# Molecular Cloning of Adipose Tissue-specific Genes by cDNA Microarray

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**ABSTRACT :** In an attempt to isolate novel molecules that may play a regulatory role in adipocyte differentiation, we devised an experimental strategy to identify adipose tissue-specific genes by modifying cDNA microarray technique. We used genefilter membranes containing approximately 15,000 rat non-redundant EST clones of which 4,000 EST were representative clones of known genes and 11,000 ESTs were uncharacterized clones. A series of hybridization of genefilter membranes with cDNA probes prepared from various rat tissues and nucleic acids sequence analysis allowed us to identify two adipose-tissue specific genes, adipocyte-specific secretory factor (ADSF) and H-rev107. Verification of tissue-specific expression patterns of these two genes by Northern blot analysis showed that ADSF mRNA is exclusive expressed in adipose tissue and the H-rev107 mRNA is predominantly expressed in adipose tissue. Further analysis of gene expression of ADSF and H-rev107 during 3T3-L1 adipocyte differentiation revealed that the ADSF and H-rev107 gene expression patterns are closely associated with the adipocyte differentiation program, indicating their possible role in the regulation of adipose tissue development. Overall, we demonstrated an application of modified cDNA microarray technique in molecular cloning, resulting in identification of two novel adipose tissue-specific genes. This technique will also be used as a useful tool in identifying novel genes expressed in a tissue-specific manner. ***(Asian-Aust. J. Anim. Sci. 2003. Vol 16, No. 12 : 1837-1841)***

**Key Words** : Microarray, Genefilter, Adipose, Tissue-specific, 3T3-L1, Differentiation

## INTRODUCTION

Adipose tissue is the major energy reservoir in higher eukaryotes; storing triacylglycerol in periods of energy excess and its mobilization during energy shortage are its primary purposes. During adipose tissue development, genes that code for the lipid transport and lipogenic and lipolytic enzymes are induced in order to carry out the adipocyte function of triacylglycerol synthesis, storage and mobilization. The role of adipose tissue mainly as an organ for energy storage and mobilization has recently been expanded by the discovery of leptin (Zhang et al., 1994; Halaas et al., 1995). Leptin is primarily made and secreted by mature adipocytes. It binds to its receptor in the hypothalamus and may function in regulating body fat mass (Maffei et al., 1995; Hotamisligil et al., 1996). Other immune system-related proteins such as TNF-α, adipsin, and ACRP30/AdipoQ along with vascular function-related molecules such as angiotensinogen and plasminogen activator inhibitor type I have been shown to be secreted by adipose tissue (Scherer et al., 1995; Hu et al., 1996; Shimomura et al., 1996). In addition, preadipocytes also secrete factors such as Pref-1 which inhibits adipocyte differentiation (Smas and Sul, 1993; Smas et al., 1997; Smas et al., 1998). Although the precise functions of these molecules are not clear, adipose tissue as a secretory organ to regulate other physiological processes as well as energy balance and homeostasis is now well established. Adipose tissue must secrete factors reflecting the nutritional status and regulating adipose tissue mass.

Among the techniques of functional genomics, DNA microarray, together with proteomics, allows to analyze the expression levels of thousands of genes simultaneously (Schena et al., 1995; Gerhold et al., 1999; Lennon, 2000). DNA microarray has been broadly used to study the alterations in the gene expression among different tissues, alterations between normal and pathological samples, and differential gene expression occurring during growth or as the result of environmental conditions. Given the fact of its higher sensitivity of detection and providing vast amount information of known and/or uncharacterized gene expressions, this technique has potential to discover novel genes specifically regulated by pathological condition, changes in environment, and tissue distribution instead of the conventional methods to genes (Ha et al., 2001; Kim et al., 2001; Kim et al., 2001b).

In an effort to clone and isolate adipose tissue specific genes, we devised an experimental strategy to accomplish our aim by modifying cDNA microarray technique. Here, we show the molecular cloning of two novel adipose tissue-specific genes whose expression patterns are correlated with the stage of adipocyte differentiation.

## MATERIALS AND METHODS

### Genefilter microarray analysis

Adipose tissue-specific genes were examined by microarray analysis using various rat Genefilter membranes (Research Genetics, USA). Five μg of total RNA isolated

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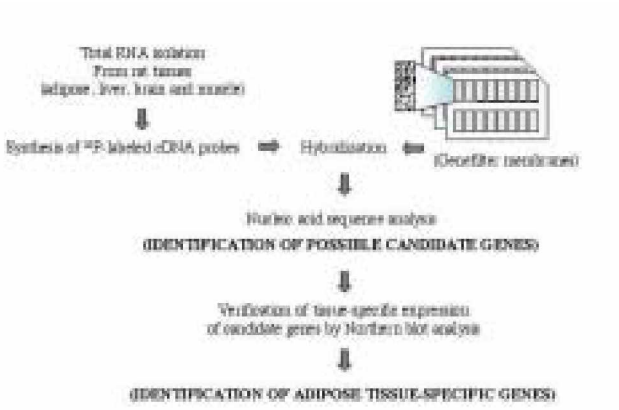


**Figure 1.** Experimental flow-chart for the identification of adipose tissue-specific genes. Various rat tissues were subjected to total RNA isolation followed by reverse transcription in the presence of $^{33}$P-dCTP for the synthesis of radiolabeled cDNA probes. After a series of hybridization of genefilter membranes with $^{33}$-P-cDNA probes, candidate adipose tissue-specific genes were analyzed by nucleic acid sequence analysis using NCBI and TIGR sequence databases. The tissue-specific expression patterns of the candidate genes were then verified by Northern blot analysis. See the "Results and Discussion" for the detail.

from various tissues of Sprague Dawley rat (Harlan, USA) including adipose, brain, liver, and skeletal muscle were used for the synthesis cDNA probe. According to the manufacturer's protocol, 2 μg of total RNA was mixed with 2 μl oligo(dT), incubated for 5 min at 65°C, and allowed to cool to room temperature for 5 min. We then added 2 μl reverse transcriptase buffer (Invitrogen, USA), 2 μl 10 mM dNTP, 10 μl α-[$^{33}$P] dCTP (approximately 30 μCi; specific activity >3,000 Ci/mmol) (ICN, USA), 300 U SUPERSCRIPT II (Invitrogen, USA). DEPC-treated water was added to obtain a total reaction volume of 20 μl. After mixing, the sample was incubated at 42°C for 1 h. Prior to hybridization, the labeled cDNA was denatured by boiling for 3 min and subsequently cooled on ice for 5 min. The genefilter membranes were prehybridized for overnight at 42°C in a hybridization oven with 5 ml hybridization buffer containing 1 μg/ml heat denatured CotDNA (Invitrogen, USA) and 100 g/ml heat-denatured salmon sperm DNA. After hybridization, the membranes were washed twice in 2xSSC/1% SDS at 50°C for 20 min, washed twice for 20 min and then once in 0.5×SSC/1% SDS at room temperature for 15 min. The membranes were stored wet in deionized water in a sealed plastic bag and exposed to X-ray films for 3-5 days. Before beginning a new hybridization, membranes were stripped using hot 0.5% SDS solution. Spots exclusively hybridized with cDNA probes prepared from adipose tissue RNA were used for further sequence analysis.

**Differentiation of 3T3-L1 cells into adipocytes**

3T3-L1 preadipocytes were maintained in DMEM containing 10% fetal bovine serum. For adipocyte differentiation, confluent cells were treated with 1 μM dexamothasone and 0.5 mM methylisobutylxanthine for 2 days. The cells then were maintained in the DMEM containing 10% fetal bovine serum for an additional 7 days.

**Isolation of RNA and northern blot analysis**

The total RNA from various rat tissues was prepared by guanidine isothiocyanate/cesium chloride centrifugation. The total RNA from the 3T3-L1 cells was prepared using TriZOL reagent (LifeTechnologies, USA). RNA was electrophoresed in 1% formaldehyde-agarose gel in 2.2 M formaldehyde, 20 mM MOPS, 1 mM EDTA, and transferred to Hybond N (Amersham Pharmacia, USA). After UV cross-linking, the membranes were hybridized with the $^{32}$P-labeled cDNA probes in ExpressHyb solution (Clontech, USA). The membranes were exposed to X-ray film with an intensifying screen and the signals were scanned using the Molecular Analyst (Bio-Rad).

## RESULTS AND DISCUSSION

**Identification of candidate genes**

Figure 1 depicts our strategy for identification of adipose tissue-specific candidate genes. To identify novel adipose tissue-specific genes, we compared expression levels of rat expressed sequence tag (EST) sequences by cDNA microarray. RNA samples were prepared from rat adipose tissue as well as liver, skeletal muscle, and brain. The total RNAs were used to label and synthesize -[$^{33}$P] dCTP labeled cDNAs for the hybridization with genefilter membranes containing approximately 15,000 rat non-redundant EST clones. Of those 15,000 EST clones, approximately 4,000 ESTs are representative clones of known rat genes and 11,000 ESTs are uncharacterized clones. After a series of hybridization, those sequences that were expressed only in adipose tissues but not in other tissues we examined were identified. Total 10 candidate EST clones were sequenced and identified by using both TIGR the Institute for Genomic Research and National Center for Biotechnology Information sequence database.

**Expression of candidate genes in adipose tissue**

In order to further verify their tissue specific gene expression, we performed Northern blot analysis. When multiple tissue blot containing total RNA from various rat tissues including white adipose tissue, brain, heart, small intestine, kidney, liver, lung, and skeletal muscle, was blotted with these 10 candidate probes, we observed that only 2 genes out of 10 candidates exhibited a significant signal in the fat pad (Figure 2). The further verification of

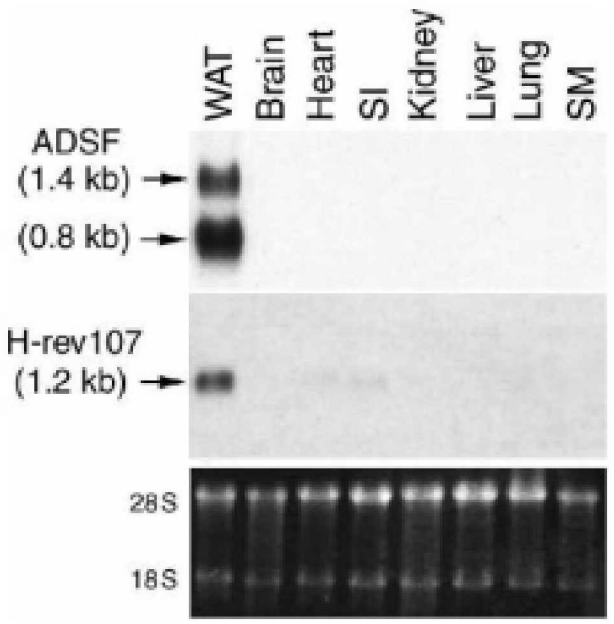


**Figure 2.** ADSF and H-rev107 expression in various rat tissues. Ten g of total RNA prepared from various tissues from rats were subjected to Northern blot analysis. The membrane was hybridized with $^{32}$P-labeled full-length ADSF or Hrev-107 cDNAs as described under "Materials and Methods". Twenty-eight S and 18 S ribosomal RNA form ethidium bromide-stained gel are shown. WAT: white adipose tissue; SI: small intestine; SM: skeletal muscle.

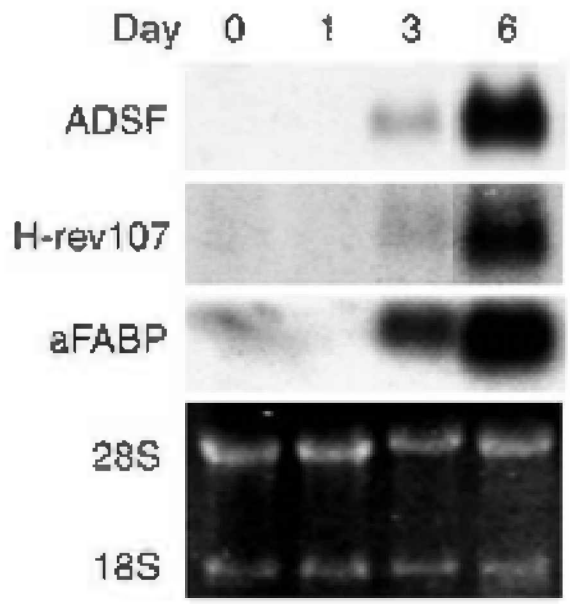


**Figure 3.** Time course of the expression of ADSF and H-rev107 during adipocyte differentiation of 3T3-L1 cells. Total RNA from cells at the indicated time points of 0 (at confluence), day 1, day 3, and day 6 were prepared, and 10 μg of RNA were subjected to Northern blot analysis for ADSF, H-rev 107 and aFABP. Twenty-eight S and 18 S ribosomal RNA form ethidium bromide-stained gel are shown.

tissue-specific gene expression pattern of these two novel genes in mouse tissues also confirmed their adipose tissue-specificity (data not shown). One of such sequences was identified as that coding for a novel adipose tissue-specific, serine- and cysteine-rich secreted protein that does not belong to any known classes of cysteine-rich proteins. We named this gene ADSF (Kim et al., 2001). Rat ADSF mRNAs showed two transcripts such as a1.4 kb and a 0.8 kb due to the multiple polyadenylation signal sequences located in 3'-untranslated region. Whereas, a single 0.8 kb mRNA was detected only in murine adipose tissue (data not shown). ADSF mRNA was exclusively expressed only in adipose tissue but not other tissues we examined (Figure 2). The gene was originally named as *FIZZ3*, which belongs to a gene family whose founding member, *FIZZ1*, is implicated as a possible mediator of neuronal function and airway hyperactivity (Holcomb et al., 2000). Simultaneously, Steppen et al. also cloned ADSF as a thiazolidinediones (TZDs), antidiabetic drugs, down-regulated factor contributing to insulin resistance, named resistin (Steppan et al., 2001). While, the second adipose tissue-specific gene has identical nucleic acid sequence to previously characterized H-rev 107. Although 1.2 kb of H-rev 107 mRNA expression is predominant in adipose tissue, lower levels of H-rev 107 mRNA expression were also detected in other rat tissues including heart, small intestine and lung from prolonged exposure of Northern blot (data not shown). H-rev 107 has originally cloned as a member of the class II tumor suppressor and it is a ubiquitously expressed gene encoding a 16 kDa protein localized in both the cytoplasm and cell membrane (Hajnal et al., 1994). Recently, it became evident that H-rev 107 is the member of a novel family of proteins involved in the control of cell proliferation. This protein family includes A-C1, predominantly expressed in skeletal muscle and TIG3/RIG1/hH-Rev107-2, a retinoid-induced gene in keratinocytes (DiSepio et al., 1998; Husmann et al., 1998; Akiyama et al., 1999; Huang et al., 2000). H-rev107 has been cloned as a gene highly expressed in a revertant cell line isolated from H-ras transformed rat fibroblasts (Schafer et al., 1991; Akiyama et al., 1999). Its overexpression in H-Ras-expressing cell lines resulted in reduction of colony formation and attenuated tumor growth in nude mice implying its tumor-suppressing activity (Sers et al., 1997). Its anti-proliferative activity was also indicated by the fact that cells overexpressing H-rev 107 could not be maintained in culture for an extended period of time (Hajnal et al., 1994; Sers et al., 1997). Similarly, the H-rev 107-related proteins, A-C1 and TIG3, significantly inhibited cell proliferation of H-ras-transformed NIH3T3 and 293 cells, respectively (DiSepio et al., 1998; Akiyama et al., 1999). By examining a variety of human and rat tumors and tumor

cell lines, Husmann et al. (Husmann et al., 1998) came to the conclusion that H-rev107 expression was blocked at the level of transcription and translation. However, the H-rev 107 gene expression and its physiological function in adipose development have not yet been examined.

**Analysis of gene expression of ADSF and H-rev107 during 3T3-L1 adipocyte differentiation**

In order to further examine the potential role of ADSF and H-rev 107 in adipocyte differentiation, we studied the time course of both ADSF and H-rev 107 expression during 3T3-L1 adipocyte differentiation. As shown in Figure 3, the ADSF mRNA was not detectable in the preadiopocyte stage (Day 0). However, levels of the 0.8 kb murine mRNA, as predicted by the murine origin of 3T3-L1 cells, were markedly increased upon treatment with 1 μM dexamethasone and 0.5 mM methylisobutylxanthine. The levels of ADSF mRNA were increased significantly after day 3. While, the H-rev 107 mRNA was also undetectable in the preadipocyte stage (Day 0). Unlike ADSF, the level of H-rev 107 mRNA was still in low at day 3 and its gene expression levels were gradually increased to highest level at day 6. As anticipated, expression of adipocyte marker such as an adipocyte fatty acid-binding protein (aFABP) was induced during adipose conversion. The results of 3T3-L1 differentiation clearly show that the mRNA levels of ADSF and H-rev107 increase during late stage of adipose tissue development, and therefore ADSF and H-rev 107 is expressed only in adipose tissue in mature rodents. Indeed, further characterization of ADSF in the regulation of adipocyte differentiation and insulin resistance indicates the critical role of ADSF in lipid and glucose homeostasis as well as a linker between obesity and obesity associated type 2 diabetes (Steppan et al., 2001). It is also interesting to further study the physiological function of H-rev 107 in adipose development in the future. Understanding its role as a tumor suppressor and anti-proliferative activity in cell culture as well as adipose tissue-specific expression pattern correlated with adipocyte differentiation, it is plausible to expect the possible involvement of H-rev 107 in the early processes of adipocyte differentiation such as growth arrest and clonal expansion of the confluent preadipocyte. However, further functional studies should prove this hypothesis.

In conclusion, we demonstrated an application of modified cDNA microarray technique in molecular cloning of adipose tissue-specific genes, which might play an important role in the regulation of adipose development. The application of cDNA microarray technology to molecular cloning provides useful tool to identify novel tissue-specific genes and saves time and efforts compared with conventional gene cloning methods. Although the efficacy of cloning of genes using this technique is totally dependent upon the number of uncharacterized EST clones spotted on the membranes, currently developed custom-designed array should overcome this limitation.

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