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Distinct expression profiles of LncRNAs between brown adipose tissue and skeletal muscle

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ABSTRACT

Both brown adipose tissue and skeletal muscle have abundant mitochondria and energy consumption capacity. They are similar in origin and gain different potential of energy metabolism after differentiation and maturation. The mechanism that cause the difference is not yet fully understood. Long non-coding RNAs (lncRNAs) which comprise the bulk of the human non-coding transcriptome have been proved to play key roles in various biological processes. Whether they will have a function on the differentiation and energy metabolism between BAT and skeletal muscle is still unknown. To identify the cellular long noncoding RNAs (lncRNAs) involved in the progress, we used the next generation transcriptome sequencing and microarray techniques, and investigated 704 up-regulated and 896 down-regulated lncRNAs (fold-change >3.0) in BAT by comparing the expression profile. Furthermore, we reported AK003288 associated with junctophilin 2 (Jph2) gene which may affect energy metabolism. This study show distinct expression profiles of LncRNAs between brown adipose tissue and skeletal muscle which provide information for further research on differentiation of adipocyte and transdifferentiation between BAT and skeletal muscle that will be helpful to find a new therapeutic target for combatting obesity.

1. Introduction

Brown adipose tissue (BAT) which is specialized for thermogenic energy expenditure has attracted people's focus as a potential target for the treatment of obesity [1]. Excess energy from food can be stored in white adipose tissue (WAT) but will be combusted in BAT. BAT have a large number of mitochondria which can use energy to release heat by oxidative phosphorylation [2], and uniquely express uncoupling protein-1 (UCP1). For a long time, BAT was thought to be only of relevance in small mammals or newborn child, but recent study prove that BAT plays an important role in human adults by using fluorodeoxyglucose positron emission tomography (FDG-PET) [3]. Skeletal muscles support the body weight during upright standing and make it possible to perform body movements. Skeletal muscle has a unique ability to rapidly increase its rate of energy consumption in situations where explosive contractions are required within a few milliseconds. Skeletal muscle also has to be able to maintain a moderate increase in energy consumptions during prolonged periods of low-intensity contractions [4]. Energy is consumed by the myosin heads or cross-bridges, and by ion pumps, mainly the sarcoplasmic reticulum (SR) Ca2+ pumps during the process of muscle contraction, and ATP is the immediate energy source during muscle contraction [5].

BAT along with white adipocytes and myocytes differentiate from mesenchymal stem cells (MSCs) [6]. Now study has shown that WAT and BAT have different precursor cells in the early developmental stages, and the differentiation lineages of brown adipose tissue and muscle tissue are much more closer [7]. Both BAT and skeletal muscle have abundant mitochondria and energy consumption capacity. Vicente Gilsanz et al. found that functional brown adipose tissue is related to muscle volume in Children. Children with visualized BAT on PET/CT examinations had significantly greater muscle volume than children with no visualized BAT [6]. Now study show that switching muscle to BAT can reduce weight gain and improve metabolism [8]. Substantial evidence concluded that BAT activation leads to increased thermogenesis and can prevent or reverse obesity and diabetes in multiple experimental models. So in this study, we will focus our vision on the relationship between BAT and muscle.

LncRNA (Long non-coding RNA, lncRNA) whose transcripts is longer than 200nt is a class of non-coding RNA molecules and itself does not encode proteins. Although in recent years, lncRNA were

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found to be associated with X-chromosome inactivation, genomic imprinting, chromatin modification, transcription activation and inhibition, RNA shear, embryonic and tissue development, but their type, quantity, functions are not clear. Lei Sun et al. have identified numerous lncRNAs that are functionally required for proper adipogenesis [9]. It was reported that a muscle-specific long noncoding RNA (linc-MD1) that displays decoy activity for two specific miRNAs and in doing so, regulates their targets in a molecular circuitry affecting the differentiation program [10]. This indicated us that lncRNA has a function on muscle differentiation. BAT and muscle are similar in origin and gain different potential of energy metabolism after differentiation and maturation. However, the study of lncRNAs, which comprise the bulk of the human non-coding transcriptome between BAT and muscle, is still in its infancy.

In this study, using next generation transcriptome sequencing and microarray techniques, we investigated 704 up-regulated and 896 down-regulated lncRNAs (fold-change >3.0) by comparing the expression profile between BAT and skeletal muscle and their possible function in differentiation and energy metabolism. In addition, we report AK003288 associated with junctophilin 2 (Jph2) gene which may affect energy metabolism. This study will be helpful to explore between brown adipose tissue and skeletal muscle having several mechanisms in common from the perspective of LncRNAs.

2. Methods and materials

2.1. Preparation of tissues

Samples of Brown adipose tissue and muscle were taken from 8-week-old C57BL/6 mice. All the mice were provided by Experimental Animal Center of Nanjing Medical University. Interscapular brown adipose tissues were carefully separated from white adipose tissue and fibrous membrane and skeletal muscles were taken from mice legs. For further study, we carefully separated four kinds of skeletal muscle of Extensor digitorum longus (EDL), Tibialis anterior (TA), Gastrocnemius (GAS) and Soleus (SOLE). Finally, 30 cases of BAT and skeletal muscles were selected. All the samples were immediately frozen in liquid nitrogen followed by storage at −80 °C prior to analysis.

2.2. RNA extraction and quality control

To isolate total-RNA from each tissue, the frozen tissues were minced with homogenizer (IKA, Germany) and resuspended in TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Total RNA obtained by Trizol extraction was purified by processing with RNeasy Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. Quantification and quality check were performed with Nanodrop and Agilent 2100 Bioanalyzer (Agilent Technologies), respectively. RNA quantity was measured by One drop OD-1000.

2.3. Microarray analysis

Arraystar Mouse LncRNA Microarray v2.0 is designed for the global profiling of mouse LncRNAs and protein-coding transcripts. The LncRNAs are carefully collected from the most authoritative databases such as RefSeq, UCSC Knowngenes, Ensembl and many related literatures. Each transcript is represented by a specific exon or splice junction probe which can identify individual transcript accurately. Positive probes for housekeeping genes and negative probes are also printed onto the array for hybridization quality control.

The sample preparation and microarray hybridization were performed based on the manufacturer's standard protocols with minor modifications. Briefly, mRNA was purified from 1 μg total RNA after removal of rRNA (mRNA-ONLY™ Eukaryotic mRNA Isolation Kit, Epicentre). Then, each sample was amplified and transcribed into fluorescent cRNA along the entire length of the transcripts without 3′ bias utilizing a random priming method. The labeled cRNAs were hybridized onto the Mouse LncRNA Array v2.0 (8 × 60 K, Arraystar). After having washed the slides, the arrays were scanned by the Agilent Scanner G2505B. Agilent Feature Extraction software (version 10.7.3.1) was used to analyze the acquired array images. Quantile normalization and subsequent data processing were performed using the GeneSpring GX v11.5.1 software package (Agilent Technologies).

2.4. Quantitative real-time reverse transcription PCR

Total RNA was isolated with the RNeasy Mini Kit (Qiagen, Valencia, CA). 1 μg of RNA from each sample was reversely transcribed to cDNA using random hexamer primer with Thermo Scientific™ RevertAid Fist Strand cDNA Synthesis kit (Thermo Scientific, Wilmington, DE). Primers for each LncRNA were designed according to Primer 3 (http://sourceforge.net/projects/primer3/) online and checked with Basic Local Alignment Search Tool (BLAST) of NCBI to ensure unique amplification product. Real-time PCR was performed on an Applied Biosystems ViiA™ 7 Dx (Life Technologies, US) using the SYBR green method according to the manufacturer's instructions. The PCR Reaction conditions were: a denaturation step at 95 °C for 10 min, followed by 40 × PCR cycles at 95 °C for 15 s and 60 for 1 min. elative gene expression levels were quantified based on the cycle threshold (Ct) values and normalized to the internal control gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). All the primer sequences used were shown in (Supplement Table 1). The $2^{-\Delta\Delta Ct}$ method was used to comparatively quantify the levels of mRNA.

2.5. Gene Ontology (GO) and pathway analysis

As a dark matter, the function and Mechanism of lncRNAs are not exactly clear. Previous studies have shown that mammalian lncRNAs are preferentially located next to genes with developmental functions. Pathway and GO analyses were applied to determine the roles of these closest coding genes in biological pathways or GO terms. The Gene Ontology is a controlled vocabulary composed of >38,000 precise defined phrases called GO terms that describe the molecular actions of gene products, the biological processes in which those actions occur and the cellular locations where they are present [11]. Fisher's exact test is used to find if there is more overlap between the DE list and the GO annotation list than would be expected by chance. The *p*-value denotes the significance of GO terms enrichment in the DE genes. The lower the *p*-value, the more significant the GO Term (*p*-value < 0.05 is recommended). Pathway analysis has become the first choice for gaining insight into the underlying biology of differentially expressed genes and proteins, as it reduces complexity and has increased explanatory power [12]. Pathway analysis is a functional analysis mapping genes to KEGG pathways. The *p*-value (EASE-score, Fisher-*p* value or Hypergeometric-*p* value) denotes the significance of the pathway correlated to the conditions. Lower the *p*-value, more significant is the pathway.

2.6. Bioinformatics analysis

By using UCSC genome browser (http://genome.ucsc.edu/) and other database, we get the sequence of lncRNAS and their associated coding genes. Many transcriptional regulatory elements exist

in the non-coding regions, and it's hard to be distinguished just using the primary sequences as a guide. In this study, we also try to map various epigenetic phenomena to aid in the identification of non-coding regulatory elements. DNA methylation is essential for normal development 1–3 and has been implicated in many pathologies [13–15]. Tri-methylation of lysine 4 (H3K4me3) and Tri-methylation of lysine 27 (H3K27me3) tends to mark promoters [16]. H3K4me3 is catalyzed by trithorax-group (trxG) proteins and associated with activation, where as H3K27me3 is catalyzed by polycomb-group (PcG) proteins and associated with silencing [17,18]. We use Broad H3 ChIPseq Track on the UCSC genome browser mapping chromatin state.

2.7. Statistical analysis

The data were analyzed using SPSS 17.0 software package (SPSS, Chicago, IL, USA). Differential expression levels of lncRNAs were compared by Independent-samples *T* test between two groups. Fisher's exact test was used in GO and pathway analysis. All values are expressed as the mean ± standard deviation (SD) from at least three independent experiments, and statistical significance was defined as $p < 0.05$.

3. Results

3.1. Profile of microarray data

Arraystar Mouse LncRNA Microarray v2.0 is designed for the global profiling of mouse LncRNAs and protein-coding transcripts. Using second-generation LncRNA microarray, 17,763 LncRNAs and 13,587 coding transcripts were detected. They are carefully collected from the most authoritative databases such as RefSeq, UCSC Knowngenes, Ensembl and many related literatures (Fig. 1A). Scatter-Plot a visualization method is used for assessing the LncRNA expression variation between the two groups of BAT and muscle (Fig. 1B). Hierarchical Clustering shows LncRNA expression patterns of samples (Fig. 1C). We identified 704 up-regulated and 896 down-regulated lncRNAs (Supplement Tables 2 and 3) in the BAT compared with muscle by set a filter of fold-change >3.0.

3.2. Real time quantitative PCR confirmation

By using Quantitative real-time reverse transcription PCR, 5 up-regulated lncRNAs (AK007267, ENSMUST00000163003, AK138356, NR_028442, NR_015486) and 4 up-regulated lncRNAs (AK009163, AK003290, ENSMUST00000126300, uc008fss.1) with fold-changes >3 were randomly selected to test and verify the microarray data in different samples of BAT and skeletal muscles (Fig. 2), Thus indicated that a series of lncRNAs constantly differentially expressed between BAT and skeletal muscles.

3.3. Expression signatures of dysregulated lncRNAs between BAT and muscle

We investigate some general signatures of dysregulated lncRNAs such as classification, length distribution, chromosome distribution. There were 258 sense overlap, 313 antisense overlap, 905 intergenic and 124 bidirectional among the dysregulated lncRNAs (Fig. 3A). The lncRNAs are mainly between 400 and 3600 bp in length (Fig. 3B). Chromosome distribution shows the numbers of up and down regulated lncRNAs location in different chromosomes (Fig. 3C and D).

3.4. Go and pathway analysis

The Gene Ontology project (http://www.geneontology.org) which covers three domains: Biological Process, Cellular Component and Molecular Function provides a controlled vocabulary to note gene and gene product attributes in any organism. Functional category enrichment based on the GO terms was evaluated for the associated coding gene of dys-regulated lncRNAs. In our study, the top 10 GO terms that the associated coding gene function of up-regulated lncRNAs involved: (1) GO: 0009987: cellular process (2) GO: 0008152: metabolic process (3) GO: 0044281: small molecule metabolic process (4) GO: 0044237: cellular metabolic process (5) GO: 0006629: lipid metabolic process (6) GO: 0044255: cellular lipid metabolic process (7) GO: 0006631: fatty acid metabolic process (8) GO: 0044238: primary metabolic process (9) GO: 0042180: cellular ketone metabolic process (10) GO: 0019752: carboxylic acid metabolic process. Meanwhile, the top 10 GO terms that the associated coding gene function of down regulated lncRNAs involved: (1) GO: 0009987: cellular process (2) GO: 0044238: primary metabolic process (3) GO: 0008152: metabolic process (4) GO: 0044237: cellular metabolic process (5) GO: 0044260: cellular macromolecule metabolic process (6) GO: 0043170: macromolecule metabolic process (7) GO: 0061061: muscle structure development (8) GO: 0032502: developmental process (9) GO: 0044267: cellular protein metabolic process (10) GO: 0065007: biological regulation (Fig. 4A, Fig. 4B).

By mapping genes to KEGG pathways, we performed pathway analysis (http://www.genome.jp/kegg/pathway.html). In our study, the top 10 pathway that the associated coding gene of up-regulated lncRNAs involved: (1) Fatty acid metabolism; (2) Peroxisome; (3) PPAR signaling pathway; (4) Valine, leucine and isoleucine degradation; (5) Fatty acid elongation; (6) Biosynthesis of unsaturated fatty acids; (7) Propanoate metabolism; (8) Pentose phosphate pathway; (9) Butanoate metabolism; (10) Glutathione metabolism. meanwhile, the top 10 pathway that the associated coding gene of down-regulated lncRNAs involved: (1) Arginine and proline metabolism; (2) MAPK signaling pathway; (3) Insulin signaling pathway; (4) Hypertrophic cardiomyopathy (HCM); (5) Wnt signaling pathway; (6) Cysteine and methionine metabolism; (7) Renal cell carcinoma; (8) Tight junction; (9) Dilated cardiomyopathy; (10) Ubiquitin mediated proteolysis (Fig. 4C, 4D).

3.5. Bioinformatics analysis

We selected dysregulated lncRNAS that have a relative high expression with a fold-change >3 in BAT and muscle and their associated coding gene with a function of developmental process, energy metabolism and so on. A under-regulated lncRNA, AK003288, was found to be located downstream of the intron of the junctophilin 2 (Jph2) gene (Supplement Fig. 1A). The red box shows the location of AK003288. The Broad H3 ChIPseq Track contain chromatin-state maps generated at the Broad Institute using ChIP-Seq. The subtracks contain the coordinates of intervals enriched for H3K4Me3, H3K27Me3 in mouse ES cells, ES-derived neural progenitors (NP) and so on. Mammal cons, UCSC track (http://genome.ucsc.edu) showing the placental mammal basewise conservation by PhyloP (Supplement Fig. 1B). Quantitative real-time reverse transcription PCR show that AK003288 was higher expressed in different kind of skeletal muscle than BAT which meet the microarray results (Supplement Fig. 1C).

4. Discussion

Although brown adipocytes, white adipocytes and myocytes can differentiate from mesenchymal stem cells (MSCs) [19]. Study indicated that brown adipocytes endogenously share a common early transcriptional program with skeletal muscle cells [20]. In contrast to white adipocytes, brown adipocytes have an abundance of mitochondria and are able to contribute positively to energy

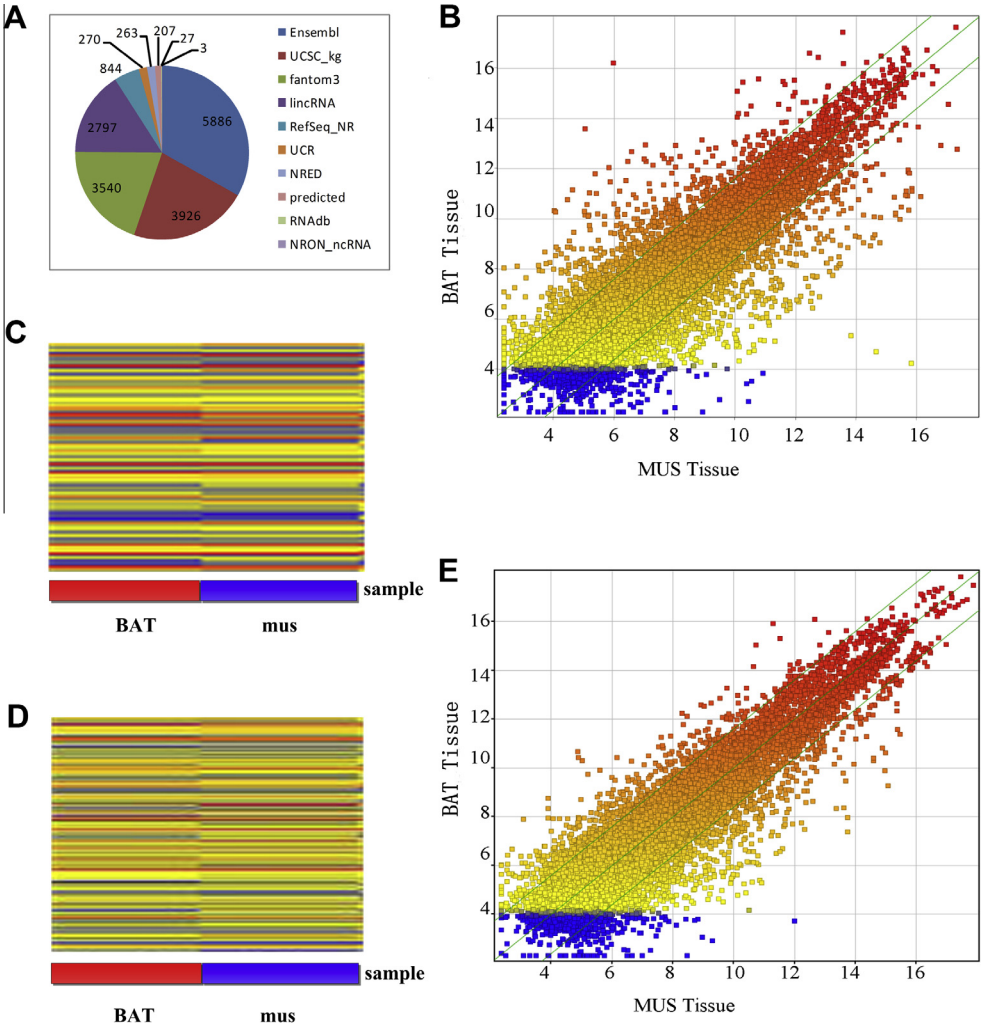

Fig. 1. Profile of microarray data. (A) Using second-generation LncRNA microarray, 17,763 LncRNAs were detected. Pie chart showing the relative numbers of LncRNAs from the most authoritative databases. (B) The LncRNAs above the top green line and below the bottom green line indicated more than 3.0-fold change of LncRNAs between the two compared groups between BAT and MUS. (C) Hierarchical Clustering shows a distinguishable LncRNA expression profiling among groups. (D) The mRNAs above the top green line and below the bottom green line indicates more than 3.0-fold change of mRNAs between the two compared groups. (E) Hierarchical Clustering shows a distinguishable mRNA expression profiling among groups. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

expenditure through the specific expression of the mitochondrial uncoupling protein, UCP1, a 32 kDa protein found in the inner mitochondrial membrane, that allows dissipation of the proton electrochemical gradient generated by respiration in the form of heat [21,22]. Study show that the precursor cells of classic brown adipocyte and muscle cells express muscle developmental gene Myf5 which does not express in white adipocyte. Seale PA [23] found that the transcriptional regulator PRDM16 (PRD1-BF1-RIZ1 homologous domain containing 16) controls a bidirectional cell fate switch of precursor cells to become muscle or brown fat cells. In the absence of PRDM16, causes precursor cells a loss of brown fat characteristics and promotes muscle differentiation, and on its increased expression, precursor cells become brown fat cells. Recent study has discovered that BAT is present and active in adults. BAT is situated predominantly around the aorta and in the supraclavicular area. BAT volume and activity are lower in individuals who are obese. This suggests that BAT significantly contributes to total energy expenditure. The activation of BAT could potentially be used to induce weight loss [24]. Study of the development and regulation of BAT has explored in the last few years that transcriptional Control of dominant brown fat regulators such as PRDM16, FOXC2, or PGC-1a, et al., that will increase differentiation or activity of BAT that exists in adult humans may offer new treatments for obesity and diabetes [25]. LncRNA were associated with transcription activation and inhibition, embryonic and tissue development and many other life activities. Gilsanz et al. found Pediatric patients with visualized BAT on PET/CT examinations had significantly greater muscle volume than patients with no visualized BAT [6]. Brown fat and skeletal muscle are particularly similar in origin and energy metabolism. Switching muscle to BAT can reduce weight gain and improve metabolism [22]. So we explored the differential expression of LncRNAs in BAT and Skeletal muscle.

Using second-generation LncRNA microarray, we detected 17,763 LncRNAs and 13,587 coding transcripts. We identified 704 up-regulated and 896 down-regulated lncRNAs in the BAT compared with muscle by set a filter of fold-change >3.0. AK003288 was found to be near the junctophilin 2 (Jph2) gene which affect energy metablism status and muscle development from ES cells [26].

The Gene Ontology is a controlled vocabulary composed of >38,000 precise defined phrases called GO terms that describe the molecular actions of gene products, the biological processes in which those actions occur and the cellular locations where they

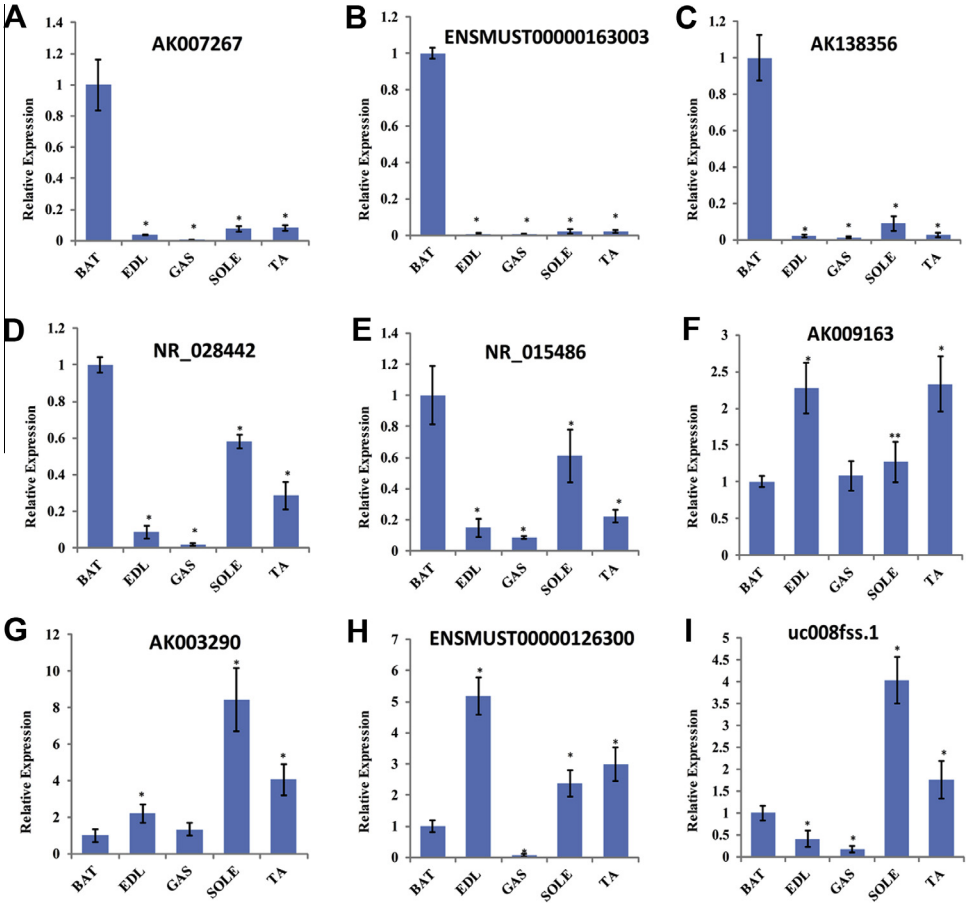

Fig. 2. The differential expression of lncRNAs between different kind of skeletal muscle (Extensor digitorum longus (EDL), Tibialis anterior (TA), Gastrocnemius (GAS) and Soleus (SOLE)) and BAT was validated by quantitative real-time PCR. $^{**}p < 0.05$, $^{*}p < 0.01$.

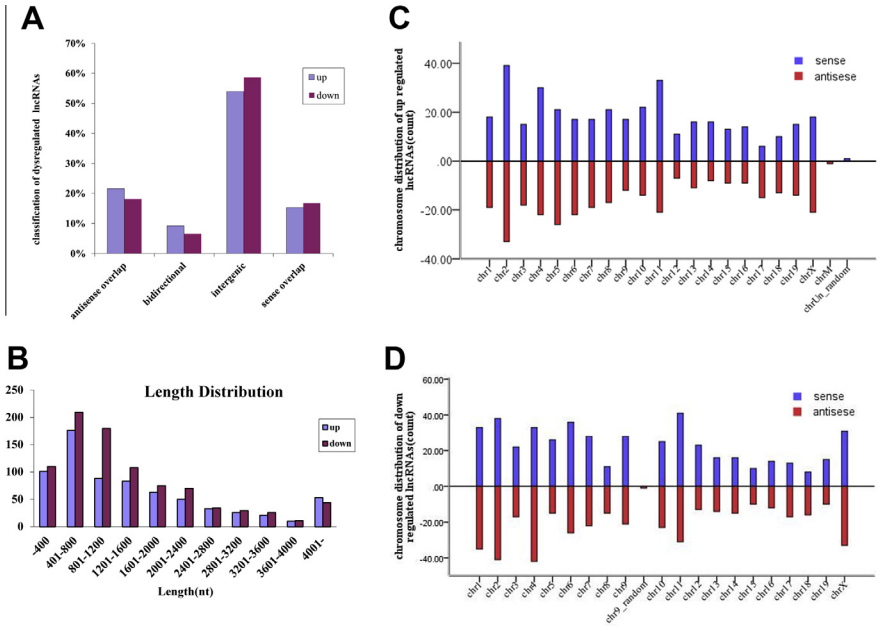

Fig. 3. (A) There were 258 sense overlap, 313 antisense overlap, 905 intergenic and 124 bidirectional among the dysregulated lncRNAs. (B) Length distribution of the dysregulated lncRNAs. The lncRNAs are mainly between 400 and 3600 bp in length. Chromosome distribution show the numbers of up (D) and down (E) regulated lncRNAs location in different chromosomes.

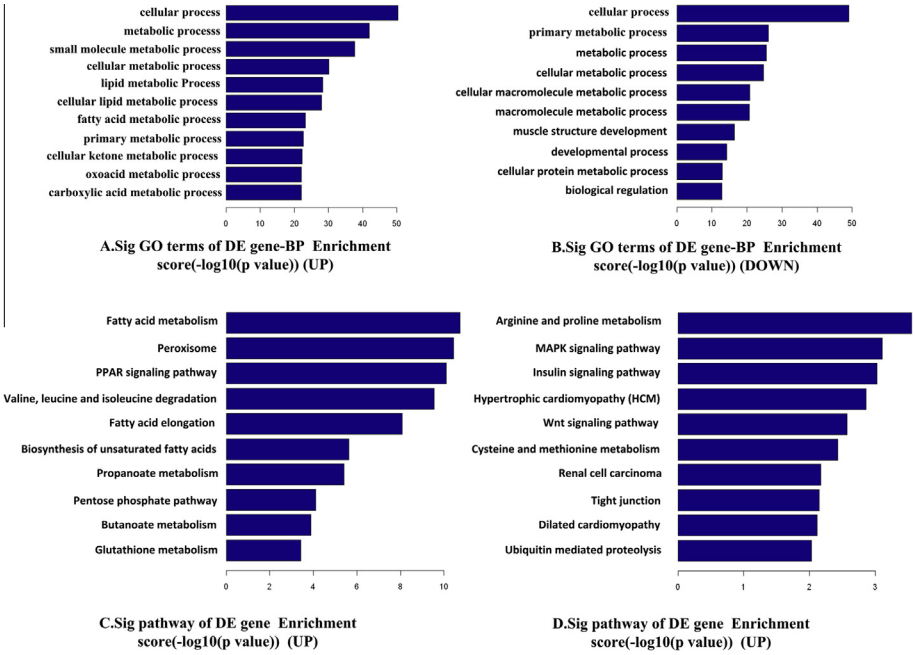

Fig. 4. GO and pathway analysis. The top 10 GO terms that the associated coding gene function of upregulated lncRNAs (A) and downregulated lncRNAs (B) are listed. The top 10 pathway that associated coding gene of upregulated lncRNAs (C) and downregulated lncRNAs (D) are listed.

are present [11]. The exist data of our survey indicate that the main biological processes involving dysregulated lncRNAs contained many closely related to development of BAT and skeletal muscle and energy metabolism, such as "cellular process"; "metabolic process"; "lipid metabolic process"; "fatty acid metabolic process"; "cellular macromolecule metabolic process"; "muscle structure development" and "biological regulation". Microarray technology show the expression profile of lncRNAs and human genes. Pathway analysis provides a method for gaining insight into the underlying biology of differentially expressed genes and proteins [12]. Pathway analysis show the associated gene of dysregulated lncRNAs between BAT and skeletal muscle mainly involved in energy metabolism and cell development such as "Fatty acid metabolism"; "Pentose phosphate pathway"; "Fatty acid elongation"; "Wnt signaling pathway" and so on. For instance, the Wnt (wingless-type MMTV integration site family members) signaling pathway is closely related to differentiation of normal and tumor cells. Recent studies have shown Wnt plays an important role in differentiation of adipose tissue, especially in the early formation of adipocytes differentiation process and is an important inhibited factor of adipocytes mature [27].

In conclusion, for the first time we report the profile of differentially expressed lncRNAs between BAT and Skeletal muscle. This indicates numerous lncRNAs are involved in development and energy metabolism of both BAT and Skeletal muscle. Further study were need to explain the biological progress and and molecular mechanisms of the dysregulated lncRNAs. This may provide a method to induce adipocytes developed to skeletal muscle cells in some muscle disease or provide a new therapeutic target for combat obesity by regulate energy metabolism progress.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.12.092.

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