

# Regulation of Global Gene Expression by Ovariectomy and Estrogen in Female Adipose Tissue

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## Abstract

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**Objective:** To study the effects of ovariectomy (OVX) and estrogen replacement on global gene expression in white adipose tissue.

**Research Methods and Procedures:** Female mice were randomly divided into four groups: 1) intact, 2) OVX, 3) OVX plus estradiol ( $E_2$ ) injection 3 hours before death ( $E_2$ -3 hours), and 4)  $E_2$ -24 hours. The serial analysis of gene expression was performed to detect the transcriptomic changes.

**Results:** A total of 15 transcripts, including several novel transcripts, were found to be modulated by OVX or  $E_2$  ( $p < 0.05$ ). Secreted acidic cysteine-rich glycoprotein, which regulates the extracellular matrix (ECM) components, was increased after OVX. Moreover, OVX up-regulated several transcripts involved in ECM, such as procollagen types I $\alpha$ 1 and 2. In cell defense, glutathione peroxidase 3 was lower in OVX than in intact mice. Cytochrome c oxidase I and three novel transcripts were up-regulated by estrogen treatment.

**Discussion:** This study underlines the importance of cell shape and ECM regulation by OVX on adiposity. Moreover, some novel transcripts may also play a relevant role in OVX-induced obesity and estrogen therapy.

**Key words:** serial analysis of gene expression, functional genomics, sex hormone, bioinformatics

## Introduction

Fat accumulation has been shown to be associated with type 2 diabetes, cardiovascular diseases, certain cancers, and other health problems. Moreover, adipose tissue has been thought to act as a steroid-metabolizing compartment, which has potential impact on the regulation of adipocyte function and other metabolic parameters (1). Evidences from both human and animal experiments suggest that estrogen plays an important role in the regulation of white adipose tissue (WAT).<sup>1</sup> Postmenopausal women have increased WAT, and estrogen therapy decreases WAT levels compared with untreated postmenopausal women (2,3). Animal experiments have also shown that ovariectomy (OVX) of rodents increases WAT, and estrogen replacement decreases WAT (4–6). In the regulation of body weight and energy balance, the effects of estrogen were found to be not only directly on the central nervous system but also on peripheral sites such as adipose tissue. However, the molecular mechanisms of the effects of OVX and estrogen on WAT still needed to be clarified.

OVX in combination with steroid hormone replacement has been established as an appropriate model for studying hormone regulation of target tissues. This model has been widely used to study the effects of estrogen on body weight and energy balance (4). This study focused on understanding the effects of OVX and estrogen on adipose tissue in the molecular aspect. Using the serial analysis gene expression (SAGE) strategy, we were able to identify the transcriptome that responds to OVX and estrogen in vivo in adipose tissue of female mice.

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<sup>1</sup> Nonstandard abbreviations: WAT, white adipose tissue; OVX, ovariectomy; SAGE, serial analysis gene expression;  $E_2$ , 17 $\beta$ -estradiol; EST, expressed sequence tag; CA III, carbonic anhydrase 3; COX, cytochrome c oxidase; ECM, extracellular matrix; SPARC, secreted acidic cysteine rich glycoprotein; FAS, fatty acid synthase.

The SAGE method has been developed and introduced as a useful tool for studying molecular events (7–10). This method can evaluate thousands of gene expressions quantitatively and simultaneously. SAGE can generate a genomic profile relying on cDNA sequences of 15 bp (SAGE tags) for transcript identification and cloning. Thus, different genomic profiles can be compared precisely. In this study, we performed the SAGE method in adipose tissues of female mice, and the gene expression profile affected by OVX and estrogen replacement was studied.

## Research Methods and Procedures

### Sample Preparation

Female C57BL6 mice, 12 to 14 weeks of age, were purchased from Charles River Canada (St-Constant, Québec, Canada). Animals were provided Laboratory Rodent Diet No. 5002 (PMI, St. Louis, MO) and water ad libitum. Intact mice ( $n = 10$ ) were sham-operated, whereas OVX mice ( $n = 10$ ) had surgery 7 days before death. Vehicle for the intact and OVX groups was injected 24 hours before death. For estrogen replacement groups,  $17\beta$ -estradiol ( $E_2 = 0.05 \mu\text{g}/\text{mouse}$ ) was injected to OVX mice separately at 3 ( $E_2$ -3 hours;  $n = 14$ ) and 24 hours ( $E_2$ -24 hours;  $n = 14$ ) before death. The retroperitoneal fat was dissected, frozen in liquid nitrogen, and stored at  $-80^\circ\text{C}$  until analysis.

### SAGE

Fat tissues were separately pooled for each group, and total RNA was extracted with TRIzol Regent (Invitrogen, Carlsbad, CA). Polyadenylated RNA was purified with the Oligotex mRNA Mini Kit (Qiagen, Mississauga, Canada). The SAGE method was performed according to the strategy described by Velculescu et al. (7,8) and the modifications of Kenzelmann and Muhlemann (9), as well as the optimizations of St-Amand et al. (10). After the annealing of biotin-5'T<sub>18</sub>-3' primer, the mRNA was converted to cDNA with an Invitrogen synthesis kit and cleaved with *Nla*III. The 3' restriction fragment was isolated with streptavidin-coated magnetic beads (Dyna, Oslo, Norway) and ligated to one of two annealed linker pairs. After extensive washing to remove unligated linkers, adjacent tags were released from the magnetic beads by cleavage with *Bsm*FI for 3 hours at  $37^\circ\text{C}$ . The blunting kit of Takara Bio (Otsu, Japan) was used for the blunting and ligation of tags, because both reactions could be performed consecutively without phenol chloroform extraction and precipitation of short tags. The produced ditags were amplified by shortened polymerase chain reaction with an initial denaturing step of 1 minute at  $95^\circ\text{C}$ , followed by 24 cycles consisting of 20 seconds at  $94^\circ\text{C}$ , 20 seconds at  $60^\circ\text{C}$ , and 2 seconds at  $72^\circ\text{C}$  (10). The polymerase chain reaction products were analyzed by polyacrylamide gel electrophoresis and digested with

*Nla*III. The band containing the ditags was excised and self-ligated to produce long concatemers. The concatemers ranging from 500 to 1500 bp were isolated by agarose gel. These products were cloned into the *Sph*I site of pUC19, and white colonies were screened by polymerase chain reaction to select long inserts for automated sequencing.

### Data Analysis

The sequence and occurrence of each of the transcript tags were determined using the software SAGEana program, a modification of SAGEparser.pl, which is freely available to academia (<ftp://ftp.pbrc.edu/public/eesnyder/SAGE/>). To identify the corresponding transcript, the sequences of 15-bp tags (*Nla*III site plus the adjacent 11 bp) were matched with UniGene and GenBank database (<http://www.ncbi.nlm.nih.gov/>). Classification of genes was done according to information from the genome directory (11). For the SAGE library comparisons, the comparative count display program (12) was used to identify the transcripts that were differentially expressed. We focused on transcripts with  $>2$ -fold difference for a statistical significance setting at  $p < 0.05$ . The data were normalized to 50,000 tags to facilitate visual comparison.

## Results

An average of 43,773 SAGE tags were sequenced and derived from an average of 22,928 transcript species for each group of female mice (Table 1). Approximately 13% of these tag species matched with well-characterized transcripts, 19% with partially characterized transcripts, which included an expressed sequence tag (EST), and 65% of the tags did not match with any known transcripts or ESTs. Transcript species expressed  $>0.1\%$  and the tags detected more than once were also calculated. From 58 to 65 transcripts were expressed in  $>0.1\%$  of the mRNA population. From 4774 to 5450 tags were sequenced more than once in one of the four groups.

### Most Abundant Transcripts in Adipose Tissue of Female Mice

The most expressed transcripts in intact adipose tissue of female mice are shown in Table 2. The relative frequency of a given tag was calculated by dividing the observed tag count by the total count of sequenced tags in each group. Eleven tag species in intact adipose tissue were expressed  $>0.5\%$ . Among them, eight transcripts were found to be expressed  $>0.4\%$  in all groups. They included the EST adipsin, two different sequences of carbonic anhydrase 3 (CA III), ATP synthase subunit 6, fatty acid binding protein 4, cytochrome c oxidase subunit III (COX 3), and stearyl-coenzyme A desaturase 1, and one tag matched with 27 transcript sequences in public databases. In addition, COX 1 was highly expressed, except for  $E_2$ -3 hours. The tags

**Table 1.** Number of tags in each group of female mice

	Intact	OVX	OVX + E <sub>2</sub>		Average
			3 hours	24 hours	
Tags sequenced	49,237	42,731	42,794	40,331	43,773
Transcript species	23,423	21,667	23,217	23,403	22,928
Well-characterized transcripts (%)	2,878 (12)	3,031 (14)	3,148 (13)	3,216 (14)	3,068 (13)
Partially characterized (%)	4,288 (18)	4,143 (19)	4,412 (19)	4,531 (19)	4,344 (19)
No match transcripts (%)	15,625 (67)	13,881 (64)	15,035 (65)	14,995 (64)	14,884 (65)
Multiple matches (%)	632 (3)	612 (3)	622 (3)	661 (3)	632 (3)
Transcript species expressed >0.1%	65	62	58	60	61
Well-characterized transcripts (%)	39 (60)	39 (63)	38 (66)	41 (68)	39 (64)
Partially characterized (%)	6 (9)	5 (8)	4 (7)	4 (7)	5 (8)
No match transcripts (%)	11 (17)	6 (10)	4 (7)	4 (7)	6 (10)
Multiple matches (%)	9 (14)	12 (19)	12 (20)	11 (18)	11 (18)
Tag detected more than once	5,027	4,774	5,092	5,450	5,086
Well characterized transcripts (%)	1,526 (30)	1,524 (32)	1,587 (31)	1,697 (31)	1,584 (31)
Partially characterized (%)	1,396 (28)	1,437 (30)	1,489 (29)	1,630 (30)	1,488 (29)
No match transcripts (%)	1,749 (35)	1,486 (31)	1,656 (33)	1,763 (32)	1,664 (33)
Multiple matches (%)	356 (7)	327 (7)	360 (7)	360 (7)	351 (7)

GAAAATGAGAA and TTTGACAATGA did not match with any known transcript in the public databases.

#### **Transcripts Regulated by OVX and E<sub>2</sub> Replacement**

Table 3 shows that several components of the extracellular matrix (ECM) were up-regulated in the OVX group compared with the intact group. Seven transcripts were differentially expressed ( $p < 0.05$ ; ratios = 2.7 to 10.7), such as procollagen type I $\alpha$ 2, secreted acidic cysteine rich glycoprotein (SPARC), and EST procollagen types I $\alpha$ 1 and 2. The expression level of these transcripts was not significantly different between OVX and estradiol replacement groups. Furthermore, fatty acid synthase (FAS), glutathione peroxidase 3, a RIKEN sequence, and the novel transcript with the sequence TTTGACAATGA were down-regulated by OVX (Table 4). The ratios for the OVX group to the intact group were from 2.5 to 4.9.

The effects of E<sub>2</sub> on transcriptome are shown in Table 5. One gene involved in energy metabolism (*COX 1*) and one potential novel transcript were down-regulated by 3-hour E<sub>2</sub> treatment. One novel transcript was down-regulated by 3- and 24-hour treatment. Another one was down-regulated by OVX and also by 24-hour treatment.

### **Discussion**

SAGE allows simultaneous and quantitative measurement of the expression of thousands of well-known and unknown genes. Using this strategy, we identified the

changes of gene expression in adipose tissue induced by OVX and estrogen replacement. Moreover, the highest expressed genes in adipose tissue of female mice are reported for the first time, to our knowledge. This study provides a better understanding of the molecular events induced by OVX and estrogen replacement in adipose tissue. The main findings of this study were the following: 1) transcripts involved in energy, lipid metabolism, and cell defense were most abundantly expressed in adipose tissue; 2) OVX up-regulated several components of ECM that are involved in cell shape and differentiation of adipocytes; 3) some transcripts involved in metabolism and cell defense were down-regulated by OVX and estradiol replacement; and 4) several novel transcripts were also differentially regulated.

#### **Most Abundant Transcripts in the Adipose Tissue of Female Mice**

This study has shown that the most highly expressed gene in adipose tissue of female mice is *EST adipsin*. In fact, adipsin is known to have a high level of expression in human fat (13) and to be deficient in several animal models of obesity (14). Although its function is not completely clear thus far, these results for adipsin show that this transcript is rich in adipose tissue and may play an important role in the physiology and pathology of adipose tissue.

The transcripts related to lipid metabolism such as stearyl-coenzyme A desaturase 1 and fatty acid binding protein 4 were also highly expressed in adipose tissue of female mice. ATP synthase subunit 6, COX 1, and COX 3,

**Table 2.** The most expressed genes in adipose tissue of female mice

Tag	Intact No. of tags (%)	OVX No. of tags (%)	OVX + E <sub>2</sub>		Description (UniGene cluster, GenBank accession no.)
			3 hours No. of tags (%)	24 hours No. of tags (%)	
CATCTGAAAAA	802 (1.60%)	571 (1.14%)	596 (1.19%)	589 (1.18%)	EST adipsin (Mm.4407,AW215391)
GAAAATGAGAA	474 (0.95%)	256 (0.51%)	13† (0.03%)	11 (0.02%)†	NM
GTGGCTCACAA	448 (0.90%)	424 (0.85%)	376 (0.75%)	429 (0.86%)	27 matches
GCTGCCCTCCA	421 (0.84%)	332 (0.66%)	121 (0.24%)	517 (1.03%)	COX I (Mm.MTG.3, NC_001569, Pos:6816)
AATTCACACC	416 (0.83%)	316 (0.63%)	283 (0.57%)	301 (0.60%)	CA III (Mm.300, NM007606)
ATAATACATAA	411 (0.82%)	310 (0.62%)	333 (0.67%)	375 (0.75%)	ATP synthase subunit 6 (Mm.MTG.6, NC_001569, Pos:8596)
TTTGACAATGA	405 (0.81%)	162 (0.32%)*	207 (0.41%)	201 (0.40%)	NM
AGCCAAAGGAA	348 (0.70%)	218 (0.44%)	252 (0.50%)	345 (0.69%)	Fatty acid binding protein 4 (Mm.582, NM024406)
AAAACCATTGC	336 (0.67%)	230 (0.46%)	242 (0.48%)	228 (0.46%)	Stearoyl-coenzyme A desaturase 1 (Mm.140785, BC007474)
ATACTGACATT	310 (0.62%)	243 (0.49%)	340 (0.68%)	296 (0.59%)	COX III (Mm.MTG.7, NC_001569, Pos:9325)
CCTATTAATAA	294 (0.59%)	312 (0.62%)	299 (0.60%)	297 (0.59%)	CA III (Mm.300, AK003671)

\* Significant decrease ( $p < 0.05$ ) compared with the intact group.

† Significant decrease ( $p < 0.05$ ) compared with the OVX groups.  
NM, no match.

which support a high  $\beta$ -oxidative flux, were highly expressed over three or four groups. The fact that these highly expressed genes are involved in energy and lipid metabolism is concordant with the evidence that adipose tissue is an organ not only for fuel storage but also for energy partitioning, channeling lipid energy to accumulation or oxidation (15,16).

Two transcript species coding for CA III were also found to be highly expressed. CA III is a member of a large family that functions in cell defense in a variety of biological processes, including respiration, calcification, and acid-base balance (17). Interestingly, CA III has previously been identified as a major constituent of murine adipose tissue and may be involved in energy balance in animals (18).

There are two novel transcripts that are among the 11 most expressed genes, and both were modulated by OVX or estrogen. These findings suggest that these two unknown transcripts may also play a critical role in adipose tissue and provide a clue to identifying new relevant genes in adipose tissue.

#### **Transcripts Regulated by OVX and E<sub>2</sub> Replacement**

It is well known that OVX of rodents increases WAT, and estrogen replacement decreases WAT (4). However, the

molecular characteristics of adipose tissue in OVX and hormone replacement are not clear. Thus, we studied the changes in transcriptome, and we identified 15 transcripts that were differentially expressed in the OVX and estradiol replacement groups. Interestingly, we found that some components of ECM were up-regulated by OVX. They included procollagen type I and several ESTs of procollagen type I, fibronectin 1, and SPARC. In multicellular organisms, ECM has a crucial role in the regulation of fundamental cellular processes such as growth, differentiation, survival/apoptosis, and gene expression (19–21). Several studies have shown that accretion of collagen and fibronectin on the fat cell surface generally increases during adipose differentiation (22–24). In addition, ECM could be produced by mouse preadipocytes during adipose differentiation in culture (25). ECM can be also secreted by microvascular endothelial cells, and this ECM derived from adipose tissue may play a role in the regulation of adipose tissue growth (26). Therefore, the increased ECM by OVX may be a key factor for fat increase in this situation.

In addition, SPARC is well known to mediate cell–matrix interactions and play a role in modulation of cell adhesion, differentiation, and angiogenesis (27,28). The transcript of SPARC was up-regulated by OVX in adipose tissue in our

**Table 3.** Components of ECM differentially expressed in the OVX group

Tag	Intact	OVX	OVX + E <sub>2</sub>		Description (UniGene cluster, GenBank accession no.)
			3 hours	24 hours	
Unique matches					
CTTTATTCCAG	3	32*	26	26	EST procollagen, type I $\alpha$ 1 (Mm.22621, BB731161)
TGGAAATGACC	12	52*	37	43	EST procollagen, type I $\alpha$ 1 (Mm.22621, BI794771)
CGCCTGCTAGC	27	85*	73	92	Procollagen, type I $\alpha$ 2 (Mm.4482, NM_007743)
GTTCCAAAGAA	3	26*	16	28	EST procollagen, type I $\alpha$ 2 (Mm.4482, AW545978)
CAAACCTCTCAC	79	211*	178	163	Secreted acidic cysteine rich glycoprotein (Mm.35439, NM_009242)
Multiple matches					
GACTTTGGAAA	18	82*	59	74	Procollagen, type I $\alpha$ 1 (Mm.22621, U08020), neuropathy target esterase (Mm.23085, NM_015801)
CCAACGCTTTA	7	34*	17	30	Fibronectin 1 (Mm.193099, BC004724); EST cellular retinoic acid binding protein I (Mm.34797, AA260205)

\* Significant increase ( $p < 0.05$ ) compared with the intact group.

study. This gene has been found to be up-regulated in three different models of obesity and is identified to be an auto-crine/paracrine factor that could affect key functions in adipose tissue physiology and pathology (29). A study on SPARC-null mice reported increased adiposity without a significant difference in overall body weight (30). Moreover, that study indicated that SPARC limits the accumulation of adipose tissue in mice, in part through its demonstrated effects on the regulation of cell shape and production

of ECM. In this study, the up-regulation of SPARC by OVX may reflect a result of compensation for the increase of adipose differentiation common in obesity rodents. The up-regulated component of cell structure and ECM by OVX in this study suggests that it may play an important role in the increase in adipose tissue of OVX rodents and post-menopausal women.

A previous study has shown that OVX decreases FAS activity in hepatic cytosol obtained from female mutant

**Table 4.** Other transcripts differentially expressed in OVX group

Tag	Intact	OVX	OVX + E <sub>2</sub>		Description (UniGene cluster, GenBank accession no.)
			3 hours	24 hours	
Metabolism					
ATGCAGGGCCA	154	43*	81	85	FAS (Mm.3760, AF127033)
Cell and organism defense					
CTATCCTCTCA	97	20*	25	44	Glutathione peroxidase 3 (Mm.7156, NM_008161)
Uncharacterized function					
TTTGCTTTAAA	85	27*	41	31	RIKEN cDNA 2810417H13 gene (Mm.45765, AK011090), expressed sequence AW538652 (Mm.25316, BC021502)
Novel transcripts					
TTTGACAATGA	405	162*	207	201	NM

\* Significant decrease ( $p < 0.05$ ) compared with the intact group.  
NM, no match.

**Table 5.** Transcripts differentially expressed in estrogen-treated groups

Tag	Intact	OVX	OVX + E <sub>2</sub>		Description (UniGene cluster, GenBank accession no.)
			3 hours	24 hours	
Metabolism					
GCTGCCCTCCA	421	332	121†	517	COX I (Mm.MTG.3, NC_001569)
Novel transcripts					
AAAAATCATCG	54	28	2†	62	NM
GAAAATGAGAA	474	256	13†	11†	NM
ATTTTCAGTTT	150	47*	7	4†	NM

\* Significant decrease ( $p < 0.05$ ) compared with the intact group.

† Significant decrease ( $p < 0.05$ ) compared with the OVX groups.

NM, no match.

analbuminemic rats, which show marked hypertriglyceridemia compared with males (31). However, no effect was observed in control rats. It is well known that sex steroid hormones influence lipid metabolism in adipose tissue (32–34). These studies have shown that estradiol treatment lowers FAS in adipose tissue of OVX rats. These results are similar to ours, suggesting that estrogen is involved in inhibition of lipogenesis.

It is well known that glutathione peroxidase 3 catalyzes the reduction of hydrogen peroxide, organic hydroperoxide, and lipid peroxides in the protection of cells against oxidative damage. A study has shown that GDX decreases glutathione peroxidase and increases oxidative damage to cardiac membranes in female but not in male rats (35). These data suggest that estrogen may have an antioxidant role in heart muscle. Interestingly, we also found that OVX down-regulated glutathione peroxidase 3 in adipose tissue.

In this study, no significant effect of estrogen replacement on the OVX-modulated transcripts was observed, although some recovery trends by estrogen replacement were found, such as with FAS, which was significantly decreased by OVX by about 3-fold and increased by estrogen nearly 2-fold compared with the OVX group. Because short-term estrogen treatment does not modify most gene expression, it will be worthwhile to more fully study the time-course and potential mechanisms. It is possible that estrogen's major effect on adipose tissue gene expression occurs through changes in adipose tissue mass. On the other hand, factors other than estrogen may be involved in the effects of OVX on adipose tissue.

In addition, we also found one novel transcript down-regulated by OVX and three transcripts down-regulated by estrogen replacement. They were 11- to 20-fold down-regulated in the estrogen replacement group compared with the OVX group. Further characterizations of these tran-

scripts need to be performed to characterize new mechanisms involved in the development of adiposity.

In conclusion, this study describes estrogen-regulated genes that are affected by OVX and estrogen treatment in the adipose tissue of female mice. A total of 11 transcript species, including 2 novel transcripts, were highly expressed in adipose tissue. They are involved mainly in energy and lipid metabolism, as well as in cell defense. The up-regulation of ECM components and cell structure by OVX suggests that they may play an important role in increased adiposity in estrogen-deprived rodents or postmenopausal women. Moreover, this study has identified novel transcripts regulated by OVX and estrogen replacement.

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