Integrated Study of Transcriptome-wide m6A Methylome Confirms a Potential Mechanism for Transcriptional Regulation During Yak Adipocytes Differentiation

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Abstract

Background: Yak (*Bos grunniens*) is considered as an iconic symbol of Tibet and high altitude, but they suffer from malnutrition during the cold season that challenges the metabolism of energy. Adipocytes perform crucial role in maintaining the energy balance and adipocyte differentiation is a complex process involving multiple changes in expression of genes. *N*⁶-methyladenosine (m⁶A) plays a dynamic role in post-transcription gene expression regulation as the most widespread mRNA modification of the higher eukaryotes. However, currently there is no research existing on the m⁶A transcriptome-wide map of bovine animals and their potential biological functions in adipocyte differentiation.

Results: We performed methylated RNA immunoprecipitation sequence (MeRIP-seq) and RNA sequence (RNA-seq) to determine the distinctions in m⁶A methylation and gene expression during yak adipocyte differentiation. In yak adipocyte and preadipocyte the content of m⁶A and m⁶A associated enzymes were substantially different. In the two groups, a total of 14,710 m⁶A peaks and 13,388 m⁶A peaks were identified. In the most part, m⁶A peaks were enriched in stop codons, 3'-untranslated regions and coding regions. The functional enrichment exploration displayed that differentially methylated genes participated in some of the pathways associated with adipogenic metabolism. In addition to that there was a positive association between m⁶A abundance and levels of gene expression, which displayed that m⁶A plays vital role in modulating gene expression during yak adipocyte differentiation.

Conclusions: In short, it could be concluded that the current study provides a comprehensive explanation of the m⁶A features in the yak transcriptome, offering in-depth insights into m⁶A topology and associated molecular mechanisms underlying bovine adipocyte differentiation which might be helpful for further understanding its mechanisms.

Background

The yak is the major livestock breed on the Qinghai-Tibet Plateau, and is the only large ruminant domestic specie that can survive in harsh environments along with high altitude (3,000 ~ 5,500 m), severe cold, strong UV radiation and in low oxygen [1, 2]. Because of outstanding adaptability and production output, yak performs a key role in the Tibetan region. Approximately, 15 million or more than 90% of the world's overall yak population are living in Chinese territory, ensuring daily necessities for local herders such as meat, milk, wool, skins, fuel and economic benefits [3, 4]. In the Qinghai-Tibet plateau, domestic yaks mainly grow on the natural herbage of native pastures below typical grazing conditions [5]. Owing to the seasonal variability of herbage, the yak must constantly undergo insufficient feeding during the harsh winter season (October-May), which leading to the large variations in seasonal body weight and circular rhythm 'live in summer, weighty in autumn, thin in winter and dead in spring' [6]. Consequently, the subcutaneous adipose layer of yak accumulates rapidly in the summer and early autumn, providing essential energy requirements and withstanding severe cold by selective fat catabolism in cold season

[7]. The adaptive mechanism allows the yak as a fascinating model for research into the adipose metabolism of plateau domestic animals.

*N*⁶-methyladenosine (m⁶A) was first discovered in the 1970s as the most prevalent internal modification of polyadenylated mRNAs and long non-coding RNAs (lncRNAs) in higher eukaryotes [8–13]. The modification of m⁶A methylation is mounted by a series of m⁶A methyltransferases labeled as writers: methyltransferase like 3 and 14 (METTL3 and METTL14), Wilms Tumor 1-associated protein (WTAP), VIRMA, vir-Like m⁶A methyltransferase associated (KIAA1429), RNA Binding Motif Protein 15 (RBM15), and zinc finger CCH domain 13 (ZC3H13) [14–21]. Beside, m⁶A demethylases eliminate methylation from RNAs to enable a delicately dynamic equilibrium modification, and named as eraser: fat mass and obesity-associated protein (FTO) and α -ketoglutarate-dependent dioxygenase alkB homolog 5 (ALKBH5) [22, 23]. Besides, a category of proteins called readers that can identify information of RNA methylation modification and engage in downstream mRNA translation, degradation, binding microRNA and RNA-protein interaction [24–27]. The specific proteins primarily contain the YTH domain family (YTHDF1-3) and IGF2BPs (IGF2BP1-3) [28–31]. Currently, m⁶A modifications have been reported in several area of RNA metabolism, such as RNA localization, transport, splicing, stability and translation [24–27]. Moreover, previous studies described that m⁶A modification of mRNA performs a significant biological function in controlling cell metabolic processes. It dominates the transition fate of cells in mammalian embryonic stem [32], regulates the differentiation and initiation of meiosis in murine spermatogonial stem cells [33], and maintains the myogenic potential in proliferative skeletal muscle progenitors [34]. In particular, FTO facilitates the differentiation of mouse pre-adipocytes by regulating alternative splicing of pre-mRNAs with adipogenesis-related genes [35]. As well as, a recent study revealed that RNA m⁶A modification has a potential function in the deposition of porcine adipose tissue [36]. Considering the notable functions of m⁶A modification mentioned above, it is logical to assume that m⁶A modification may also refer to bovine adipocyte differentiation. Previously, there is no any documented relationship lies between m⁶A modification and bovine adipocyte differentiation.

Therefore, it is necessary to detect m⁶A sites on transcriptome-wide level for identification the potential biological function of RNA m⁶A modification. In 2012, two independent researches established a method of m⁶A RNA immunoprecipitation accompanied with high-throughput sequencing (MeRIP-seq), followed the first discovery of the *N*⁶-methyladenosine modification map to methylomes of m⁶A RNA with a resolution of 100-nucleotides [37, 38]. Simultaneously, MeRIP-seq has been used to identify the m⁶A profile in humans and mice. These results revealed that m⁶A is predominantly located close to stop codons, 3'-untranslated regions (3'-UTRs), and also in long internal exons and transcription start sites, indicating it performs a crucial role in the regulation of gene expression after transcription. These innovative researches proved that the construction of transcriptome-wide m⁶A methylome maps is suitable for further exploring the possible biological modification features in a new area of research.

In order to further explore the role of m⁶A modification in bovine adipose metabolism, and to facilitate m⁶A studies in plateau domestic livestock, we initially extracted preadipocytes from yak adipose tissue and induced them into mature adipocyte. Subsequently, the first known transcriptome-wide m⁶A methylome profile was obtained in yak with MeRIP-seq, and the m⁶A modification features were elucidated during yak adipocyte differentiation. Differential m⁶A RNA modifications between yak preadipocyte and mature adipocyte were verified that regulate gene expressions and pathways related to energy metabolism of adipose. For our research, it can be conjectured that the m⁶A modification could be a breakthrough point for exploring the yak energy metabolism.

Results

The yak preadipocyte induced differentiation and global m⁶A quantification

The result of Oil Red O showed that the visibility of lipid droplets in adipocytes increased significantly at day 12 compared to day 0 after induction with adipogenic agents (Fig. 1a-b). Meanwhile, the expression of adipocyte differentiation specific marker genes (*PPAR γ* , *C/EBP α* and *FABP4*) was significantly elevated on day 12 (adipocyte) than day 0 (preadipocyte) (Fig. 1c), suggesting preadipocyte full differentiation into adipocyte. Subsequently, to overview the m⁶A methylation during yak adipocyte differentiation, the expression of RNA methylation-related genes were contrasted by quantitative real-time PCR (qRT-PCR) detected, including *METTL3*, *WTAP*, *METTL14*, *FTO*, *ALKBH5* and *YTHDF1/2/3*. Comparing the group of preadipocytes (Pread0) and adipocytes (Ad), the findings showed that the expression level of methyltransferases (*METTL14*, *WTAP* and *METTL3*) and *ALKBH5* were dramatically upregulated, while *FTO* was substantially downregulated and m⁶A-binding proteins (*YTHDF2* and *YTHDF3*) was drastically upregulated (Fig. 1d). Furthermore, the content of m⁶A in the group of adipocyte was significantly higher compared with the preadipocyte group (Fig. 1e). Thereby, we hypothesized that during yak adipocyte differentiation the difference of m⁶A methylation may exist, in which furtherly discovered using MeRIP-seq.

Transcriptome-wide m⁶A-Seq revealed global m⁶A modification patterns during yak adipocyte differentiation

The yak adipocyte and preadipocyte of three biological replicates were used for transcriptome wide m⁶A-sequencing (m⁶A-seq) and RNA-sequencing (RNA-seq) assays. In total, 12 libraries were sequenced, comprising of three replicates of preadipocyte and adipocyte for input and MeRIP samples (Additional file 1). With each MeRIP library, an average of 9.22 giga base-pair (Gb) of high-quality data was produced, and 9.49 Gb per input library (RNA-seq dataset). Then, we eliminated reads containing adapter pollutants, low-quality and indeterminate bases, an average of 7.17 Gb and 7.11 Gb obtained from per MeRIP and

input libraries, respectively. The valid data were mapped to *Bos_mutus* genome (Version: BosGru_v2.0) using HISAT2. The proportions of mapped reads ranged from 87.96 to 96.57%, correspondingly (Additional file 1). In the yak Ad group, R package exomePeak found a total of 14,710 m⁶A peaks, containing transcripts of 9633 genes. Likewise, 13,388 m⁶A peaks were found in the Pread0 group corresponding to transcripts of 9142 genes (Fig. 2a-b). In addition, 5848 peaks were consistently observed in two groups, and 3964 genes within the groups were modified by m⁶A. Compared to the Pread0 group, the Ad group had 9226 new peaks occurring with the absence of 7904 peaks, reflecting the significant difference between Pread0 and Ad groups in global m⁶A modification trends (Fig. 2a-c). m⁶A methylomes were ulteriorly mapped by HOMER software to define whether RRACH motif (R represents purine, A is m⁶A and H is U, A or C) were ubiquitous in our detected m⁶A. The results of the enrichment analysis in both groups showed that m⁶A RRACH consensus sequences (Fig. 2d) accorded with previous studies, in which strengthens the credibility of the m⁶A peaks and confirms the presence of a prevailing methylated modification mechanism.

Analysis of m⁶A modification distribution in yak transcriptome

We analyzed metagene models of m⁶A peaks in the global transcriptome to identify the differential distribution of m⁶A in transcripts. Our findings indicated that m⁶A peaks were strongly associated with two different co-ordinates: enriched instantly the end of the 5' untranslated regions (5' UTRs), coding sequence (CDS), stop codon and starting of the 3' untranslated region (3' UTRs) in Ad and Pread0 (Fig. 3a); whereas, the end peaks were noticeable abundant than beginning in CDS. Subsequently, to systematically calculate the enrichment, we investigated no overlapping transcript segments per m⁶A peak with 5' UTR, CDS and 3' UTR (Fig. 3b), in which most of them were abundant in CDS. Afterwards, we explored the distribution of m⁶A modified peaks with each gene, finding that almost of 60% affected genes hold only one m⁶A peak and most genes contain one to three m⁶A peaks (Fig. 3c).

Analysis of the GO and KEGG pathways of differentially methylated genes

Comparison was performed for the abundance of m⁶A peaks between preadipocytes and adipocytes. These findings exposed that 118 markedly hypermethylated m⁶A peaks and 51 substantially hypomethylated peaks were obtained ($|\log_2(\text{fold change})| > 1, p < 0.05$) (Fig. 4a). The residual peaks of the m⁶A were viewed as unaltered peaks. According to Integrative Genomics Viewer (IGV) software, the differentially methylated sites in these two groups showed altered intensity, with the GGACU motif associated m⁶A peaks (Fig. 4b). Moreover, differentially methylated m⁶A peaks represented genes were investigated by GO and KEGG pathway analysis, revealing the biological significance of m⁶A methylation

during yak adipocyte differentiation. GO analysis revealed that differentially methylated genes were mainly implicated with DNA-templated and regulation of transcription by RNA polymerase II (ontology: biological process), cytoplasm, nucleus and integral component of membrane (ontology: cellular component), and transcription factor and microtubule binding (ontology: molecular function) (Fig. 4c, Additional file 2). Meanwhile, the biological enrichment of KEGG indicated that the genes differentially methylated were substantially related to the adipogenic metabolism regulation pathways, NOD-like receptor signaling pathway, FoxO signaling pathway, Ether lipid metabolism, cAMP signaling pathway and Hippo signaling pathway (Fig. 4d, Additional file 3).

RNA-seq identification of genes differentially expressed in both groups

An analysis of the RNA-seq dataset (m⁶A-seq input library) displayed that the trends of global mRNA expression between preadipocyte and adipocyte were considerably different. There were 648 significantly different mRNAs, including 300 up-regulated and 348 down-regulated ($|\log_2(\text{fold change})| > 1$, $p < 0.05$), as shown in Fig. 5a. Then, we conducted a clustered heat map to further explore the potential roles of the genes (Fig. 5b, Additional file 4). Furthermore, GO ontology and KEGG pathway were performed to analyze the differentially expressed genes. As Fig. 5c and Additional file 5 displayed, the top 20 most notable functional annotations included regulation of glucose metabolic process, canonical Wnt signaling pathway, positive regulation of cell proliferation and insulin-like growth factor ternary complex, which influenced adipocyte differentiation. Meanwhile, the pathway exploration revealed that signaling pathways regulating pluripotency of stem cells, ECM-receptor interaction, PI3K-Akt signaling pathway and FoxO signaling pathway were significantly enriched (Fig. 5d, Additional file 6), revealing that differentially expressed genes were potentially participated in adipogenic metabolism.

Conjoint analysis of RIP-seq and RNA-seq data with both groups

We found an interesting relationship of differentially methylated m⁶A peaks and gene expression patterns in preadipocytes and adipocytes through cross-analysis the m⁶A-seq and RNA-seq results, in which a positive correlation existed in differentially methylated m⁶A peaks and gene expression level (Fig. 6a). Otherwise, all genes were segregated mainly four types: 8 hypermethylated and upregulated genes termed 'hyper-up'; 7 hypomethylated and downregulated genes termed 'hypo-down'; 12 hypermethylated and whilst downregulated genes termed 'hyper-down'; and 2 hypomethylated and whilst upregulated genes termed 'hypo-up' (Fig. 6b). There were slightly more 'hyper-up' and 'hypo-down' than 'hyper-down' and 'hypo-up'. Table 1 lists the expression of genes were significantly differently ($|\log_2(\text{fold change})| > 1$, $p < 0.05$), comprising significantly differently methylated peaks. Then, both groups were evaluated the overall expression levels of the m⁶A-methylated and non-m⁶A-methylated transcripts (Fig. 6c). These

suggested that in yak adipocyte differentiation, m⁶A modifications appear to have a positive association of mRNA expression. Further, we were wondering if the position of m⁶A peaks on RNA transcripts or the number of m⁶A peaks per transcript is correlated with the levels of gene expression. Based on m⁶A modification sites, RNA transcripts were classified into subgroups. As shown in Fig. 6d, m⁶A modifications of RNA transcripts in CDS, 5'UTR or 3'UTR do not clearly difference with gene expression. Through studying m⁶A-modified sites and relative expression levels of genes, revealing that the genes have three or four modified sites appears to be more abundant in contrast with other m⁶A-modified sites (Fig. 6e).

Table 1

List of 29 genes with significant changes in m⁶A and mRNA transcript abundance in yak adipocyte as compared to preadipocyte.

Gene name	Pattern	m ⁶ A level change			mRNA level change		
		Peak region	Peak start	Peak end	diff.p	log2(fc)	p-value
QPRT	Hyper-up	Exon	491048	491299	0.03	2.30	0.00
BCL2L11	Hyper-up	5' UTR	1175002	1175378	0.01	2.15	0.04
PER1	Hyper-up	3' UTR	235431	236437	0.01	2.22	0.00
KLHL29	Hyper-up	3' UTR	4950507	4950957	0.01	1.44	0.02
KLF9	Hyper-up	5' UTR	1298633	1299052	0.01	2.18	0.00
ZNF395	Hyper-up	3' UTR	730872	731331	0.01	2.25	0.00
ZNF608	Hyper-up	Exon	980374	980553	0.01	2.38	0.00
MTERF4	Hyper-up	3' UTR	726115	726234	0.01	1.32	0.02
CD247	Hypo-down	3' UTR	33979	34575	0.01	-1.78	0.02
SLC05A1	Hypo-down	Exon	1066324	1066623	0.01	-2.00	0.01
AFAP1L2	Hypo-down	Exon	854733	856548	0.01	-3.48	0.02
CENPF	Hypo-down	Exon	1363460	1363640	0.01	-3.67	0.00
USP43	Hypo-down	3' UTR	129792	130001	0.01	-3.08	0.00
ARHGEF28	Hypo-down	Exon	175098	178009	0.01	-2.81	0.00
ARAP3	Hypo-down	Exon	442290	442350	0.01	-3.53	0.00
PHF19	Hyper-down	3' UTR	273548	273938	0.00	-1.29	0.04
ADAMTSL1	Hyper-down	3' UTR	142570	142929	0.00	-1.99	0.01
PLD3	Hyper-down	3' UTR	118422	118482	0.01	-1.16	0.05
CDCA8	Hyper-down	3' UTR	4260517	4260782	0.01	-2.38	0.04

PLEKHA6	Hyper-down	Exon	333151	338353	0.01	-1.76	0.02
SHANK1	Hyper-down	Exon	403394	404020	0.01	-2.14	0.00
SHANK1	Hyper-down	Exon	406035	406274	0.01	-2.14	0.00
CENPF	Hyper-down	Exon	1341226	1357574	0.01	-3.67	0.00
B4GALNT1	Hyper-down	3' UTR	320399	320607	0.01	-2.38	0.04
TEAD4	Hyper-down	3' UTR	240855	242598	0.01	-1.50	0.03
RHBDF2	Hyper-down	3' UTR	274815	275085	0.01	-1.86	0.02
UHRF1	Hyper-down	Exon	560816	563952	0.01	-2.62	0.01
FOXO1	Hypo-up	3' UTR	475509	475778	0.01	1.21	0.05
LOC102267107	Hypo-up	3' UTR	311297	311596	0.01	1.49	0.02

The Verification of m⁶A modified Differentially Expressed Genes by Quantitative Real Time PCR

We implemented qRT-PCR to confirm the expression of differentially methylated genes between adipocyte and preadipocyte. The mRNA expression pattern was consistent with the RNA-seq data (Fig. 7a-b), which confirms the validity of our transcriptome results.

Discussion

The harsh environment of Qinghai-Tibet plateau encourages yak to develop a special mechanism for energy metabolism. As an organ for energy metabolism, adipose tissue plays an crucial role in this process. To date, it has been found that epigenetic regulation is engaged in various biological processes including embryo development, stem cell self-renewal, DNA damage response, primary miRNA processing and energy metabolism [39–43]. In recent years, as the most extensive and plentiful internal modification on mRNAs, m⁶A modification has been a major focus in the area of epigenetic regulation [44].

Furthermore, the potential roles of m⁶A modification in most domestic animals, and especially for adipogenic differentiation, remained largely unknown. For the first time, our study established a comprehensive transcriptome-wide pattern of m⁶A modification in yak preadipocyte and adipocyte using MeRIP-Seq technology to explore the function of m⁶A modification in bovine adipogenic differentiation.

Our findings showed that yak mRNA m⁶A sites were primarily located in CDS, 5'UTRs and 3'UTRs and the distribution semblable with humans and mice [28, 45], suggesting that in mammalian transcriptomes the overall distribution of m⁶A sites is in similar manner. According to previous studies, the consitent motif pattern of "RRACH" was over-represented the m⁶A motif sequence area [38, 46, 47]. Accordingly, in comparison with previous studies [37, 38], the consensus motif (GGACU) sequence in the yak transcriptome was appropriately identified revealing that RNA adenosine methylation was conserved in mammals.

Earlier studies indicated that m⁶A modification is closely related to gene expression [45, 48–50]. Jean-Michel Fustin et al. reported that *METTL3* depletion inhibited the export mRNA [51] and Guanqun Zheng et al. reported that depletion of *ALKBH5* increased the export of mRNA to cytoplasm [23], suggesting m⁶A promotes the export of mRNA and modulates gene expression [25]. Above results indicating a potential positive association between the degree of m⁶A methylation and the transcript level. In the present study, the majority of modified m⁶A genes were expressed at a medium level with a positive relationship exists in gene expression and m⁶A methylated modification. Our fondings are in agreement with Chen et al., they revealed that m⁶A modifications tend to have positive correlation with mRNA expression in clear cell renal cell carcinoma [49]. These findings show that m⁶A methylation affects gene expression by controlling post-transcription regulation.

GO analysis explored the differentially methylated genes, which participated in the transcript regulation with a variety of transcription factors by RNA polymerase II. For example, FOXO1 identified as a Forkhead transcription factor controlling the differentiation of adipocytes [52], and many members of the ZNF family considered as the crucial eukaryotic transcription factors involved in adipogenic metabolism [53], indicating m⁶A methylation participate in lipid metabolism. The KEGG pathway analysis revealed that the signaling pathway of differentially methylated genes closely related to adipose metabolism, such as FoxO signaling pathway, Ether lipid metabolism, Glycerophospholipid metabolism and Hippo signaling pathway-multiple species. Especially, *FOXO1* furtherly found involved in FoxO signaling pathway which demonstrated important for adipocyte differentiation [52]. As a TEA domain family transcription factor, *TEAD4* selected from Hippo signaling pathway-multiple species, in which recruits the cofactors VGLL4 and CtBP2 to inhibit murine adipogenesis [54]. To summarize the above findings, we concluded that activating the FoxO signaling pathway and Hippo signaling pathway through m⁶A methylated gene may perform a key function during differentiation of yak adipocyte.

Integrated analysis of m⁶A-seq and mRNA-seq data exposed that 29 significant change genes existed in adipocyte group with differently methylated m⁶A sites compared to preadipocyte. Several of the genes have been confirmed to regulate adipose metabolism and adipogenic differentiation, such as *ZNF395*, *KLF9*, *TEAD4*, *FOXO1*, and *UHRF1*. *ZNF395*, the mRNA of which is hypermethylated and the expression upregulated in adipocyte group compared to the preadipocyte group. As a member of the C2H-type Zinc finger proteins, *ZNF395* is classified to Papillomavirus-binding factor and Huntington disease gene regulatory regionbinding protein 2 [55]. Experiments of loss and gain function demonstrated that *ZNF395*

interacts with *PPARG2* to modulate the transcriptional regulatory pathway that may be necessary for preadipocyte differentiation [56]. Besides, previous literature reported that mesenchymal stem cells were co-transduced with *ZNF395* and *PPARG2* enhanced the endogenous expression of *PPARG2* and *C/EBPα* which are necessary for adipocyte differentiation [56, 57]. In addition to that it was reported that Krüppel-like factor 9 (*KLF9*), deemed to the basic transcription element-binding protein-1 (BTEB1) could transactivate *PPARγ2* to regulate adipogenesis in the 3T3-L1 cell line [58]. Besides, Kimura Hiroko et al. found that *KLF9* triggered the early stage of adipogenesis by promoting the *C/EBPβ* gene expression in 3T3-L1 cells [59]. Ubiquitin-like with PHD and RING Finger domains 1 (*UHRF1*) has been widely documented to promote cell proliferation [60]. Additionally, a study revealed that *UHRF1* facilitates the proliferation of human adipose-derived stem cells and represses adipogenesis via inhibiting peroxisome proliferator-activated receptor γ [61]. These findings suggested that m⁶A modifications may perform an essential role during yak adipocyte differentiation.

Conclusions

Current findings displayed that the m⁶A distribution patterns in the yak transcriptome and close association between m⁶A methylation and adipose metabolism. Furthermore, we have also explore the correlation between the m⁶A methylation and the level of gene expression, indicating a regulatory role for m⁶A in adipocyte differentiation. These results provides additional knowledge of m⁶A methylation in adipose tissues and it set the foundation for further understanding its possible roles and regulatory mechanisms, which could be helpful for exploration the yak adaptive mechanism in harsh environment.

Methods

Pre-Adipocyte Isolation

The Datong Yak Breeding Center (Datong County, Qinghai, China) provided three healthy 3-day-old Datong yaks. The night before slaughter, yaks were not fed. On the next morning, the yaks were humanely sacrificed by the way of electrical stunned (90 V, 10 s, and 50 Hz) at a commercial slaughter facility and exsanguinated for necessary to ameliorate the suffering, according standard approved industry protocols. The subcutaneous adipose tissue was harvested according to the protocols and guidelines for animal ethics of the People's Republic of China. Yak adipocytes were isolated from subcutaneous fat tissue that was flushed with penicillin (200 U/mL) and streptomycin (200 U/mL) added to the phosphate saline buffer (HyClone, Thermo Fisher Scientific, Carlsbad, CA, USA). After that they were finely minced into about 1 mm³ pieces with an aseptic setting. The segments were digested by Type I collagenase in a continuously agitated water bath at 37°C for 60-90 min. With a 40μm nylon mesh film, indigestible material was screened and the filter liquor was resuspended for 5 min at 1400g. The sediment was subsequently hatched at room temperature for 10 min with the erythrocyte lysis buffer (0.154 M NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA). The cells were then filtered with 200μm nylon mesh film, and rinsed twice with a serum-free medium. After 5 minutes of centrifugation at 1400g, preadipocytes were harvested and

solubilized in the growth media, including DMEM-F12 (Hyclone, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, USA).

Adipogenic differentiation and staining of Oil red O

To induce adipogenic differentiation, pre-adipocyte was induced for 2 days by adipogenic compounds composed with 3-isobuty-methylxanthine (MIX) (Sigma, USA), dexamethasone (Sigma, USA), rosiglitazone (Sigma, USA), and insulin (Sigma, USA) after cells confluence approached 70% in growth media. The medium was replaced after two days with DMEM-F12 containing 10% FBS, penicillin (200 U/mL), streptomycin (200 U/mL) and 5 ng/mL of insulin, and updated with cycles of two days, until day 12. The cells were usually flushed twice with PBS and set for 1 h in 4% formalin. Cells were then reacted at room temperature for 30 min with a saturated solution Oil Red O. And then, cells were rinsed three times with sterile water, and photographs were acquired from light microscopy.

Quantitative real-time PCR

Real-time RT-PCR was accomplished in a CFX Link Real-Time PCR Detection System, and 10 μ L volume of reaction consisting of 5 μ L 2xSYBR Premix Ex Taq II, 0.4 μ L primers (10 μ M), and 0.8 μ L cDNA. The reaction condition was as follows: denaturation for 30s at 95°C followed by 35 additional cycles for 15s at 94°C, annealing for 30s at 72°C. A melting procedure with a heating rate of 0.5°C /10s was performed to create melting curves ranging from 95 °C. The gene expression levels were estimated using the $2^{-\Delta\Delta C_t}$. Additional file 7 lists the sequences used for the primers.

Measuring the m⁶A Content

The overall content of mRNA m⁶A was measured by a methylation quantification kit of EpiQuik RNA (Epigentek, P-9005, NY, USA). In short, a standard curve was constructed at concentrations of 0.01-0.5 ng/ μ L by positive control. The equivalent RNA solution (1-8 μ L) and negative control were applied to the strip wells. The plate was wrapped with parafilm, hatching for 1.5 h at 37 °C. Then, the wells were washed three times and added the 1:1000 diluted capture antibody at room temp for 1 h. After washing thrice, the detection antibody (1:2000 dilution) and enhancer solution were applied to every well hatched at room temp for 30 min. After five washes, detection solutions were placed on each well and hatched for 10 minutes at room temperature to protect from light. Finally, stop solution applied to each wells and absorbance read with microplate reader at 450 nm.

MeRIP-seq and mRNA sequencing

According to manufacturer's protocol, the total RNA was extracted using Trizol reagent (Invitrogen, CA, USA). Bioanalyzer 2100 and RNA 6000 Nano LabChip Kit (Agilent, CA, USA) with RIN number > 7.0 were used to evaluate the total RNA quality and quantity. Nearly over 200 ug total RNA was performed to isolate Poly (A) mRNA through magnetic beads (Invitrogen) attached poly-T oligo. After purifying, poly (A) mRNA fractions are broken into 100nt long oligonucleotides using divalent cations at high temperatures. Then, the fragmentation of broken RNA were incubated in an IP buffer (50 mM Tris-HCl, 750 mM NaCl and 0.5 % Igepal CA-630) supplied with BSA (0.5-1 µg / µl) for 2 hours at 4 ° C with m⁶A-specific antibody (No. 202003, Synaptic Systems, Germany). The blend was further hatched with protein-A beads and eluted with the extraction buffer (1×IP buffer and 6.7 mM m⁶A), following precipitated by 75% ethanol. DUTP method was used to transform eluted m⁶A-containing fragments (IP) and undisposed input control fragments into final cDNA library according to a strand-specific library preparation. The paired-end libraries of insert sizes were 100 ± 50bp. Afterwards, we implemented the paired-end sequencing on an Illumina NovaseqTM 6000 system at LC-BIO Bio-tech ltd (Hangzhou, China), using a suggested protocol from the manufacturer.

Sequencing data analysis

First of all, in-house perl scripts and Cutadapt [62] were performed eliminate the reads containing contaminants of the adapter, bases of low quality and indeterminate. Meanwhile, the quality of the sequence was validated using fastp. The reads were mapped to the *Bos_mutus* genome (Version: BosGru_v2.0) by HISAT2 [63] with default parameters. Using R package exomePeak [64] identify the m⁶A peaks from mapped reads of IP and input libraries with bed or bam format so as to configure for viewing on IGV software (<http://www.igv.org/>) or the UCSC genome browser. *De novo* and defined motif were identified by MEME [65] and HOMER [66], accompanied by perl scripts in the house seeking the motif with respect to peak. Called peaks were annotated using ChIPseeker [67] by intersection with gene architecture. StringTie [68] calculated the expression level of all mRNAs from input libraries, in which normalized with FPKM (FPKM=[total exon fragments/mapped reads (millions)]). The differentially expressed mRNAs were collected by R package edgeR [69] with the $|\log_2(\text{fold change})| > 1$ and $p\text{-value} < 0.05$. GO seq R package was performed to the Gene Ontology (GO, <http://www.geneontology.org/>) enrichment analysis for the differentially expressed genes [70]. Kyoto Encyclopedia of Genes and Genomes (<http://www.genome.jp/kegg/>) database is a major resource for learning high-level functions and utilities of biological systems. The statistical enrichment tests for genes of differential expression in the KEGG pathways were used the KOBAS software [71].

Statistical analysis

The SPSS 22 software package was used to evaluate statistics. A one-way test of variance assessed the significance of the differences between all of the groups. Statistically significant was the degree of probability * $p < 0.05$; ** $p < 0.01$. Values are shown as mean ± SEM.

Abbreviations

m⁶A: N⁶-methyladenosine; CDS: Coding sequence; 3' UTRs: 3' untranslated regions; 5' UTRs: 5' untranslated regions; IGF2BPs: insulin-like growth factor 2 mRNA-binding proteins; IGV: Integrative Genomics Viewer; GO: Gene ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; FOXO1: forkhead box 1; TEAD4: transcriptional enhancer activation domain 4; VGLL4: vestigial like family member 4; CtBP2: C-terminal-binding protein 2; ZNF395: zinc finger proteins 395; PHF19: PHD finger protein; MTERF4: mitochondrial transcription termination factor-4

Declarations

All authors have reviewed and agreed the version of the manuscript.

Ethics approval and consent to participate

Animal treatment during research was carried out in complete accordance with the protocols and guidelines for animal ethics of the People's Republic of China, and all operations were approved by the Animal Administration and Ethics Committee of Lanzhou Institute of Husbandry and Pharmaceutical Sciences , Chinese Academy of Agricultural Sciences (Permit No. SYXK-2014-0002).

Consent for publication

Not applicable.

Availability of data and material

The data was submitted to the data base of the Sequence Read Archive (SRA). The appropriate number for accession is PRJNA649748.

Competing interests

The authors have no conflicts of interest.

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Authors' contributions

The experiments were conceived and designed by Y.Z. and P.Y. The experiments were performed by Y.Z. The experiments were assisted with J.P., X.W., X.G., M.C., P.B., X.D., C.L. The paper was written by Y.Z. and revised by Q. K. All authors have read and agreed to the published version of the manuscript.

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Figures

Figure 1

Preadipocyte induced differentiation and detection of m6A level between preadipocyte and adipocyte. (a-b) Preadipocytes and adipocytes dyed with Oil red O. (c) Relative expression of PPAR γ , C/EBP α , and FABP4 between preadipocyte (day 0) and adipocyte (day 12). (d) The mRNA expression level of methyltransferases (METTL14, WTAP and METTL3), demethylases (FTO and ALKBH5), and m6A-binding proteins (YTHDF1, YTHDF2 and YTHDF3). (e) m6A abundance between the preadipocytes and adipocytes. (SEM, * $p<0.05$; ** $p<0.01$).

Figure 2

m6A-seq transcriptome-wide, and m6A peak analysis. (a) Number of common and specific m6A peaks in groups of preadipocyte and adipocyte. (b) Venn diagram showing the m6A peaks for transcripts of two group. (c) Profile of m6A-modified genes discovered in m6A-seq. (d) The top motifs enriched from m6A peaks identified from the groups of adipocyte and preadipocyte.

Figure 3

m6A methylome distributed on yak transcripts. (a) m6A peaks enriched with transcripts. Transcript is separated into three parts comprising 5' untranslated region, coding sequence and 3' untranslated region. (b) The proportion of m6A peak distribution in the adipocyte and preadipocyte regions (c) Proportion of genes possessing m6A peaks with different numbers.

Figure 4

The alteration of global m6A modification in adipocyte compared with preadipocyte. (a) Volcano plots showing different m6A peaks (fold change ≥ 2 and $p < 0.05$) between preadipocyte and adipocyte. (b) The abundance of m6A on KLF9 and FOXO1 mRNA transcripts observed by m6A-seq in preadipocyte and adipocyte. (c) GO terms of genes with differential m6A peaks between preadipocyte and adipocyte. (d) The top20 markedly enriched pathways for the genes of differential peaks between preadipocyte and adipocyte.

Figure 5

RNA-seq analyzes of genes differentially expressed between preadipocyte and adipocyte. (a) Volcano plots illustrating the differentially expressed genes. (b) Clustered heat map for comparing preadipocyte and adipocyte with the top 100 most differentially expressed genes. (c) The top 20 significantly GO enrichment terms for significantly differentially expressed genes. (d) The top 20 enriched pathways of dramatically differentially expressed genes.

Figure 6

Conjoint analysis of differentially methylated genes and differentially expressed genes. (a) Dot plot of Log2 fold change (FC) (mRNA expression) versus Log2 FC (differential m6A methylation) revealing a positive association between total m6A methylation and level of mRNA expression (Pearson $r = 0.2135$; p-value = 1.109×10^{-11}). (b) Four quadrant plot showing differentially expressed genes with differentially methylated m6A peaks. (c) The holistic expression levels of the transcripts with m6A-methylated and non-m6A-methylated in different groups. (d) For all m6A peaks, the position of m6A peaks for RNA transcripts and gene relative expression level. (e) Relative mRNA transcript expression level with different number of m6A peaks. * $p < 0.05$ compared with the m6A peak = 1.

Figure 7

qPCR tests of eight differentially expressed genes modified by m6A in preadipocyte and adipocyte. (a) The relative mRNA levels were determined by qPCR of eight genes in both groups and (b) the genes change levels based on RNA-seq data.

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