Integrative Transcriptome Analyses of Metabolic Responses in Yak Liver Define Putative Pivotal LncRNAs

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Abstract

Background

The yak (*Bos grunniens*) is a crucial resource to supply meat and milk to the people localized in Tibetan plateau area. To systemically identify lncRNAs regulating metabolism in yak, we performed transcriptome analyses to simultaneously profile mRNAs and lncRNAs of liver in yak under three representative age (**LD**: Liver 1 Day, **LM**: Liver 15 Month, **LY**: Liver 5 Year) conditions.

Result

Of 288 differentially regulated lncRNAs, function-oriented filters yield 88 regulated metabolically related lncRNAs that are differentially expressed at least two age conditions. These lncRNAs predicted by lncRNA-mRNA correlation analyses to function in diverse aspects of metabolism. Specific regulations of liver metabolically related lncRNAs were further defined by qRT-PCR.

Conclusion

Combining genome-wide screens, bioinformatics function predictions, and qRT-PCR analyses, this study supports that a class of lncRNAs function as important metabolic regulators and establishes a framework for systemically investigating the role of lncRNAs in physiological homeostasis of yak.

Introduction

The yak (*Bos grunniens*) lives throughout the Tibetan plateau in western China at high altitudes where hostile environmental factors exist including low humidity, temperature, and oxygen levels; strong winds; and UV radiation. The yak has adapted to thrive under these harsh conditions, where few other domestic animals could survive. The yak is a crucial resource to supply meat and milk to the people localized in Tibetan plateau area. For these reasons, the yak is one of the most critical domestic animals for the 6.5 million Tibetan people [1]. Based on the fact that the yak represents the primary source of meat and milk for the people of this region, it is imperative to understand the genetic makeup of the yak and how this can influence its development.

While, metabolic process is one of the most basic activity required for life, and metabolism is related to nearly all essential biological and physiological activities in cells and whole animals [2]. Liver, the key metabolic organs, engage in constant dialogs using endocrine factors to regulate overall metabolic balance. And it is a complex digestive gland in ruminant animals including yak, plays an important role in the metabolism of carbohydrates, fats, proteins, vitamins, hormones, and other substances. Nutrients absorbed from the digestive tract pass through the liver, enter the circulatory system of yak. During the developmental period, liver plays a critical role [3–5]. Dorland et al. reported the period of transition from

new born to young cattle involved considerable metabolic adaptation in dairy cows and the liver [3]. So, in the present study, the liver of new born (Day 1), young yak (5 months) and mature yak (5 years) were chosen to be sampled.

Although great efforts have been made in studying the regulation of individual metabolic pathways in recent years, there are still considerable unknown fields in our knowledge of the complex regulatory networks governing metabolic physiology, and it remains very difficult to design effective treatment strategy against metabolic disorders such as obesity, diabetes that have reached epidemic proportion globally [6–8]. In contrast to the hypothesis that mammalian genomes mainly encode protein-coding genes, nearly two thirds of all transcripts are noncoding RNAs derived primarily from regions previously regarded as junk gene [9]. Among the noncoding RNAs identified so far, the largest and also the least understood group is lncRNAs, which are transcripts of 200 nt or longer that lack coding potential. lncRNAs have been identified in all model organisms [10], and their number has been growing continuously [11–13]. It has been demonstrated that lncRNAs affect several aspects of cellular function, including chromatin modification and transcription regulation, RNA stability, and translational control [14, 15]. Evolution studies suggest that more than 1,000 lncRNAs could have conserved functions in mammals [16]. There are also many reports in bovine indicates lncRNAs function in embryo development [17], skeletal muscle [18] and mammary glands [19]. In mouse, *in silico* method had been used to discover functional lncRNAs in metabolic process [20]. However, it is still unknown fields in ruminant animals like bovine or yak.

Therefore, to systemically illustrate the importance of lncRNAs in metabolic homeostasis in yak, we have established an integrative roadmap to identify functional lncRNAs in metabolic regulation by combining genome-wide screens of lncRNAs transcriptomes in liver under diverse age of yak, extensive lncRNA-mRNA correlation analyses for lncRNA function prediction, and qRT-PCR assays connecting lncRNAs to specific metabolic pathways. Overall, our data support that lncRNAs are a critical component of metabolic responses, and our work provides a framework for systemically identifying and predicting functional lncRNAs that regulate metabolic homeostasis.

Material And Methods

Animal

Yak from the Research Institute of Qinghai-Tibet Plateau of Southwest Minzu University were used for all experiments. For lncRNA functional test, yaks were sacrificed at three age conditions including 1 Day, 15 Month and 5 Years old, weighing 10.55 ~ 14.24 kg, 96.38 ~ 101.37 kg and 240.73 ~ 296.36 kg, and the liver tissue were sampled of each age yak. Yaks with different age were established for profiling the transcriptomes in liver tissue.

RNA quantification and qualification

RNAiso Plus (TaKaRa, Japan) was used to extract total liver tissue RNA. The extent of RNA degradation and contamination was monitored on 1% agarose gels. RNA purity was checked using the NanoPhotometer[®] spectrophotometer (IMPLEN, CA, USA). RNA concentration was measured using Qubit[®] RNA Assay Kit in Qubit[®] 2.0 Fluorometer (Life Technologies, CA, USA). RNA integrity was assessed using the RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies, CA, USA). If the OD_{260/280} value of total liver tissue RNA is between 1.8 ~ 2.2, the quality inspection is qualified (Supplementary Table 1) and the next analysis can be carried out.

Library preparation for high-throughput sequencing

A total amount of 3 µg RNA per sample was used as input material for the RNA sample preparations. A chain-specific library was constructed by removing rRNA, that was, sequencing libraries were generated using NEBNext[®] UltraTM RNA Library Prep Kit for Illumina[®] (NEB, USA) [21]. Briefly, mRNA was purified from total RNA using poly-T oligo-attached magnetic beads. First strand cDNA was synthesized using random hexamer primer and RNase H. Second strand cDNA synthesis was subsequently performed using DNA Polymerase I and RNase H. The purified double-stranded cDNA was subjected to end repair, was added poly-A tail, and was connected to a sequencing adapter. In order to select cDNA fragments of preferentially 150~200 bp in length, the library fragments were purified with AMPure XP system (Beckman Coulter, Beverly, USA). Then 3µL USER enzymes (NEB, USA) were used to degrade the second strand of U-containing cDNA, and perform PCR amplification to obtain the library. After the library was constructed, Qubit2.0 Fluorometer (Invitrogen, USA) was used for preliminary quantification. PCR products were purified (AMPure XP system) and library quality was assessed on the Agilent Bioanalyzer 2100 system. qRT-PCR (Bio-Rad, USA) was used to accurately quantify the effective concentration of the library to ensure library quality. The library preparations were sequenced on an Illumina Hiseq platform and 150 bp paired-end reads were generated.

Transcriptome sequencing data analysis

The quality-controlled Clean Reads was compared with the yak reference genome (BosGru_v2.0: ftp://ftp.ensembl.org/pub/release-97/fasta/bos_mutus/) quickly and accurately by the HISAT2 v2.0.5 software (<http://ccb.jhu.edu/software/hisat2>) to obtain Mapping Reads for subsequent analysis [22]. At the same time, the comparison results were evaluated for quality. By analyzing the different regions and chromosome distributions of Mapping Reads in the reference genome, to obtain the comparison efficiency and mapping information about Mapping Reads for each sample [23]. The new transcripts for Mapping Reads were assembled and quantified by StringTie (1.3.3b) software [24]. Sequencing depth and gene length were corrected using FPKM [25]. Differential expression analysis was performed using the DESeq2 R package (1.10.1). DESeq2 provide statistical routines for determining differential expression in digital gene expression data using a model based on the negative binomial distribution. Genes with an adjusted padj<0.05 found by DESeq2 were assigned as differentially expressed [26].

Functional enrichment

ClusterProfiler (3.4.4) software was used to perform GO function enrichment analysis and KEGG pathway enrichment analysis on differential gene sets, to predict the biological processes and functions that they may participate in, and to make corresponding classification and statistics [27]. All lncRNAs are used for target gene prediction, that was, the target genes of lncRNA are predicted by the positional relationship (co-location) and expression correlation (co-expression) of lncRNA and protein-coding genes [28]. Then, functional enrichment analysis (GO / KEGG) was performed on the target genes of differential lncRNA to predict the main function of lncRNA.

Real-time fluorescence quantitative PCR

Measurement of gene expression with qRT-PCR has been applied in our studies. Briefly, total RNA was extracted from liver tissue of each group using a RNAiso Plus (TaKaRa, Japan). RNA is complete without degradation, good quality and high purity, which meet the requirements of subsequent experiments. qRT-PCR was performed in a total volume of 10 μ L containing 5.2 μ L of TB GreenTM Premix Ex TaqTM (TaKaRa, Japan), 1 μ L of cDNA, 0.8 μ L of each primer (10 mM) and 2.2 μ L of double-distilled water. The reaction conditions used were as follows: 95 °C for 3 min, followed by 39 cycles of 95 °C for 10 s, 53.4/52 °C (β -actin/verification genes) for 20 s and 72 °C for 20 s, with the dissolution curve increasing from 0.5 °C to 95 °C every 5 s. The assays were performed on a real-time fluorescence quantitative PCR System (Bio-Rad, USA). For each sample, the cycle threshold (CT) values were obtained from three replicates. β -actin mRNA was employed as an internal reference. The primers used for amplification of target and internal reference genes are presented in Supplementary Table 2. The relative expression levels of target genes were analyzed using the $2^{-\Delta\Delta CT}$ method.

Result

Dynamic regulation of lncRNAs and mRNAs in key metabolic organs under multiple age conditions

Because all coding and noncoding transcripts were quantified in parallel, our expression profile allows the assessment and comparison of the developmental stage on the lncRNA and mRNA transcriptomes in the liver. We first performed hierarchical clustering analyses on all transcripts expressed. As expected, mRNA expression profiles readily separate all samples into three distinct groups on the basis of their age (1 day, 15 months, 5 years), and samples cluster tightly within each treatment group (Figure 1A, top left). Interestingly, a nearly identical pattern of sample clustering was observed for lncRNAs (Figure 1A, top right), indicating that expression profiles of lncRNAs could serve as a metabolic signature in a manner similar to those of protein coding mRNAs. Using RNA-seq platform, we detected 35,216 mRNA and 10,073 non-redundant lncRNA transcripts (Figure 2B). From transcriptomes in the livers of yak aged from three diverse age (LD: Liver 1 Day, LM: Liver 15 Month, LY: Liver 5 Year) (Figure 2B).

Consistently, PCAs on all regulated transcripts, either for mRNAs or lncRNAs, readily separate all samples into distinct groups (Figure 1C). These patterns suggest that regulated lncRNA and mRNA transcriptomes might function coordinately in related physiological processes, and their intrinsic functional connections could be defined by performing correlation analyses of samples under multiple metabolic conditions in which the lncRNA-mRNA networks are sufficiently dynamically regulated. 433 mRNAs and 152 lncRNAs were identified to be regulated by LD versus LM, as well as 412 mRNAs and 160 lncRNAs by LD versus LY (Figure 1D). And 263 mRNAs and 66 lncRNAs were completely LM versus LY (Figure 1D), suggesting that their expression levels are stringently governed by age states of the animals.

Functional analysis of differentially expressed mRNAs in each age condition in liver

At LD versus LM, the differentially expressed genes of many functional gene ontology categories enriched for terms related to metabolism, ion binding, developmental process and so on. While in KEGG pathway analysis, differentially expressed genes were also enriched for terms such as metabolism, biosynthesis, and ECM-receptor interaction so on (Figure 2A). At LD versus LY, there were functional gene ontology categories enriched for metabolism, tissue remodeling and developmental process and so on. While in the KEGG pathway analysis, differentially expressed genes were also enriched for terms like metabolism, biosynthesis, PI3K-Akt signaling pathway and so on, indicating that (Figure 2B).

Similar functional gene ontology categories of metabolism, biosynthetic process and oxidation reduction process and so on were enriched at LM versus LY. While in the KEGG pathway analysis, differentially expressed genes were also enriched for terms like metabolism, biosynthesis, focal adhesion and so on, indicating that (Figure 2C). All these results suggest metabolism related function are developed during the aged process (Figure 2).

Functional prediction of metabolically related differentially expressed lncRNAs by lncRNA-mRNA co-expression correlation under at least two age conditions

Those more dynamically regulated lncRNAs would be ideal for functional prediction by lncRNA-mRNA expression correlations across different age conditions. Therefore, we filtered and selected all lncRNAs that are regulated by at least two of the three age conditions from the total 288 regulated lncRNAs, and obtained 88 highly regulated non-redundant lncRNAs (Figure 1D; Figure 4). To provide an example of examining the specific roles of these metabolically sensitive lncRNAs, we have selected six metabolically sensitive lncRNAs for further analyses.

Our work exemplified six lncRNAs that our correlation analyses provide insights into their functions in cell differentiation and metabolism. ENSBMUG00000000490 and XLOC_045379 was predicted to be

associated with fat single organism metabolic process (Figure 3A and 3B), and XLOC_021536 and XLOC_041441 associated with collagen metabolic processes and protein metabolic processes in liver, respectively (Figure 3C and 3D). Additionally, ENSBMUG00000026019 and XLOC_183608, have been shown to be enriched in cell proliferation and transport (Figure 3E and 3F). These results indicate that the co-expression network we built can efficiently predict the potential metabolic functions for age regulated and metabolically sensitive lncRNAs. In addition, the dynamic regulation of several randomly selected lncRNAs by different age conditions was confirmed by qRT-PCR, attesting to the quality of this method (Figure 4).

Discussion

Using genome-wide screens method, we have established a comprehensive catalog of lncRNAs implicated in metabolic regulation and a bioinformatics workflow to develop a roadmap for identifying and characterizing functional lncRNAs in different age conditions of yak. Our results support that lncRNAs are a critical component of metabolic responses. lncRNA expression patterns change systemically and coordinately according to different age status, in a way similar to those of mRNAs. In addition, key groups of metabolism-associated lncRNAs are regulated in liver by age conditions, and they often exhibit strikingly changed expression in yak with different age, supporting their potential physiological significance.

It is still very difficult to identify and functionally characterize lncRNAs regulating metabolic homeostasis in animals, and the very small number of lncRNAs shown to regulate energy metabolism *in vivo* precludes an objective assessment of the role of lncRNAs in metabolic physiology. To overcome this obstacle, we developed a functional lncRNA detection pipeline by comprehensively profiling lncRNAs in liver under different age conditions. This integrative approach allows us to effectively reduce 288 lncRNAs regulated by representative age conditions to a concise and annotated catalog of 88 putative lncRNA metabolic regulators.

Only a few lncRNAs have been shown to be able to significantly regulate an important metabolic pathway *in vivo* in mouse [29–32] and no reports about the function of lncRNAs had been illustrated in yak. We expect that there are more lncRNAs critical to metabolic awaiting to be discovered and characterized, and we hope that our lncRNA detection workflow could speed up the process of identifying and mechanistically characterizing these lncRNA metabolic regulators, which could provide insights into the complex network of metabolic regulation.

Understanding lncRNAs engage in metabolic pathways in different age conditions could fill in the unknown fields in our understanding of the complexity of metabolic physiology. A comparative genomics method based on information of homologous genes or protein motifs has been very successful in uncovering functions of novel protein-coding genes but so far has proved to be ineffective in identifying lncRNA functions [33]. This is because most lncRNAs have not been functionally studied, and more critically, lncRNAs are much less conserved than mRNAs, even between closely related model organisms

such as rat and mouse [34]. Since most lncRNAs are mammal specific, it is difficult to infer their functional roles on the basis of their evolutionary histories [35].

Lack of a practical approach to predict potential functions for lncRNAs makes it very challenging to identify and characterize lncRNAs that regulate metabolism *in vivo*. Because metabolism is centrally important to most biological systems, all organisms, especially mammals, use delicate mechanisms to maintain metabolic homeostasis, and any perturbation to key metabolic pathways in an organ is often strongly compensated by rewiring of metabolic fluxes in the same organ or systemically. So, a strict *in vivo* function test needed to identify important nodes in metabolic regulation. Considering the efforts required to generate an lncRNA animal model, information on inferred functions of lncRNAs is needed to choose the right target to start with. Moreover, such information can also help in designing targeted and sensitive assays to identify specific metabolic phenotypes in a given lncRNA animal model that are often embedded in complex networks of organ communications or dampened by compensatory mechanisms. Thus, an effective approach to predict metabolic functions for lncRNAs or associate them with metabolic pathways could greatly accelerate the pace of identifying important lncRNA metabolic regulators.

Conclusions

The present study has systemically illustrated metabolic related lncRNA expression in different age of yak and established a roadmap to identify and characterize metabolism-associated lncRNAs. We have also generated a catalog of potential lncRNA metabolic regulators with predicted functions. This work could offer useful information for delineating the metabolic functions of lncRNAs in yak.

Abbreviations

RNA: Ribonucleic acid; lncRNAs: Long noncoding RNAs; mRNAs: Messenger RNAs; LD: Liver 1 day; LM: Liver 15 month; LY: Liver 5 year; PCR: Polymerase chain reaction; qRT-PCR: Real-time fluorescence quantitative PCR; UV: Ultraviolet; DNA: Deoxyribonucleic acid; cDNA: Complementary DNA; RNase H: M-MuLV reverse transcriptase; FPKM: Fragments per kilobase of transcript sequence per millions base pairs sequenced; padj: Corrected p-value for multiple hypothesis test; GO: Gene ontology; KEGG: Kyoto encyclopedia of genes and genomes; PCAs: Principal-component analyses; ECM: Extracellular matrix; PI3K-Akt: Phosphatidylinositide 3-kinases-serine/threonine kinase

Declarations

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Authors' contributions

LW and XL conceived and designed this study. WX and FF performed experiments, analyzed data and wrote the manuscript. FF and JG collected sample collection. LW and XL contributed to the revisions. All authors reviewed and approved the final manuscript.

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Availability of data and materials

All data generated or analysed during this study are included in this published article and its supplementary information files.

Ethics approval and consent to participate

All animal experiments were performed in accordance and with approval from Southwest Minzu University Animal Care and Use Committee.

Consent for publication

All of the authors have approved the final version of the manuscript, agree with this submission to Journal of Animal Science and Biotechnology.

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

Dynamic Regulation of LncRNAs and mRNAs in Key Metabolic Organs under Multiple Age Conditions (A) Hierarchical clustering (top) and PCAs (bottom) of differentially expressed mRNAs (left) and lncRNAs (right) in yak livers at different age conditions. The differentially expressed genes were defined by one-way ANOVA to be significantly different. (B) Experimental outline. Each metabolic condition contains three yak, and the expression profiles of mRNAs or lncRNAs from 9 liver samples were analyzed. (C) PCAs (bottom) of differentially expressed mRNAs (left) and lncRNAs (right) in yak livers. (D) Numerical distribution of age-specifically and commonly regulated mRNAs (left) and lncRNAs (right) in liver.

Figure 2

gene ontology (GO) and KEGG pathway terms for differentially expressed mRNAs in each age condition in liver (A) Gene ontology (left) and KEGG pathway(right) terms for differentially expressed mRNAs of LD vs LM in yak liver. (B) Gene ontology (left) and KEGG pathway(right) terms for differentially expressed mRNAs of LD vs LY in yak liver. (C) Gene ontology (left) and KEGG pathway(right) terms for differentially expressed mRNAs of LM vs LY in yak liver.

Figure 3

Functional Prediction of Metabolically related Differentially expressed LncRNAs by LncRNA-mRNA Co-expression Correlation under at least two Age Conditions (A) Gene ontology terms for differentially expressed lncRNA ENSBMUG0000000490 in yak liver. (B) Gene ontology terms for differentially expressed lncRNA XLOC_045379 in yak liver. (C) Gene ontology terms for differentially expressed lncRNA XLOC_021536 in yak liver. (D) Gene ontology terms for differentially expressed lncRNA XLOC_041441 in yak liver. (E) Gene ontology terms for differentially expressed lncRNA ENSBMUG00000026019 in yak liver. (F) Gene ontology terms for differentially expressed lncRNA XLOC_183608 in yak liver.

Figure 4

qPCR validation of selected Differentially expressed LncRNAs and mRNAs (A) Validation of lncRNA RNA-seq results by quantitative real-time PCR analysis. Expression levels of 6 lncRNAs were quantified. Error bars are SEM, $n=4$. * $P < 0.05$ (LD vs LM), # $p < 0.05$ (LD vs LY). (B) Validation of mRNA RNA-seq results by quantitative real-time PCR analysis. Expression levels of 7 mRNAs were quantified. Error bars are SEM, $n=4$. * $P < 0.05$ (LD vs LM), # $p < 0.05$ (LD vs LY). (C) Pearson correlation of log2-transformed fold changes between FPKM and RNA-seq for differentially expressed mRNA(left) and lncRNAs(right).

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