasing trend during development of the mouse lung except for a slight decrease on postnatal d 10. In particular, there is a dramatic increase in expression after the mouse is born (P<0.0001). GDS4583 shows that expression of CTLA2A in the lungs of C57BL/6 mice increased 1.6 times (P<0.05) after intratracheal instillation of *Escherichia coli* (*E. coli*) (Figure 5.5B). In another case, GDS4582 suggests that CTLA2A expression is promoted more than 2 times (P<0.05) 24 h after infection by wildtype *Staphylococcus aureus* (*S. aureus*). On the other hand, deletion of Alpha-hemolysin (Hla) - an essential lethal factor secreted by *S. aureus*-
reduced expression of CTLA2A to a medium level (Figure 5.5C). In GDS4914, expression of CTLA2A in the lungs of C57BL/6 mice increased 1.3 times (P<0.01) after exposure to 50 ppm of arsenate for 90 d compared to the control group (Figure 5.5D). A similar change occurred in GDS2709 as well (Figure 5.5E). In a rat strain resistant to ventilator-associated lung injury (VALI), expression of CTLA2A is significantly higher (P<0.05) than that in a strain sensitive to VALI under the same treatment. In the VALI sensitive strains, high tidal volume ventilation induced expression of CTLA2A 1.4 times (P<0.001) compared to the control group.

The correlation between MUP19 and the GH/IGF-1 signaling pathway is demonstrated by GDS1261 and GDS1053. According to GDS1261, expression of MUP19 was reduced more than 90% in Ames dwarf mice with mutant pituitary specific transcription factor 1 (PIT-1) - one key regulator in the GH/IGF-1 signaling pathway (Figure 5.6A). In GDS1053, knockout of the growth hormone receptor (GHR) led to a 60% decrease in MUP19 expression (P<0.001) in the liver of 42-d old mice. In addition, when the GHR was truncated at residue 596, there was not a significant decrease in MUP19; when GHR was truncated at residue 391, the decrease in MUP19 became 65% (P<0.0001) compared to the wild type mice (Figure 5.6B). Expression of MUP19 is also affected by deficiency of low density lipoprotein (LDL) receptors. In GDS279, expression of MUP19 in the liver was repressed by 33% in C57BL/6 mice lacking LDL receptors compared to the normal mice after high fat diet treatment for 12 wk (Figure 5.6C). The correlation between MUP19 and Stearoyl-CoA desaturase 1 (SCD1) seems to be affected by food intake. In GDS1517, expression of MUP19 decreased 50% in livers of mice with SCD1 deficiency compared to wild type mice under treatment of a low fat, high carbohydrate diet (P<0.001, Figure 5.6D). However, in GDS1374, where the SCD1-/- mice were fed a chow diet, the expression of MUP19
was increased 1.2 times compared to the wild type mice (P<0.01, Figure 5.6E).

SERPINA1F, WFDC15B and DEFB29 are all highly expressed in the kidney. From GDS2031/2030 (Figure 5.7A) and GDS4316 (Figure 5.7B), it can be seen that the expressions of all 3 genes were at a very low level during the embryonic and neonatal stages and only increased after the mouse was born and stayed at a high level or kept increasing after postnatal 4 wk, indicating that these 3 genes are important for normal function of the developed kidney.

SERPINA1F encodes one isoform of serine peptidase inhibitor, clade A, member 1 (SERPINA1). In GDS3675, it seems that expression of SERPINA1F is positively related to blood pressure in the kidney (Figure 5.7C). Expression in the hypertension mouse strain is about 2 times that of normotension mice (P<0.0001), while the expression in the hypotension mouse strain dropped 30% compared to that of normotension mice (P<0.0001).

As was the case for SERPINA1F, expression of WFDC15B in the kidney is also positively correlated with blood pressure as shown in GDS3675 (Figure 5.7C). Expression in the hypertension mouse strain is about 1.1 times that of normotension mice (P<0.05), while expression in the hypotension mouse strain also dropped 30% compared to normotension mice (P<0.0001). In GDS2817, expression of WFDC15B is downregulated by 36% (P<0.01) in the kidney of mice with severe kidney atrophy and fibrosis caused by loss of the GLIS2 gene compared with normal mice (Figure 5.7D). In GDS1583, expression of WFDC15B at the ureteric bud tip is 2 times and 10 times higher respectively than in the ureteric bud stalk and the metanephric mesenchyme in the embryonic kidney (Figure 5.7E). In GDS4449, in kidneys of 12 d mouse embryos with defective ureteric branching morphogenesis and nephrogenesis due to overexpression of β-catenin, WFDC15b was downregulated by 54% (P<0.01)
compared to the wild type group (Figure 5.7F).

As was the case for WFDC15B, DEFB29 shows higher expression in the ureteric bud tip than in the ureteric bud stalk (P<0.0001) in the mouse embryonic kidney in GDS1583, but its expression in the metanephric mesenchyme is not significantly lower than in the ureteric bud tip (Fig 5.7E). In GDS4839, expression of DEFB29 is prompted by mutation of methylmalonyl-CoA mutase (MUT). In the homozygous mutant mice, expression of DEFB29 increased 1.4 times compared to that in wild type mice (P<0.05). In the heterozygotes, expression of DEFB29 also showed an increasing tendency (Figure 5.7G). In GDS3612, another interesting pattern occurs in expression of DEFB29, as it seems positively correlated with expression of functional Claudin 16 (CLDN16). In mice with the homozygous mutant CLDN16, the expression of DEFB29 is 40% less than in wild type mice (P<0.05); in the heterozygous mice, expression of DEFB29 also shows a decreasing trend (Figure 5.7H).

DISCUSSION

In this study, we successfully applied the powerful approach established by Song et al. (2013) to select novel tissue-specific genes in the mouse. Among the 31 tissue-specific genes selected by microarray data analysis and semi-quantitative PCR analysis, there are 3 main categories: i) genes that were reported by previous publications to be secretory genes in the tissues in which we detected high expression; ii) genes that were reported by previous publications to be secretory genes, but secretion had not been reported in the tissues in which we detected high expression; and iii) genes that were not previously reported to be secretory genes.
Lung-Specific Genes

Among the 11 lung specific genes, CHIL4 (Chitinase-like protein 4), LYZ1 (lysozyme) and SEMA3G (semaphorin 3G) belong to category i. CHIL4 is a member of the Chitinase-like proteins, which are indicators of inflammation and cancer in humans (Kzhyshkowska et al., 2007). CHIL4 itself is also suggested to play an important role in airway inflammation (Song et al., 2008). LYZ1 is the primary antibacterial component secreted by submucosal glands in tracheobronchial airways in lung (Dajani et al., 2005). SEMA3G is a member of Class 3 semaphorins, which are chemorepellants for growing neurons and axons (Tran et al., 2007). However, there is also accumulating evidence for involvement of some semaphorins in the immune system (Kikutani and Kumanogoh, 2003). Therefore, SEMA3G may also be involved in both the nervous and immune systems in the lung.

NDNF (neuron-derived neurotrophic factor), NPNT (nephronecin), PCOLCE2 (procollagen C-endopeptidase enhancer 2), LGI3 (leucine-rich glioma inactivated 3), EGFL6 (epidermal growth factor - like protein 6), HILPDA (hypoxia inducible lipid droplet-associated protein) and SPON1 (F-spondin) belong to category ii. They are reported to be secreted in some cell cultures or tissues, but not to be secreted or specific to the tissues in which we detected with high expression. NDNF was recently reported to be a regulator that promotes endothelial cell function and its secretion is reported to be stimulated by hypoxia in the vascular system (Ohashi et al., 2014). So, it may play a similar role in the lung. NPNT is a ligand of integrin subunit α8β1, which is an important regulator that promotes branching morphogenesis in the lung (Benjamin et al., 2009) and kidney (Kim and Nelson, 2012). The interaction between NPNT and integrin α8 has proved to be important for kidney development (Kim and Nelson, 2012). Therefore, NPNT may play an important role in lung
morphologenesis. PCOLCE2 was reported to be highly expressed in the adult human heart rather than in the lung. It is an enhancer for sufficient procollagen processing that is required for myocardial collagen deposition in the adult human heart (Baicu et al., 2012). Thus, it may also play the same role in the extracellular matrix of lung. LGI3 is a member of the secreted leucine-rich repeat LGI family. This protein family was reported to be crucial for development and function of the nervous system (Kegel et al., 2013). LGI3 has been proven to induce neurite outgrowth in the brain (Park et al., 2010) and promote metabolic inflammation in adipose tissue (Kim et al., 2013). Therefore, it may also be involved in the nervous and immune systems in the lung. EGFL6 was reported to promote endothelial cell migration and angiogenesis (Chim et al., 2011). Therefore, it may be important for the endothelium of vasculature, including that in pulmonary circulation. HILPDA was reported to be a target protein of hypoxia-inducible factor 1 (HIF-1) and upregulated by hypoxia (Gimm et al., 2010), indicating that it is involved in metabolism regulation and oxygen delivery in the lung. SPON1 was reported to inhibit human umbilical vein endothelial cell migration and angiogenesis (Klar et al., 1992). Therefore, SPON1 may be important in the stabilization of endothelial cells in the mature lung as well.

CTLA2A (cytotoxic T lymphocyte-associated protein 2 alpha) belongs to category iii. It is mostly expressed in cytotoxic T lymphocytes, and its expression is induced upon lymphocyte activation (Brunet et al., 1988). Therefore, it is tightly correlated with immunological response. Because of the massive surface of lung epithelium and continuous exposure to external environment, the lung has many T lymphocytes to regulate host defenses against viruses, fungal pathogens and bacteria (Chen and Kolls, 2013). Therefore, high expression of CTLA2A in the lung may be essential for normal T cell response and pulmonary host defense. As indicated by the
cell culture and western blot analysis, alternative splicing of CTLA2A, which can barely be detected by PCR, leads to a non-secretory protein without signal peptide. Therefore, the secretion of this protein is predominant with only a tiny amount maintained inside the cell. In GEO data analysis, expression of CTLA2A increased dramatically after birth due to exposure to the external environment, after infection with *E. coli* and wild type *S. aureus*, and after long-term exposure to 50 ppm of arsenate and high tidal volume ventilation. On the other hand, its expression is also moderately elevated in a rat strain that is resistant to VALI and in mice infected by *S. aureus* lacking lethal factor. All of these data suggest that CTLA2A plays an important role in protecting the lung from harmful invasion.

**Liver-Specific Genes**

All of the liver-specific genes except MUP19 belong to category i, since the secretion of their products has been reported in the liver. SERPINA1C (Serpin Peptidase Inhibitor, Clade A, member 1C) is one member of SERPINA1. Liver injury triggered by accumulation of mutant SERPINA1 in ER of hepatocytes is the most common genetic cause of hepatic disease in children (Pastore *et al.*, 2013). CPB2 (Carboxypeptidase B2) is secreted to plasma from the liver and is upregulated during the inflammatory response and inhibits fibrinolysis and inactive inflammatory peptides after activation by the thrombin-thrombomodulin complex on the vascular endothelial surface (Campbell *et al.*, 2001). ITIH1 and ITIH3 are 2 heavy chains of ITI. ITI is also a plasma protein secreted by the liver and is well-known for its anti-inflammatory function and stimulatory function in endothelial cells (Balduyck and Mizon, 1991). C8B is a subunit of complement component 8 (C8) protein. C8 is a component of the membrane attack complex (MAC), which plays a key role in the innate and adaptive immune system by mediating cell lysis (Sonnen and Henneke,
KLKB1 (kallikrein B) is also a plasma protein secreted by the liver. It can stimulate release of neutrophil elastase release during blood coagulation, and also participates in production of plasmin from plasminogen, the kallikrein-kinin system, the renin-angiotensin system and the alternative complement pathway (Björkqvist et al., 2013).

MUP19 (major urinary protein 19) belongs to category iii, although according to its name, it belongs to the MUP family, which is a group of lipocalins secreted from the liver and excreted to urine after circulation (Shaw et al., 1983). Its secretion and function have not been reported. Therefore, we can only speculate as to its function based on GEO data analysis and the function of other members of the family. From GEO data analysis, we predict that MUP19 is positively regulated by growth hormone (GH), since mutation in the PIT-1 gene causing reduced GH production and deletion of GHR, both dramatically reduced expression of hepatic MUP19. In addition, the intensified decrease in expression of MUP19 is also concordant with the exaggerated truncation of GHR (Rowland et al., 2005). In addition, decrease of MUP19 in mice with LDL receptor deficiency indicates expression of MUP19 is regulated by lipid homeostasis in the liver, because mice with LDL receptor deficiency cannot intake lipids to the liver from blood circulation (Huszar et al., 2000). Finally, we predict that MUP19 is a protein regulated by lipid metabolism in liver due to modulation of its expression by dietary treatment in mice lacking SCD1. Because SCD1 is a key enzyme in lipid metabolism that catalyzes the de novo synthesis of monounsaturated fatty acids (MUPA), which are components of triglycerides, its ablation impairs not only MUFA synthesis but also lipid metabolism in the liver induced by low-fat, high-carbohydrate diets (Flowers et al., 2006). Therefore, the expression of MUP19 is reduced. However, when enough dietary MUPA can be
obtained from food, there is no compensatory increase in fatty acid synthesis. Instead, the activation of lipid oxidation and reduction in triglyceride synthesis produced an anti-obesity effect (Ntambi et al., 2002). Therefore, the expression of MUP19 increased. The reduction of MUP19 expression by low-fat, high-carbohydrate diets and induction of its expression by a chow diet indicates that MUP19 may play a positive role in lipid metabolism promotion and may be related to lipid deposition in the liver. This role is similar to that of MUP1, which has been extensively studied. Liver-specific overexpression of MUP1 significantly reduced triglyceride levels in livers, and chronic treatment with MUP1 also reduced plasma lipids in db/db mice (Zhou et al., 2009).

Heart/Muscle-Specific Genes

The 2 heart-specific genes, FNDC5 (fibronectin type III domain-containing protein 5) and IL15 (interleukin 15) are in category i. The secreted protein of FNDC5 - irisin is induced after physical exercise, and increases caloric expenditure by converting white adipose tissue to brown adipose tissue, and, thus, opposes the formation of atheromata (Aydin et al., 2014). IL15 is a widespread cytokine regulating immune response. It can stimulate the proliferation of T cells, and promote development and function of natural killer cells. However, according to the GEO data analysis and semi-quantitative PCR analysis, IL15 is expressed more in the heart than in the other 5 tissues, indicating that it may have a special function in the heart. It has been proven that elevated circulating levels of IL15 can reduce body fat significantly and inhibit obesity (Quinn et al., 2009). Therefore, high expression of IL15 may be helpful in preventing excessive lipids in the cardiovascular system. GPC1 (glypican-1) is an important protein in both skeletal and cardiac muscle, since it is specifically expressed in both the muscle and heart. As suggested by Velleman et al. (2006),
GPC1 promotes myogenic satellite cell proliferation and responsiveness of fibroblast growth factor 2 (FGF2), which is a stimulator of muscle cell proliferation and an inhibitor of differentiation.

**Kidney-Specific Genes**

Except for SERPINA1F, WFDC15B and DEFB29, the other 4 kidney-specific genes belong to category ii because there are no publications about their secretion in kidney. However, the high expressions we detected in mouse kidney suggest their potential functions in the kidney. SPINK3 (serine peptidase inhibitor, kazal type 1) is highly expressed in the pancreas and has mainly been studied in the pancreas and sex glands. It is known as a protease inhibitor, a growth factor for acinar cells in the pancreas and an inhibitor of calcium uptake on the sperm head (Assis et al., 2013). Therefore, SPINK3 may also function as a regulator of calcium transport or as a growth factor in the kidney. C1QTNF3 (c1q and tumor necrosis factor related protein 3) is specifically expressed in cartilage and kidney in adult mice (Maeda et al., 2001). It is known as an adipokine opposing leptin (Peterson et al., 2010) and an enhancer for skeletal development (Maeda et al., 2001). However, its function in the kidney is still unclear. CGREF1 (cell growth regulator with EF-hand domain 1) is a protein that mediates cell adhesion (Devnath et al., 2009) and is an inhibitor of cell growth (Madden et al., 1996). SECTM1B (secreted and transmembrane 1b) is one isoform of SECTM1. It is a chemoattractant for monocytes (Wang et al., 2014) and a stimulator for T cell activation (Wang et al., 2012) and, thus, may play an important role in the kidney immune system.

SERPINA1F, WFDC15B and DEFB29 belong to category iii. SERPINA1F was reported to be predominantly expressed in the epididymis of male mice and is reduced after castration and recovered by administration of testosterone (Yamazaki et
However, our study indicates that SERPINA1F is also highly expressed in the kidney, although there are no reports concerning the expression and function of this gene in the kidney. SERPINA1F encodes one isoform of serine peptidase inhibitor clade A member 1 (SERPIN1). Expression of SERPIN1 is up-regulated by acute kidney injury and can protect injured proximal tubule cells by reducing activity of neutrophil elastase (Zager et al., 2014). Therefore, SERPINA1F may also play a role in protection of the kidney. The increase of SERPINA1F expression in a hypertension mouse strain and decrease in a hypotension mouse strain indicates that it may be involved in regulation of response to renovascular blood pressure. The increase in SERPINA1 excretion has been reported in patients with arterial hypertension (Lisowska-Myjak et al., 1999). One patient with SERPINA1 deficiency also developed uncontrolled hypertension (Os et al., 1997). Therefore, SERPINA1F may play a critical anti-hypertension role in mice as does AAT in humans. This may also be attributed to its role in kidney protection since renal hypertension usually causes vascular injury and inflammatory response in the kidney. As shown in cell culture and western blotting, the peptide translated by alternative splicing lacking 55 residues seems to be retained inside the HEK293 cells rather than secreted. This situation is similar to that of soluble secreted endopeptidase (SEP) and cholesteryl ester transfer protein (CETP). In SEP, deletion of 23 residues following the transmembrane domain at the beginning of the luminal domain resulted in retention of the protein in ER (Raharjo et al., 2001). In CETP, deletion of 60 amino acids in the central region also resulted in its retention within ER (Quinet et al., 1993). According to prediction of the transmembrane protein with the TMHMM server (http://www.cbs.dtu.dk/services/TMHMM/), the deleted region in SERPINA1F also closely flanks one transmembrane domain. Therefore, the short peptide translated by
alternative splicing may also stay in the ER and the retention may be also mediated by the luminal domain as SEP. In addition, inhibition of secretion of full-length protein by the short protein for CETP through complex association also provides a clue for future study of the association between the 2 isoforms of SERPINA1F.

WFDC15B encodes single whey acidic protein (WAP) motif protein 1 (SWAM1) in the mouse, which is an antibacterial protein. Its expression was detected in the kidney and epididymis and its antibacterial property has also been observed for *E. coli* and *S. aureus* (Hagiwara et al., 2003). Therefore, it may participate in the immune system. GEO profiles indicate that WFDC15B is important for both the health of the mature kidney and development of the embryonic kidney, because its expression was reduced significantly after the occurrence of severe kidney atrophy and defective ureteric branching morphogenesis. In addition, high expression of WFDC15B in ureteric bud tip cells may indicate its contribution to development of nephrons as a secretory factor from the ureteric bud tip, because the ureteric bud tip cells play an important role in inducing conversion of metanephric mesenchyme into nephrons by secreted factors (Schmidt-Ott et al., 2005). In addition, WFDC15B may be also associated with blood pressure as it shows the same expression pattern as SERPINA1F in hypertension and hypotension mouse strains compared with the normotension strain.

DEFB29 (defensin, beta 29) is one member of the β-defensin family, which is a group of small antibiotic proteins involved in host defense by disrupting the cytoplasmic membrane of microorganisms (Yang et al., 1999). Therefore, DEFB29 may contribute to the immune response of infections and inflammatory disease. GEO data analysis indicates that expression of DEFB29 is increased concordantly with the loss of methylmalonyl-CoA mutase (MUT). Because MUT is an enzyme that
mediates metabolism of carbon skeletons through the Krebs cycle, deficiency of MUT usually causes increased oxidative stress in the renal tubule, which leads to chronic tubulointerstitial nephritis (Manoli et al., 2013). Therefore, the increase in DEFB29 may reflect the immune response caused by renal tubular dysfunction. In addition, DEFB29 may also be related to renal Ca\textsuperscript{2+} and Mg\textsuperscript{2+} homeostasis, because its expression decreases concordantly with the loss of Claudin 16 (CLDN16). As reported by Will et al. (2010), CLDN16 is a critical regulator of Ca\textsuperscript{2+} and Mg\textsuperscript{2+} transport in the thick ascending loop of Henle. Finally, DEFB29 may play an important role in development of the ureteric bud and metanephric mesenchyme since it is highly expressed in both structures in embryonic kidneys.

**Adipose and Liver-Specific Genes**

The 2 adipose and liver-specific genes, LRG1 (leucine-rich alpha-2-glycoprotein) and SERPINA3N (serpin peptidase inhibitor, clade A, member 3N) belong to category i, but they are only reported to be secreted from liver to plasma. LRG1 in plasma can sequester cytochrome C (Cyt C) released by apoptotic cells and protect lymphocytes from the toxic effects of Cyt C (Codina et al., 2010). In addition, LRG1 can promote angiogenesis by regulating transforming growth factor β (TGF-β) signaling (Wang et al., 2013). Adipose stromal cells have been reported to alleviate tissue damage and their secreted media has also been proven to protect neuronal cells from apoptosis (Wei et al., 2009). In addition, preadipocytes also become relatively resistant to apoptosis during differentiation (Sorisky et al., 2000). Therefore, LRG1 may be secreted in adipose tissue as a survival factor. In addition, angiogenesis is very important during adipogenesis, providing new vasculature to supply nutrients to and remove metabolic waste from growing and proliferating adipocytes (Corvera and Gealekman, 2014). Therefore, this evidence provides support for prospective studies
on the role of LRG1 in adipose tissue.

SERPINA3N is one isoform of Serpin Peptidase Inhibitor, Clade A, member 3 (SERPINA3). This protein is a typical acute phase protein that is upregulated dramatically in response to inflammation. As an important inhibitor of leukocyte cathepsin G, SERPINA3 can prevent excessive or prolonged activity of cathepsin G that may lead to tissue damage at an inflammation site (Wiedow et al., 2005). Therefore, secretion of SERPINA3 may also reflect function of adipose tissue related to regulation of metabolic inflammation and immune response.

From the above review of the selected genes, it can be noticed that functions of most genes in category ii are rarely studied in the lung and kidney; so, we can only speculate as to their possible functions in these tissues based on studies of their functions in other tissues. However, the possibility of special functions cannot be ruled out for these genes in the lung and kidney. Therefore, further research in these tissues is important for a thorough understanding of the functions of these genes. In general, our study developed a powerful approach to identify novel tissue-specific secretory genes by GEO data analysis, and to confirm it at the mRNA and protein levels by showing predominant expression of genes in specific tissues and secretion of these proteins. With the same strategy, more novel genes sharing common cellular locations, common physiological functions, or common pathological mechanisms can be explored in other animals, tissues and protein groups. In addition, the prediction of gene function by integrating valuable microarray data and the literature is also an efficient way to identify novel secretory genes related to different systems such as immune response, developmental regulation and homeostasis regulation.

ACKNOWLEDGEMENTS
We appreciate the contribution of Michelle E. Milligan in proofreading and modifying the manuscript.

**AUTHOR CONTRIBUTIONS**

Table 5.1. Primer sequences for semi-quantitative PCR

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Table 5.3. Gene expression values of selected genes in GDS3142

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Table 5.3 Continued

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The highest expression value for each gene is highlighted in bold font. A/O: ratio between average expression of certain gene in tissue with specific expression of that gene and expression in other tissues. EM: Extracellular matrix; ER: Endoplasmic reticulum.

\(^{a-e}\) Different superscripts within a row indicate significant difference (P<0.05).
Figure 5.1. Expression of adult mouse gene transcripts detected by semi-quantitative PCR. Expressions of selected genes in fat (F), muscle (M), heart (H), lung (Lu), liver (Li) and kidney (K) were detected by PCR reaction and 1% agarose gel electrophoresis. Housekeeping gene - cyclophilin (CYC) - was used as a loading control. Genes without reported subcellular locations are marked with asterisks.
Figure 5.2. Different isoforms of CTLA2A and their expression in HEK293 cell culture.

A, alternative splicing of CTLA2A that leads to change in protein sequence. B, cell lysates and conditioned medium from HEK293 cells transiently transfected with HA-tagged murine expression vector of long isoform (CTLA2AL) and short isoform (CTLA2AS) and empty pcDNA3.1 vector as negative control (NC) were detected using western blotting.
Figure 5.3. Different isoforms of SERPINA1F and their expression in HEK293 cell culture. A, alternative splicing of SERPINA1F that leads to double bands in gel electrophoresis of PCR product and change in protein sequence. B, cell lysates and conditioned medium from HEK293 cells transiently transfected with HA-tagged murine expression vector of long isoform (SERPINAL) and short isoform (SERPINAS) and empty pcDNA3.1 vector as negative control (NC) were detected using western blotting.
Figure 5.4. Detection of protein expression of MUP19, WFDC15B and DEFB29 in HEK293 cell culture. A, Cell lysates and conditioned medium from HEK293 cells transiently transfected with HA-tagged murine expression vector of MUP19 and WFDC15B and empty pcDNA3.1 vector as negative control (NC) were detected using western blotting. B, Cell lysates and conditioned medium from HEK293 cells transiently transfected with HA-tagged murine expression vector of DEFB29 and empty pcDNA3.1 vector as negative control (NC) were detected using western blotting.
Figure 5.5. Expression profile in lung for CTLA2A in microarray DataSets obtained from NCBI website. 

A, Expression in mice on embryonic day 12 (E12), 14 (E14), 16 (E16) and 18 (E18) and postnatal d 2 (P2), 10 (P10) and 30 (P30) in GDS3950 (n=2 per time point).

B, Expression in mice infected by E.coli and control group in GDS4583 (n=3 per group).

C, Expression in mice after 24-h infection by S.aureus deficient in alpha-hemolysin (Hla-/-) and wild type S.aureus (WT) with saline treated mice as control group (n=3 per group).

D, Expression in mice after 90-d exposure to 50 ppm arsenate (AS) with untreated mice as control in GDS4914 (n=5 per group).

E, Expression in resistant rat strain (VR) to ventilator-associated lung injury (VALI) and sensitive rat strain (VS) to VALI under treatment of high tidal volume ventilation (HV) with untreated mice as control (C) group in GDS2709 (n=3 per group). Each bar represents mean ± SEM. Statistical difference is indicated by different letters (P<0.05), * (P<0.05) and ** (P<0.01) above the bars.
Figure 5.6. Expression profile in liver for MUP19 in microarray DataSets obtained from NCBI website.  

**A**, Expression in Ames Dwarf mice (n=8) and normal mice (n=7) with free access to standard food in GDS1261.  

**B**, Expression in 42-d old mice with growth hormone receptor knocked out (KO), truncated at amino acid residue 569 (ST) and 391 (LT) with wild type (WT) mice as control group in GDS1053 (n=3 per group).  

**C**, Expression in C57BL/6 mouse with null mutant low-density lipoprotein receptor (LDLR) under the treatment of high fat diet for 12 wk along with WT mice as control group in GDS279 (n=3 per group).  

**D**, Expression in mice with stearoyl-CoA desaturase 1 deficient mutants (Scd1-/-) under treatment of low-fat, high-carbohydrate diet along with WT mice as control group in GDS1517 (n=5 per group).  

**E**, Expression in 6-wk Scd1-/- mice fed with chow diet along with WT mice as control group in GDS1374 (n=5 per group). Each bar represents mean±SEM. Statistical difference is indicated by different letters (P<0.05), ** (P<0.01) and *** (P<0.001) above the bars.
Figure 5.7. Expression profile in kidney for kidney specific genes in microarray DataSets obtained from NCBI website. A, Expression of SERPINA1F, WFDC15B and DEFB29 in mice on embryonic d 12 (E12), 13 (E13) and 16 (E16) and postnatal 10 wk in GDS2030/2031 (n=2 per time point). B, Expression of SERPINA1F, WFDC15B and DEFB29 in postnatal C57BL/6 mice at 1, 4 and 8 wk of age in GDS4316 (n=5 per time point). C, Expression of SERPINA1F and WFDC15B in hypertensive (HBP, n=5), normotensive (NBP, n=5) and hypotensive (LBP, n=4) mice strain in GDS3675. D, Expression of WFDC15B in mice with null mutant GLIS family zinc finger 2 (GLIS2/-) and wild type (WT) mice in GDS2817 (n=3 per group). E, Expression of WFDC15B and DEFB29 in ureteric bud tips (UBT) and stalks (UBS) and metanephric mesenchyme (MM) from Embryonic 12-d mice in GDS1583 (n=2 per section). F, Expression of WFDC15B in mice with b-catenin overexpression (CTNNB1+) and WT mice on embryonic d 12 in GDS4449 (n=3 per group). G, Expression of DEFB29 in homozygous mice for mutant methylmalonyl-CoA mutase (Mut/-/-), heterozygous mice (Mut+/-) and WT mice in GDS4839 (n=4 per group). H, Expression of DEFB29 in WT mice (n=4), heterozygous mice (Cldn+/-, n=3) and homozygous mice for mutant claudin 16 (Cldn-/-, n=4) in GDS 3612. Each bar represents mean ± SEM. Statistical difference is indicated by different letters (P<0.05) and ** (P<0.01) above the bars.
CHAPTER 6
IDENTIFICATION OF HPS4 AS A NOVEL ADIPOSE-SPECIFIC GENE IN CHICKEN ADIPOSE TISSUE

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ABSTRACT

Adipose-specific genes are potential targets for regulation of fat deposition in animals and treatment of obesity in human, because they commonly reflect special properties of adipose tissue and have important functions in adipose tissue. In this study, we identified a novel adipose-specific gene in broiler chickens through microarray analysis. Higher expression of HPS4 was detected in chicken adipose tissue than in muscle, heart, lung, liver and kidney and also higher expression in differentiated fat cells than in preadipocytes. In addition, its expression is upregulated during in vitro primary cell culture and in vivo adipose development. Finally, two alternative transcripts were identified by PCR with cDNA from chicken adipose tissue, and two protein sizes were identified by western blotting. The smaller protein is between 50kDa and 75 kDa and shows increasing expression with age. However, the larger protein is between 75kDa and 100kDa, and continues to decrease with age until
it nearly disappears by posthatch day 33. The size of these proteins corresponds to the molecular weight of proteins translated by the two alternative transcripts. However, further research needs to be conducted to confirm whether the proteins are produced by the two transcripts. To the best of our knowledge, this is the first study of HPS4 in adipose tissue, providing a basis for follow-up functional studies that may provide a new foundation for adipose biology.

INTRODUCTION

Normal development and function of adipose tissue is regulated by coordination of numerous genes involved in many biological activities such as metabolism, inflammation, proliferation and differentiation. Therefore, identification of master and specific genes in adipose tissue is important to unravel the mysteries in this tissue and find a better way to cure obesity in human and regulate fat accumulation in domestic animals. Some adipose-specific genes such as AdipoQ and Adipose triglyceride lipase (ATGL) have been discovered as important genes in lipid metabolism and adipose development (Hu et al., 1996; Zimmermann et al., 2004). Two adipose-specific genes, ADSF/resistin and ISG12b1, were also discovered in our previous studies (Kim et al., 2001; Li et al., 2009). In addition, these studies showed that microarray analysis is an effective strategy for discovery of adipose-specific genes.

In this study, we detected through microarray analysis higher expression of the Hermansky-Pudlak syndrome 4 (HPS4) gene in chicken adipose tissue than in muscle, heart, lung, liver, kidney, spleen and brain, and proposed its potentially specific expression and important function in adipose tissue. The name of HPS4 originates from the fact that its mutations cause a subtype of Hermansky-Pudlak syndrome (HPS)
HPS is a collection of autosomal recessive disorders of vesicle formation in humans. Generally, patients suffer from oculocutaneous albinism due to defective biogenesis of melanosomes and prolonged bleeding due to a deficiency of platelet dense granules (Shotelersuk and Gahl, 1998). Some patients also suffer from more serious diseases, such as pulmonary fibrosis and granulomatous colitis (Gahl et al., 1998). Nine HPS-causing genes have been identified in human (Carmona-Rivera et al., 2013). Most of these genes play a role in membrane and protein trafficking and produce subunits of Biogenesis of Lysosome-related Organelle Complexes (BLOC) whose defects can affect lysosome-related organelles (LROs) (Wei ML, 2006). Among these genes, HPS4 dimerizes with another family member - HPS1 - to produce one functional protein - BLOC3, which plays a critical role in trafficking of proteins to newly formed organelles (Chiang et al., 2003). Although HPS4 has been extensively studied in terms of its genetic correlation with HPS disease, its expression and function in adipose tissue has not been studied. The objective of this study was to investigate the expression of HPS4 in different fractions of adipose tissue, as well as during primary cell culture and adipose development, and to discuss the possible functions of the HPS4 gene in adipose tissue.

MATERIALS AND METHODS

Experimental Animals

Commercial broiler eggs (Ross 708) were hatched, and chicks were raised at The Ohio State University Poultry Facility in Columbus until 33 days of age with ad libitum access to a diet provided by the Ohio Agricultural Research and Development Center (OARDC, Wooster, OH). The diet met NRC nutrient recommendations for chickens (NRC, 1994) and contained 21% crude protein and 3,121 kcal of
metabolizable energy per kilogram. Four chickens were sacrificed at embryonic day (E) 15 and 17 and at posthatch day (P) 1, 5, 11, 21 and 33, and subcutaneous fat pads on the legs were collected at the location shown in a previous paper (Chen et al., 2014). At P21, abdominal fat, thigh and pectoral muscles, heart, lung, liver, kidney, spleen and brain were also collected in addition to the subcutaneous fat. The collected tissues were then snap-frozen immediately in liquid nitrogen and stored at -80°C (Lee et al., 2009; Lee et al., 2012). Posthatch chickens were euthanatized by CO₂ inhalation. All procedures were approved by The Ohio State University Institutional Animal Care and Use Committee.

**Separation of Stromal-Vascular and Fat Cell Fractions**

After the abdominal fat tissues were collected at P21 from four chickens, one part of the tissue for each chicken was further separated into stromal-vascular (SV) and adipocyte cell (FC) fractions according to the procedures described in previous papers (Deiuliis et al., 2006 and 2008). In brief, the adipose tissues were washed with PBS, minced with a razor blade and then incubated for 1 hour in a shaking water bath (180 rpm) with digestion buffer containing 3.2 mg/mL of collagenase II (Sigma-Aldrich, St. Louis, MO) at the ratio of 5 mL per gram of tissue. The suspension was then filtered using a nylon cell strainer (BD Falcon, Franklin Lakes, NJ) with mesh pores of 100 μm diameter to remove the undigested tissue debris. After centrifugation at 200×g for 5 min, the cells were further separated into FC in top layer and SV pellets.

**Primary Stromal-Vascular Cell Culture**

After SV cells were isolated from the abdominal fat tissues in four chickens, the cells of each chicken were further cultured in one culture plate according to the description in a previous report (Oh et al., 2011). Briefly, the cells were first washed
with Dulbecco’s modified Eagle’s medium (DMEM) and then centrifuged at 500×g for 10 min at room temperature. After centrifugation, the pellet was resuspended in DMEM containing 10% fetal bovine serum (FBS) to an approximate concentration of 6×10^6 cells/mL. The cells were then seeded in 12-well plates (Greiner Bio-one, Monroe, NC) at a density of 3×10^6 cells/mL in culture medium and maintained in a humidified incubator filled with 5% CO₂ at 37 °C. After the cells reached 50% confluence, differentiation was induced by a medium containing 10% FBS, 50mM linoleic and oleic acids, and 10 μg/mL of insulin in DMEM. Cells were then collected at day 1 before differentiation (day -1), initiation of differentiation (day 0), and day 1, 2 and 3 after differentiation.

**Total RNA Isolation and cDNA Microarray**

Total RNA was isolated from collected tissues and cells using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. After adding Trizol, tissue samples were homogenized with a Tissuemiser homogenizer (Fisher Scientific, Pittsburgh, PA), while the cells were vortexed to isolate total RNA. The quality and quantity of isolated RNA were assessed through gel electrophoresis and analysis using a Nanodrop machine (NanoDrop Technologies, Wilmington, DE, USA), respectively. RNA samples from each tissue of subcutaneous adipose tissue, thigh muscle, heart, lung, liver, kidney, spleen and brain in four P21 chickens were pooled with the same amount from each individual so that one sample for each tissue was used for microarray analysis. The RNA samples were then frozen and delivered to The Ohio State University Microarray Core Facility where they were converted to cDNA and hybridized to an Affymetrix GeneChip Chicken Genome array chip. A total of 32,773 transcripts corresponding to 28,000 chicken genes were covered by the chip. Background correction and quantile normalization of raw data were conducted by Dr.
Lianbo Yu from The Center for Biostatistics at The Ohio State University. After these procedures, the raw data were converted to relative gene expression per sample (Hassan et al., 2014).

**cDNA Synthesis and Quantitative Real-time PCR**

After RNA isolation from tissues and cells, 1 mg of total RNA was reverse transcribed to make cDNA with M-MLV reverse transcriptase (Moloney murine leukemia virus RT, Invitrogen). The conditions for reverse transcription were 65 °C for 5 min, 37 °C for 52 min and 70 °C for 15 min. After reverse transcription, gene expression of HPS4 (forward 5’- CACGCTGATCCAC TCTGACTTCA-3’ and reverse 5’- CATTATTGCTGAGATACGCTACCTCA-3’; GenBank: XM_004945639) was measured by quantitative PCR (qPCR) in an ABI 7300 real-time PCR Instrument (Applied Biosystems, Foster City, CA). The reaction consisted of 2.5 μL of GeneAmp 10×PCR buffer II containing 100mM Tris-HCl, pH 8.3 and 500mM KCl, 2 μL of 25mM MgCl₂, 0.5 μL of 10mM deoxynucleoside triphosphate mix (dNTP), 0.5 nM of each of the forward and reverse primers, 0.385 μL of 0.2% Rox as reference dye, 0.034 μL of 0.2% SYBR green as detection dye, 0.125 μL of AmpliTaq Gold polymerase (Applied Biosystems), 1 μL cDNA and UltraPure DNase/RNase-free distilled water (Invitrogen) up to 25 mL. The conditions of qPCR were 95°C for 10 min, 40 cycles of 94°C for 15 s, 58°C for 40 s, 72°C for 30 s and 82°C for 32 s. Correct single PCR product and optimized thermal cycling procedures were confirmed by a normal amplification curve, one major peak in melting point analysis using qPCR software (Applied Biosystems) and a single target band in gel electrophoresis. Efficiency of qPCR amplification was calculated through a standard curve determined by serial dilutions (1:1, 1:2, 1:4 and 1:8) of each template cDNA sample. After qPCR, the relative expression level was calculated using the
comparative 2^ΔΔCt method for relative quantification (Livak and Schmittgen, 2000). Average expression stability values of four reference genes were calculated using the geNorm program in qbasePLUS software to evaluate their stability (Biogazelle, Zwijnaarde). Finally, β-actin (gACTB) was selected as an internal control (housekeeping gene) for expression normalization in different tissues and cell fractions and at different primary cell culture time points, whereas ribosomal protein S13 (gRPS13) was selected as an internal control for expression normalization among different age groups, because their stability values were lower than 1.5. The gene expressions after normalization were then displayed as change compared to the calibrator, which is usually the lowest expression value in the comparison. In addition to HPS4, expressions of one preadipocyte marker - delta-like 1 homolog (DLK1) - and one adipocyte marker - peroxisome proliferator-activated receptor gamma (PPARγ) - were also measured in the stromal-vascular and fat cell fractions and during differentiation of preadipocytes in vitro. The primers were designed to span genomic introns (>1Kb) to avoid amplification of possible genomic DNA contamination. Primer sequences of chicken RPS13 (GenBank: NM_001001783), ACTB (GenBank: NM_205518), DLK1 (GenBank: EU288039) and PPARγ (GenBank: NM_001001460) have been described in our previous reports (Lee et al., 2009; Yang et al., 2013; Hassan et al., 2014).

**Western Blot Analysis**

In order to detect protein expression of HPS4 among different tissues and during development of adipose tissue, western blot analysis was conducted following procedures described in previous reports (Zhang et al., 2014; Chen et al., 2014). Protein samples were isolated from subcutaneous and abdominal fat, thigh and pectoral muscles, heart, lung, liver and kidney of one randomly selected 21-day old
chicken for detection of tissue distribution. For expression during adipose
development, protein samples were isolated from subcutaneous adipose tissues of two
randomly selected chickens at each time point among E15, E17, P1, P5, P11 and P33.
For protein extraction, about 100 mg of tissues were first homogenized in 1mL of
lysis buffer containing 62.5 mM Tris, pH 6.8 and 1% SDS. After vortexing to ensure
complete lysis, the lysates were centrifuged at 13,000 rpm for 5 min at 4°C, and the
supernatant containing protein was transferred to another clean centrifuge tube.
Eighty milliliter protein samples were mixed with 80 mL 2 × Laemmli buffer
containing 62.5 mM Tris, pH 6.8, 1% SDS, 5% 2-mercaptoethanol, 12.5% glycerol
and 0.05% bromophenol blue (Bio-Rad Laboratories, Hercules, CA) and then boiled
at 98°C for 5 min. The remaining samples were then stored at -80°C for future use.
The boiled samples were loaded onto a 10% gel for coomassie staining to ensure the
same amount of protein was loaded for each sample. After the protein amount was
adjusted, proteins were transferred to Immobilon polyvinylidene difluoride transfer
membrane (Millipore, Billerica, MA) after separation in SDS-PAGE by the Mini
Protean 3 western transblot system (Bio-Rad Laboratories). The membranes were
blocked at room temperature in 4% nonfat dry milk in Tris-buffered saline Tween-20
(TBST) containing 0.1% Tween 20, 20 mM Tris and 150 mM NaCl, pH 7.4. After 30
min, the membranes were transferred with clean forceps to 4% nonfat dry milk
containing a HPS4 primary antibody raised in rabbit (1:5000 dilution; AbClon, Seoul,
Korea). The antibody was raised against EVHLRETLPKD in chicken HPS4 protein,
which is also a homologous region in human and mouse HPS4. After incubation
overnight at 4°C with the primary antibody, the membranes were washed in TBST 7
times for 4-5 min each time, and then incubated with an anti-rabbit horseradish
peroxidase-conjugated secondary antibody (R&D systems, Minneapolis, MN) in 4%
nonfat dry milk (1:5000 dilution). After being washed 7 times again in TBST, proteins were visualized on Amersham Hyperfilm ECL (GE Healthcare, Piscataway, NJ) in a dark room after applying Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare) to the membrane.

**Statistical Analysis**

Multiple comparisons among different groups were performed using the MIXED procedure in SAS software (version 9.3, SAS Institute Inc., Cary, NC, USA) with the DIFF option for detection of significant differences between each pair of least squares means. Student’s t-test in SAS was used in comparisons between only two population means. Differences with p-values lower than 0.05 were treated as significant. All of the statistical results are shown as least squares means plus or minus standard errors of the least squares means (SEM). For the microarray data, only one expression value was obtained for each sample, so there are no statistical results.

**RESULTS**

**Adipose Tissue-specific Expression of HPS4 in Broiler Chickens**

Microarray data showed the highest expression of HPS4 in adipose tissue compared to muscle, heart, lung, liver, kidney, spleen and brain in broiler chickens (Figure 6.1A). Although there was no significant statistical evidence, the pooled sample for each tissue alleviated possible bias. In addition, the highest expression of HPS4 in adipose tissue was also confirmed at mRNA and protein levels by conducting qPCR and western blotting. The qPCR results indicated that expression of HPS4 was significantly higher in both subcutaneous adipose tissue and abdominal adipose tissue (P<0.001) than in thigh and pectoral muscle, heart, lung, liver and kidney (Figure 6.1B). In the western blot analysis, the target bands of HPS4 were the most intense for
subcutaneous and abdominal adipose tissue, whereas the target band for lung was much weaker compared to adipose tissue. Target bands for thigh and pectoral muscle, heart, liver and kidney are not detectable (Figure 6.1C).

**mRNA Expression of HPS4 is Higher in Fat Cells than in Preadipocytes**

As suggested by the qPCR results, mRNA expression of HPS4 was more than 50 times higher (P<0.001) in the fat cell (FC) fraction than in the stromal vascular (SV) fraction (Figure 6.2A). On the other hand, expression of the DLK1 gene, which is a preadipocyte marker, was more than 4 times greater (P<0.05) in the SV fraction than in the FC fraction, because the SV fraction contains mostly preadipocytes. On the other hand, expression of the PPARγ gene, which is an adipocyte marker, was more than 6 times greater (P<0.05) in the FC fraction than in the SV fraction (Figure 6.2A), because the FC fraction consists mainly of mature fat cells.

During primary culture of SV cells, HPS4 was lowly expressed before day 1 of differentiation. However, after day 1 of differentiation, its expression increased significantly each day. The expression on day 2 and day 3 increased about 2 times (P<0.01) and 3 times (P<0.01), respectively, compared to that at day 1 (Figure 6.2B). PPARγ showed a similar expression pattern as HPS4. It was also the lowest before day 1 of differentiation and increased more than 3 times (P<0.01) by day 3 compared to the expression on day 1. However, expression of DLK1 showed the reverse pattern. It was highly expressed before initiation of differentiation, but decreased significantly (P<0.05) 1 day after induction of differentiation and then stayed at the lowest level from day 2 to day 3 (Figure 6.2B).

**Expression of HPS4 during Development of Chicken Adipose Tissue**

To detect mRNA and protein expression of HPS4, both qPCR and western blotting were conducted with cDNA and protein samples from subcutaneous adipose
tissues of broiler chickens on embryonic day 15 and 17 (E15 and E17), as well as on posthatch day 1, 5, 11 and 33 (P1, P5, P11 and P33). Both mRNA and protein expression showed a decreasing trend from E15 to P1 and an increasing trend from P1 to P11 (Figure 6.3). The mRNA expression on P11 was more than 9 times greater (P<0.05) than expression on P1 (Figure 6.3A). Another interesting point in the western blot results was the existence of a band between 75 kDa and 100 kDa in addition to the major band between 50 kDa and 75 kDa, which was possibly translated from alternative transcripts. Different from the major band, the larger band showed a continuous decreasing trend from E15 to P33 (Figure 6.3B).

**Alternative Splicing of HPS4 in Chicken Adipose Tissue**

In order to check whether the two bands shown in the western blot analysis are different isoforms of HPS4, alternative splicing of HPS4 was searched in GenBank. Indeed, two alternative transcripts were found in GenBank (accession No.: XM_415198 and XM_004945639). As shown in Figure 6.4A, chicken HPS4 has 14 exons in total. One transcript, XM_415198, lacks exon 4, but has a longer coding sequence starting from exon 1. Another transcript, XM_004945639, is longer, but has a shorter coding sequence starting from exon 6, because there is another stop codon located in exon 4 that makes the translation stop early and restart from another start codon in exon 6. In order to detect the two different alternative transcripts, PCR was conducted with forward primer located in exon 3 (5’-CAGGAGCTCCTCCAAGCCTTA-3’) and reverse primer spanning exon 7 and 8 (5’-GTGCTCACAATCAAGCCTTTGTAG-3’). A cDNA sample from one E17 chicken and one P33 chicken was used in the PCR. As shown in Figure 6.4A, both randomly selected samples showed two bands in electrophoresis of their PCR product. However, the embryonic sample seemed to have more short transcripts than long transcripts. In
order to quantify the longer transcripts, qPCR was conducted with a forward primer at exon 4 (5’-AGGGTGTAAGCATTGCAATGCA-3’) and the same reverse primer spanning exons 7 and 8. As shown in Figure 6.4B, expression of the long transcript was at a low level before P1. From P1 to P11, it showed a continuous increasing trend, the same expression pattern as detected in Figure 6.3A with primer sets for both transcripts. However, unlike total expression of the two transcripts, the long transcript showed the highest expression on P33 rather than on P11.

**DISCUSSION**

In this study, significantly higher expression of HPS4 was detected in adipose tissue than in other tissues in broiler chickens, suggesting that this gene may have a special function in adipose tissue. Further study showed that expression of HPS4 is much higher in the FC fraction than in the SV fraction and increases during differentiation of adipocytes and development of adipose tissue. Therefore, it is highly possible that HPS4 may play a more important role in mature fat cells than in preadipocytes. In addition, the existence of a fairly rich amount of HPS4 in the lung in western blot analysis (Figure 6.1C) may also indicate its importance in lipogenesis, since there is also dynamic fat accumulation in the lipofibroblasts and surfactant phospholipids production in alveolar type II cells during lung development (Torday et al., 1995).

Western blot analysis showed two different proteins detected by the antibody, and two alternative transcripts were also found in GenBank, in which the longer transcript encodes a small protein and the shorter transcript encodes a large protein. As predicted by the Protein Molecular Weight program (http://www.bioinformatics.org/sms/prot_mw.html), the molecular weights of their translated
proteins are respectively 63.41kDa and 77.26kDa, which are similar to the molecular weights detected in the western blot analysis. In addition, the changes in expression of the long transcript at different ages (Figure 6.4A) also agreed with the changes of the small protein in western blotting (Figure 6.3B). Therefore, we speculate that the two proteins detected in western blotting may be products of the two transcripts. However, due to the difficulty of designing a primer specifically for the short transcripts, we cannot detect their expression at different ages using qPCR. In order to verify this speculation, we still need to construct expression vectors for the two transcripts and detect their encoded proteins by cell transfection and protein collection.

If the different proteins were verified to be the products of the alternative splicing, the short transcript translating a larger protein may be important for early development of chicken adipose tissue because the protein amount gradually decreased with age (Figure 6.3B) and disappeared in the adipose tissues of 21-day old broiler chickens during the process of tissue distribution detection (Figure 6.1C).

As a subunit of biogenesis of lysosome-related organelles complex 3 (BLOC3), HPS4 is involved in the biogenesis of lysosome-related organelles (LGOs) (Chiang et al., 2003). LGOs include melanosomes, MHC class II compartments, platelet-dense granules, basophil granules, lytic granules and azurophil granules (Dell’Angelica et al., 2000). Except for the melanosomes and platelet-dense granules, all of the other organelles are mainly related to the immune system and are present in antigen-presenting cells, such as dendritic cells, mononuclear phagocytes, granulocytes and lymphocytes. This leads to our thought that high expression of HPS4 in adipose tissue may be related to increasing inflammation during adipogenesis, because many inflammatory factors such as interleukin (IL-6), tumor necrosis factor alpha (TNF-α) and monocyte chemoattractant protein 1 (MCP-1) are produced by the adipocytes to
modulate immunity and inflammation (Fantuzzi, 2005).

Another possible reason for the high expression of HPS4 in chicken fat cells and adipose tissue is that it may be related to the formation of lipid droplets. Lipid droplets have been suggested to be fat storage organelles in *C. elegans*, and they are also evolutionarily conserved. However, unlike animals with dedicated adipose tissue, *C. elegans* uses its intestine as the main site for fat storage. In the intestine cells, the fat is stored in gut granules, which are lysosome-related, terminal endocytic organelles (Schroeder *et al*., 2007). The gut granules were originally called “lipofuscin granules”, because the fluorescent pigments accumulated with age, and the granules are similar to lipofuscin granules in vertebrates (Clokey and Jacobson, 1986). Therefore, lipid droplets in animals may be evolutionarily homogeneous to the lipofuscin granules in *C. elegans*. Lipofuscin is an introlysosomal pigment that consists of oxidized proteins and lipids. It can not be degraded by lysosomes and thus accumulates with age due to oxidative stress and finally leads to dysfunction of lysosomes and apoptosis (Höhn and Grune, 2013). Therefore, high expression of HPS4 in fat cells may be necessary to regulate proper storage of lipofuscin, as well as apoptosis of fat cells and macroautophages in fat tissue. In addition, fat storage by intestinal cells in *C. elegans* seems analogous to the function of lamellar bodies, which are lysosome-related organelles for storage and release of lung surfactants (Weaver *et al*., 2002). This may also be the reason for the existence of HPS4 in lung. However, to confirm these hypotheses, more functional research, such as gene overexpression or gene knockdown and knockout needs to be done in the future. In general, our research initiated the study of a novel adipose specific gene that may be an important step in the complete understanding of adipocytes and adipose tissue.
AUTHOR CONTRIBUTIONS

Figure 6.1. Expression of HPS4 in different tissues in 21-day broiler chickens. A, microarray data with pooled cDNA from fat (F), muscle (M), heart (H), lung (LU), liver (LI), kidney (K), spleen (S) and brain (B). B, mRNA expression of HPS4 was detected in subcutaneous fat (SF), abdominal fat (AF), thigh muscle (TM), pectoral muscle (PM), heart (H), lung (LU), liver (LI) and kidney (K) (n=4 for each tissue) using qPCR with Chicken β-actin (gACTB) as a control for normalization. C, western blot results with protein isolated from the same tissues in one broiler chicken. Coomassie staining results are provided to show equal amounts of protein in each lane.
Figure 6.2. Comparison of expression of DLK1, PPARγ and HPS4 between preadipocytes and fat cells in chicken adipose tissue. A, relative gene expression between stromal vascular (SV) and fat cell (FC) fractions (n=4 for each group) detected by qPCR with gACTB as internal control. Each bar represents mean ± SEM. Statistical significance is indicated by * (P<0.05) and ** (P<0.01). B, relative gene expression was detected at 1 day before differentiation induction (day -1), initiation of differentiation (day 0), and 1, 2 and 3 days after differentiation induction and normalized by gACTB gene. Each bar represents mean ± SEM. Significant differences (P<0.05) between different time points are indicated by different letters (a - c).
Figure 6.3. Expression of HPS4 during development of adipose tissue. A, mRNA expression detected in subcutaneous adipose tissues on embryonic day 15 (E15) and day 17 (E17), post-hatch day 0 (P0), 5 (P5), 11 (P11) and 33 (P33) (n=4 for each age group) and normalized by chicken ribosomal protein S13 (gRPS13) gene. Each bar represents mean ± SEM. Significant differences (P<0.05) between different time points are indicated by different letters (a - b). B, protein expression detected by western blotting in subcutaneous adipose tissues of two randomly selected broiler chickens in each age group. Coomassie staining results are provided to show equal amounts of protein loaded to each well.
Figure 6.4. Alternative splicing of chicken HPS4. A, two alternative transcripts for chicken HPS4 in GenBank were confirmed by PCR with forward primer at exon 3 and reverse primer spanning exons 7 and 8. PCR products with cDNA from one fat sample on embryonic day 17 (E17) and another fat sample on posthatch day 33 (P33) were run on 1% agarose gel and two fragments (406 bp and 481 bp) were detected for both PCR products. B, relative expression of long transcript of HPS4 detected in subcutaneous adipose tissues on embryonic day 15 (E15) and day 17 (E17), post-hatch day 0 (P0), 5 (P5), 11 (P11) and 33 (P33) (n=4 for each age group) and normalized by chicken ribosomal protein S13 (gRPS13) gene. Each bar represents mean ± SEM. Significant differences (P<0.05) between different time points are indicated by different letters (a - c).
CHAPTER 7

CONCLUSIONS

The development and normal functions of different tissues and their coordination in living organisms depend on proper regulation and communication of numerous genes and their products. To unravel the complex network and solve the mysterious problems in life science, it is necessary to distinguish the master genes in various pathways. Since the master gene with important function in one tissue is usually synthesized more abundantly in that tissue, gene expression is usually a reliable index to determine the importance of one gene in one tissue, and identification of specific gene expression in one tissue usually leads to critical findings about that tissue. However, high expression of one gene in one tissue does not always mean that it has an important biological function in that tissue. For example, most proteins produced in the liver are transported to blood and other tissues where they play more important roles than in the liver itself. Therefore, identification of the secretion of one gene’s product is also beneficial for us to make better judgement about the gene’s function. Finally, because gene expression in one tissue usually changes during development due to change of composition in the tissue and alteration of received signals, temporal investigation of gene expression is also indispensable for complete understanding of one gene’s regulatory mechanism.

For a large number of genes in various tissues and time points in development, a microarray facilitates simultaneous detection of their expressions, which can be
compared after normalization. Therefore, microarray analysis is very useful for selection of important genes and novel genes in one tissue or cell type. If one microarray study is not adequate for accurate selection of important genes, several microarray studies with similar purpose will be able to support each other and provide more elaborate filtration in the selection process. In addition, the enormous information provided by a microarray study can hardly be completely covered in one paper. Therefore, the Gene Expression Omnibus (GEO) in the National Center for Biotechnology Information (NCBI) provides a useful tool with free access to a huge repository of microarray data from previous studies. With the availability of such a valuable resource, we can not only conduct initial selection of important genes, but can also predict the general function of the selected genes by combining results from multiple microarrays with similar treatments or conditions.

In this dissertation, the four studies, despite their different objectives, utilized one common strategy and methodology. First, novel or important target genes were selected based on analysis of GEO data or microarray data. Second, the properties of selected genes were then investigated using molecular technologies such as quantitative real-time PCR (qPCR), western blotting, immunostaining and cell culture.

In the first study, three important cell cycle inhibitors - CCNG2, CDKN2C and PMP22 - were selected based on their higher expression in adipose tissue than in other tissues in GEO datasets. After qPCR and western blot analysis, their expressions were found to be higher in the fat cell (FC) fraction than in the stromal vascular (SV) fraction, and increased during fat cell differentiation and fat tissue development. Therefore, the three genes were proposed to be important during adipocyte differentiation.
In the second study, the same strategy and GEO datasets were utilized to select three cell cycle activators – CCNA1, CCND3 and ANAPC5 – with lower expression in adipose tissue than in other tissues. In addition to the same qPCR studies in broiler chickens for the three cell cycle inhibitors and three cell cycle activators, cellularity studies including H&E staining and PCNA immunostaining were also combined for in depth study of development of chicken adipose tissue. In addition, the expressions of the three cell cycle inhibitors during primary cell culture and adipose development were found to be different from that in pigs, suggesting different regulation of these genes in different animals.

In the third study, tissue specific secretory genes were selected based on literature study and analysis of the same GEO dataset in mouse as in the previous study (Song et al., 2013). The secretion of five genes with predicted but unreported secretion was confirmed by expression vector construction, cell transfection and western blotting. In addition, alternative splicing that inhibits secretion was identified in CTLA2A and SERPINA1F. Finally, the functions of the five novel genes in specific tissues were predicted based on meta-analysis of various GEO profiles.

In the fourth study, a novel adipose specific gene – HPS4 – was identified by DNA microarray analysis of broiler chickens. Its higher expression in adipose tissue than in other tissues was then confirmed by qPCR and western blotting. Based on its higher expression in FC than in SV, as well as its increasing expression during primary cell culture and adipose tissue development, HPS4 was proposed to play an important role in adipocytes and adipogenesis. In addition, two different proteins possibly produced by alternative splicing were identified and showed different expression patterns during development of adipose tissue.
In all of these studies, target genes were first screened and filtered by GEO or microarray analysis, and then their expressions were characterized and functions were proposed. This powerful methodology can be extensively applied as an initial step for functional genomics studies.
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