Identification, functional prediction, and key lncRNA verification of cold stress-related lncRNAs in rats liver

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Cold stimulation reduces the quality of animal products and increases animal mortality, causing huge losses to the livestock industry in cold regions. Long non-coding RNAs (lncRNAs) take part in many biological processes through transcriptional regulation, intracellular material transport, and chromosome remodeling. Although cold stress-related lncRNAs have been reported in plants, no research is available on the characteristic and functional analysis of lncRNAs after cold stress in rats.

Here, we built a cold stress animal model firstly. Six SPF male Wistar rats were randomly divided to the acute cold stress group (4 °C, 12 h) and the normal group (24 °C, 12 h). lncRNA libraries were constructed by high-throughput sequencing (HTS) using rat livers. 2,120 new lncRNAs and 273 differentially expressed (DE) lncRNAs were identified in low temperature environments. The target genes of DElncRNA were predicted by cis and trans, and then functional and pathway analysis were performed to them. GO and KEGG analysis revealed that lncRNA targets were mainly participated in the regulation of nucleic acid binding, cold stimulation reaction, metabolic process, immune system processes, PI3K-Akt signaling pathway and pathways in cancer. Next, an interaction network between lncRNA and its targets was constructed. To further reveal the mechanism of cold stress, DElncRNA and DEmRNA were extracted to reconstruct a co-expression sub-network. We found the key lncRNA MSTGR.80946.2 in sub-network. Functional analysis of key lncRNA targets showed that targets were significantly enriched in fatty acid metabolism, the PI3K-Akt signaling pathway and pathways in cancer under cold stress. qRT-PCR confirmed the sequencing results. Finally, hub lncRNA MSTGR.80946.2 was characterized, and verified its relationship with related mRNAs by antisense oligonucleotide (ASO) interference and qRT-PCR. Results confirmed the accuracy of our analysis. To sum up, our work was the first to perform detailed characterization and functional analysis of cold stress-related lncRNAs in rats liver. lncRNAs played crucial roles in energy metabolism, growth and development, immunity and reproductive performance in cold stressed rats. The MSTGR.80946.2 was verified by network and experiments to be a key functional lncRNA under cold stress, regulating ACP1, TSPY1 and Tsn.

lncRNAs are non-coding RNAs of greater than 200 nt in length. Unlike mRNAs, the primary structure of lncRNAs are not highly conserved, and a considerable number of lncRNAs are transcribed from introns, exons, intergenic regions and overlapping regions1. lncRNAs are the scientific frontier of the genome era and may reveal new RNA-mediated genetic expression regulatory networks, which could clearer clarify function of genome from the perspective of non-coding RNAs. lncRNA works mainly in following ways: lncRNA remodels chromatin2, DNA binding3, promoting chromatin formation4, acting effects on RNA processing5, affecting mRNA stability6 and lncRNA can directly regulate the function of proteins7. With the application of high-throughput sequencing, lots of biologically functional lncRNAs have been discovered. In mammals, lncRNAs participate in regulating important physiological processes such as individual neurodevelopment8, cell cycle9, cell protection10, and tumor development and metastasis11.

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In cold regions, animals are prone to cold stress and their physical development is affected. In pregnant animals, cold stress can result in symptoms such as miscarriage and even infertility. The stress response caused by cold stimulation can cause damage to the nervous, cardiovascular, and immune systems. However, reports on the function of cold stress-related IncRNA are rare. Su et al. studied overexpression of IncRNA TUG1 (tau- rine up-regulated gene 1) in mice can prevent cold-induced damage. IncRNAs associated with cold stress have also been reported recently in cabbage and cassava. Kidokoro found that soybean (C repeat binding factor) CBF/DREB1 (dehydration response element binding protein 1) regulates gene expression in cold response process. This process activates many defense mechanisms, including molecular chaperones, metabolite biosynthesis enzymes and so on. Freezing and low temperature stress can cause plant metabolic disorder and increase the production of various reactive oxygen species. H2O2 can regulate gene expression under cold stress, affecting transduction in wild type and catalase (ΔkatG) thioredoxin peroxidase (tpx) cells treated by cold stress. Keeping body temperature constant under cold environment needs heat production and protection. These mechanisms are affected by various neurotransmitters and hormones, and regulated by the nervous system. Numerous studies have shown that cold stress affects multiple metabolic and molecular regulatory processes in vivo. PACAP (Pituitary adenylate cyclase activating polypeptide) acts a pivotal part in peripheral and central physiological stress responses. Cline found that PACAP is involved in thermostimulated sympathetic signaling and may be a crucial regulator of lipid metabolism. Environmental factors such as cold stress may lead to mammal hippocampus apoptosis in late pregnancy, and in a caspase-3-independent manner to enhance phosphorylation of Ser536 by P65. In addition, some IncRNAs can regulate cell function through other pathways. For example, Kiang et al. found that energy-induced IncRNA HAND2-AS1 (heart and neural derivatives expressed 2-antisense 1) inhibits HIF-1 (hypoxia inducible factor-1) α-mediated energy metabolism and inhibits osteosarcoma development. However, the regulation of IncRNA involvement in cold stress in rats remains unclear.

As an important organ of heat production in the body, the liver increases its activity and heat production during acute cold stimulation to maintain the body's normal temperature. In this process, how IncRNAs play regulatory roles needs further study. Here, we analyzed and identified the characteristics of IncRNAs in liver of cold-stressed rats, predicted the target genes of these differential IncRNA by cis and trans, and explored the roles of IncRNAs under cold stress in rat liver. Our data will help to better understand the mechanisms of IncRNA in rat liver under cold stress.

**Results**

**Identification of IncRNAs in liver of rats.** Six rats were randomly selected respectively in the normal group (L01, L02 and L03) and the stress group (L04, L05 and L06). Then total RNA was extracted from the liver samples and six cDNA libraries were constructed. After quality control of raw data from each sample, high quality data remained nearly 21.10 Gb, accounting for approximately 96.39% of the total. Afterwards, screening of dependable candidate IncRNAs from assembled transcripts based on process pipelines for high-throughput sequencing data (Fig. 1). Clear statistics on the quality of data and the proportion of raw data are shown in Fig. 2A. The distribution of IncRNAs on each chromosome is shown in Fig. 2B. These IncRNAs are evenly distributed on all chromosomes. It is worth noting that the number of IncRNAs on chromosomes 1 and 2 were relatively high, respectively 513 (8.5%) and 304 (5.04%). Four different tools, Coding-Potential Assessment Tool (CPAT), Coding-Non-Coding Index (CNCI), Pfam-scan and Coding Potential Calculator (CPC) were used to calculate the ability of transcripts to encode proteins (Fig. 2C).

**Characterization of cold stress-related IncRNAs.** We described the genomic characteristics of the acquired cold stress-related IncRNAs in rats liver. A large number of the IncRNAs contained two exons (Fig. 3A). Consistent with the size distribution pattern of the IncRNA library, the length of library IncRNAs at 300–500 nt was most distributed, and the number of IncRNAs for this length was 1,611. There were 723 IncRNAs with tags larger than 3000 nt (Fig. 3B). Most IncRNAs (85%) contained a short ORF (open reading frame) (approximately 20–60 amino acids), which is shorter than for cDNA (Fig. 3C). From Figs. 3D, 6,025 candidate IncRNAs were captured, including 3,729 lincRNAs (61.9%), 889 antisense IncRNAs (14.8%), 1,129 intronic IncRNAs (18.7%) and 278 sense IncRNAs (4.6%). Of these IncRNAs, 317 (5.2%) were identified as known using BLAST alignment with the rat IncRNAs in NONCODEv5 database, and we obtained a grand total of 451 novel IncRNAs.

We assessed the conservation of rat IncRNAs (Fig. 4A). Most IncRNAs scores ≤0.4, indicating poor conservation. Moreover, the overall distribution of IncRNAs expression was presented by the FPKM (fragments per kilobase million) density distribution comparison chart and the FPKM box plot. By detecting the expression pattern of IncRNAs, it was shown that the expression profiles of the three biological replicates in the cold stress group and the normal group were relatively close, indicating that the experimental repeated data was will (Fig. 4B). The FPKM value of IncRNAs expression levels spanned 10−2 to 104 six orders of magnitude (Fig. 4C). In Fig. 4D, we quantified the expression level of the IncRNAs using Stringtie. The clustering plots showed significant differences in IncRNA expression between the groups, but the differences within each group were small.

**Differential expression of IncRNAs.** Expression levels were analyzed using the ‘ballgown’ R package to screen differential expressed (DE) IncRNAs between the cold stress group and the normal group. We identified 146 up-regulated IncRNAs (53.5%) and 127 down-regulated IncRNAs (46.5%), a total of 273 significant DElncRNAs. The top 20 significant DElncRNAs were demonstrated in Table 1. The volcano plot in Fig. 5A depicted the approximate distribution of DElncRNAs. In our study, 2120 novel IncRNAs were acquired, including 435 antisense IncRNAs, 1618 lincRNAs and 67 intronic IncRNAs. The targets of DElncRNAs were predicted based on trans and cis-acting. Furthermore, the targets of 58 IncRNAs had functional annotations. From the correlation plot, it can be seen that IncRNA expression is highly correlated within the group (Fig. 5B).
Functional and pathway analysis of DElncRNA targets. The DAVID software was used to predict functions of DElncRNA targets. GO (Gene Ontology) analysis included 19 cell component (CC) terms, 21 molecular function (MF) terms and 22 biological process (BP) terms. Among them, the cell part, membrane part; the binding, catalytic activity, signal transduction activity and biological regulation, metabolic process, response to stimulation had high percentages of genes (Fig. 6). GO results showed that regulation of nucleic acid acid binding, cold stimulation response, regulation of cytokines, regulation of protein complex stability, regulation of protein ubiquitination, metabolic processes, multicellular biological processes and the like. In order to clarify the specific signal pathways affected by the targets, the KEGG (Kyoto Encyclopedia of Genes and Genomes) analysis was conducted. As shown in Fig. 7, the target genes were divided into 50 KEGG pathways. The PI3K-Akt signaling pathway, insulin signaling pathway, T cell receptor signaling pathway, pathways in cancer, fatty acid metabolism, HIF-1 signaling pathway, glucose metabolism and lipid metabolism were mainly involved in the regulation of liver in cold stress rats. Results suggested that these DElncRNAs targets may play crucial roles in energy metabolism, immunity, growth and development, proliferation and apoptosis.

To compare the functions of targets predicted in different ways, GO and KEGG analyses were performed on cis and trans targets respectively from DElncRNAs. GO classification of cis and trans targets from up and down-regulated lncRNAs respectively showed in Supplementary Figs. S1–S4. KEGG classification of cis and trans targets from up and down-regulated lncRNAs showed in Supplementary Figs. S5–S8. The key pathways and functions in red both appear in up- and down-regulate lncRNAs. It indicated that targets were mainly involved in metabolic processes, responses to stimuli, multicellular biological processes, immune system processes, reproductive processes, transport activity, molecular function regulation, nucleic acid binding transcription factor activity, fatty acid metabolism, pathways in cancer and the PI3K-Akt signaling pathway under cold stress.

Construction of co-expression network to reveal hub lncRNAs. First, a interaction network of lncRNAs and their targets was constructed using limma platform (Fig. 8). The results showed that a total of 723 interactions were identified among 273 DElncRNA and 415 target genes. There were 517 positive interactions and 206 negative interactions among pairs within the network, and most lncRNA-gene pairs were positively correlated. In addition, one lncRNA can be associated with 1 to 35 mRNAs, and one mRNA can be associated with 1 to 30 lncRNAs. To reveal the most significant hub DElncRNA (P < 0.01), we extracted the junctions between DElncRNA and DEMRNA to reconstitute a co-expression network. As shown in Fig. 9, the two lncRNAs MSTRG.80946.2
and MSTRG.7147.72 were in the center of the network. Compared with MSTRG.7147.72, the MSTRG.80946.2 had higher node degrees and more interactive pairs. This suggested that MSTRG.80946.2 may be a key functional lncRNA in rats liver under cold stress.

In order to understand clearer the functions of hub MSTRG.80946.2, the functional analyses were performed on its co-expressing DEMRNAs. GO analysis revealed the top three significantly enriched in “Biological Processes” (BP), including regulation of protein complex stability, metabolic processes and multicellular biological processes (Fig. 10). In addition, the top ten pathways of genes were listed as the most significant p-values. As shown in Fig. 11, KEGG enrichment analysis showed that the top three pathways enriched with most targets were pathways in cancer, the PI3K-Akt signaling pathway and fatty acid metabolism. They respectively included mRNA TSPY1, ACP1, Tsn, Hsp90ab1 and so on. Pathways in cancer is closely related to immunity, cell...
proliferation and apoptosis. PI3K-Akt signaling pathway involves many biological processes, such as protein synthesis, glycolysis, and apoptosis. Studies have reported that lncRNA TUG1 (taurine up-regulated1) inhibits mice apoptosis and thus has a protective effect on cold-induced liver injury. Translin was a regulator that responds to metabolic changes. Metabolic status has a major impact on the regulation of biological rhythms. Hsp90ab1 was an ATP-dependent highly conserved molecular chaperone. It interacted with some epidermal growth factor receptor (EGFR), human epidermal growth factor receptor-2 (HER2), which played an important role in cancer pathway and participates in various pathophysiological processes of cells. ACP1 was a marker enzyme for lysosomes. As an organelle for digestive function in cells, it contained a large amount of acidic hydrolase, which played an important role in the metabolism of substances inside and outside the cell. These results showed that lncRNA targets were prominent in metabolic disorders and cancer pathways under cold stress.

Quantitative analysis verified sequencing accuracy. We selected 10 significantly DElncRNAs (MSTRG.488.1, MSTRG.73505.5, MSTRG.7147.72, MSTRG.69299.2, MSTRG.4553.16, MSTRG.52070.1, MSTRG.29045.2, MSTRG.55788.4, MSTRG.487.14 and MSTRG.80946.2) from cold-stressed rat livers to verify the accuracy of sequencing results by qRT-PCR (Fig. 12). The results illustrated that the relative expressed changes of lncRNAs in conformity with high-throughput sequencing results, indicating that expressed assessment and identification of lncRNAs were persuasive. In all DElncRNAs, MSTRG.80946.2 was the most significantly DE (P < 0.001) under cold stress. Therefore, further functional verification of this key lncRNA was performed.

Characteristic analysis of the key lncRNA. We compared MSTRG.80946.2 with the known rat sequence in NONCODEv5 database, which was closest to NONNATT021477.2 in length and chromosomal location with 99% homology. So far, there was little information about NONNATO21477.2, only its sequence length was 712 bp and located in chr4. The full length of MSTRG.80946.2 was amplified by RACE (rapid amplification of the cDNA ends). As shown in Fig. 13, the length of the 5' RACE was 583 bp, and the length of the 3' RACE was 383 bp. After
the linker sequence was removed, the full-length was spliced to 746 bp. The RACE products were subjected to agarose gel electrophoresis. The results showed a distinct band at 746 bp. Next, we performed BLAST alignment using the full length of MSTRG.80946.2 and the known rat sequence from NCBI Genebank. It was found that its sequence was inversely complementary to the sequence of acid phosphatase 1 (ACP1) (Supplementary Fig. S9).

In a comparison with the co-expression network (Fig. 9), ACP1 may be a target gene of MSTRG.80946.2 by Cytoscape.3.60. This illustrated that our analysis was aligned with the sequencing results. Regulating the expression of adjacent genes is one way in which lncRNAs act. Thus, we hypothesized that MSTRG.80946.2 may play a part in rats liver under cold stress by regulating ACP1 expression. Then the subcellular localization of MSTRG.80946.2 in BRL (Rat liver cell) was verified by fluorescence in situ hybridization (FISH). The Fig. 14 showed that MSTRG.80946.2 was mostly expressed in the nucleus, and expressed at a low level in the cytoplasm.

| IncRNA         | P-Value     | FDR        | log2FC      | regulated | length (bp) |
|---------------|-------------|------------|-------------|-----------|-------------|
| MSTRG.56167.3 | 8.44E-11    | 4.61E-07   | 11.10088491 | up        | 2858        |
| MSTRG.487.14  | 2.45E-10    | 6.70E-07   | −9.10749482 | down      | 1414        |
| MSTRG.65671.4 | 2.10E-09    | 3.82E-06   | −10.51898195| down      | 258         |
| MSTRG.23974.3 | 1.26E-08    | 1.72E-05   | 10.44064717 | up        | 6389        |
| MSTRG.4553.16 | 7.33E-08    | 8.44E-05   | −9.45919615 | down      | 3451        |
| MSTRG.65602.6 | 1.91E-07    | 0.00017409 | 9.287886208 | up        | 3182        |
| MSTRG.30092.2 | 2.66E-07    | 0.00020722 | 9.54994454  | up        | 6710        |
| MSTRG.7147.36 | 4.07E-07    | 0.00027635 | −9.09401165 | down      | 1800        |
| MSTRG.488.3   | 1.35E-06    | 0.00081959 | −14.1999886 | down      | 1498        |
| MSTRG.7147.38 | 1.79E-06    | 0.00097992 | −9.20439527 | down      | 1862        |
| MSTRG.55788.4 | 3.26E-06    | 0.00161810 | −8.40699362 | down      | 1230        |
| MSTRG.81485.14| 9.94E-06    | 0.00452224 | 8.117395911 | up        | 2558        |
| MSTRG.75398.3 | 1.36E-05    | 0.00571481 | −9.07490696 | down      | 1327        |
| MSTRG.7146.36 | 1.73E-05    | 0.00631420 | −10.7718630 | down      | 1897        |
| MSTRG.7146.37 | 1.73E-05    | 0.00631420 | −6.58325236 | down      | 1015        |
| MSTRG.52429.7 | 3.65E-05    | 0.01215520 | −8.07726431 | down      | 1380        |
| MSTRG.7146.42 | 3.78E-05    | 0.01215520 | 10.14630706 | up        | 1963        |
| MSTRG.60605.1 | 5.38E-05    | 0.01632447 | 8.05143068  | up        | 6118        |
| MSTRG.25014.4 | 9.39E-05    | 0.02700417 | 9.57666706  | up        | 6097        |
| MSTRG.7147.7  | 0.000103965 | 0.0270409  | −9.41843048 | down      | 2206        |

Table 1. Differentially expressed lncRNAs in liver samples.

Figure 5. Differentially expressed lncRNAs. (A) The Volcano plot of DElncRNAs between the cold stress group and the normal group. Red color represents significant up-regulated and green color represents significant down-regulated. (B) The correlation plot, three repeated samples, the points are concentrated in the diagonal, indicating a high correlation coefficient. Correlation was evaluated by Pearson's correlation coefficient of total lncRNAs expression levels.
Validation of key lncRNA and its targets in vitro. In order to verify the relationship between MSTRG.80946.2 and its targets, we transfected ASO targeting MSTRG.80946.2 into BRL cells. The Fig. 15(A) confirmed the silencing effect of MSTRG.80946.2 compared to control ASO-NC, and interference efficiency at

Figure 6. GO classification of target genes of DElncRNAs in rats. The abscissa was the GO classification, the left side of the ordinate was the percentage of genes, and the right side is the number of genes.

Figure 7. KEGG classification of target genes of DElncRNAs in rats. The abscissa is the annotated genes, the ordinate was pathway term.
Figure 8. Differential lncRNA interacts with target genes. lncRNAs and coding transcripts were presented as squares and circles, respectively.

Figure 9. The co-expression network of DElncRNAs and DEmRNAs. IncRNAs and mRNAs were presented as diamonds and squares, respectively. And green and blue indicated down-regulate, red indicated up-regulate.

Figure 10. GO classification of co-expressing mRNAs of lncRNA MSTRG.80946.2.
have opposite effects on cell proliferation and degradation of viral HBx (Hepatitis B virus X protein) oncoprotein.

Signaling, due to amino acid deficiency. There is increasing evidence that Igf2 cold stress, and can target two cis genes, genes through cis mechanism. For example, lncRNA MSTRG.7147.72 (1910 bp, chr 1) was down-regulated under study, it was identified as a known lncRNA with 99% homology to NONNATT021477.2, and had 10 target gene transcription of these target genes requires further investigation. MSTRG.62962.1, ENSRNOG00000042133 and ENSRNOG00000059588 showed strong co-expression, but annotation of co-expressed genes. For example, lncRNA MSTRG.15671.1 was identified as a novel lncRNA with 99% homology to NONNATT021477.2, and had 10 target gene of the identified lncRNAs were not found in public data, and there was little information to describe the functional annotations of co-expressed genes. For example, MSTRG.62962.1, ENSRNOG00000042133 and ENSRNOG00000059588 showed strong co-expression, but annotation of these target genes requires further investigation.

MSTRG.80946.2 was the hub lncRNA in the net. Currently, there were few reports on this lncRNA. In this study, it was identified as a known lncRNA with 99% homology to NONNATT021477.2, and had 10 target gene pairs (Fig. 9), including TSPY1, ACP1, Tsn, Il2rb, SZT2, Lpin1, EPAS1, Hsp90ab1, Alb and Ccdc107. Testis-specific protein Y-encoded (TSPY) is expressed in sperm cells of adult animal testes (33). TSPY1 is related to male testis and fertility. TSPY and its homologous TSPX act as protooncogenes and tumor suppressor genes, respectively. They have opposite effects on cell proliferation and degradation of viral HBx (Hepatitis B virus X protein) oncoprotein (34). Recently, human TSPY has been reported to inhibit USP7 (ubiquitin-specific peptidase 7) -mediated p53 function and promote spermatogonia proliferation A (35). The deletion of Il2rb causes mice to develop immune disease and NK cell dysfunction, including severe autoimmunity (36). Loss of function of SZT2 leads to over-activation of mTORC1 signaling, due to amino acid deficiency (37). There is increasing evidence that SZT2 is associated with neurological diseases such as growth retardation and epilepsy. SDC belongs to the family of HSPG (heparan sulfate proteoglycans). Giuseppina et al. found that SDC4 may regulate lipid homeostasis and play a key role in human health and longevity (38). Studies have shown that translin/trax RNase complex can degrade microRNA, thereby regulating energy metabolism (39). Lack of Lpin1 can lead to severe metabolic homeostasis, such as fatty liver and cardiovascular disease. It regulates cellular triacylglycerol levels and liposomes in cellular signaling pathways (40). Lipoproteins as a target for inflammation diseases or metabolic therapies require further investigation. Herui et al. established a mouse model to confirm that the EPAS1 mutation is a causative gene for somatostatin (41). Studying the HIF-2α function associated with EPAS1 helps to discover the role of tumors. HSP (Heat Shock Protein) can form a multi-protein chaperone complex involved in the proliferation of animal cells and the folding of apoptotic substrates (42). Inhibition of Hsp90 leads to ubiquitination of the proteasome pathway (43). Hsp90a1 is one of its isoforms. Ccdc107 is one of the family of helical coil domains (Ccdc). Ccdc has many important biological functions and can regulate various biological behaviors such as invasion and metastasis of malignant tumor cells (44). It has been confirmed that Ccdc protein is abnormally expressed in prostate cancer, breast cancer and so on (45). And it has a direct link with tumor cell migration.
and invasion. These target gene reports above suggested that lncRNA may affect the physiological processes of energy metabolism, reproductive performance, immunity, apoptosis and proliferation in rats under cold stress. It is well known that the effects of low temperature environments on the energy metabolism of living organisms are considerable large\(^47\). Cold stress can damage macromolecules such as proteins, nucleic acids and lipids in body cells. These molecular deletions lead to metabolic disorders and changes in redox potential\(^48\).

In further study, lncRNA MSTRG.80946.2, which is closely related to cold stress and has a significant expression level, was selected for verify in cells to elucidate its mechanism of action. We found that MSTRG.80946.2 is 99% homologous to NONNATT021477.2 from NONCODEv5 database. MSTRG.80946.2 is mainly expressed in the nucleus of BRL by FISH (fluorescence in situ hybridization), so subsequent gene silencing experiments prefer ASO technology.

The full length of MSTRG.80946.2 was amplified by RACE. It was found to be an antisense lncRNA of rat ACP1 compared with NCBI database. ACP1 is a lysosomal marker enzyme that is involved not only in intracellular digestion and endocytosis of phagocytic cells, but also in important life activities, such as nucleic acid and protein metabolism, immune regulation and signal transduction\(^49\). Wang et al. found that ACP has strong activity in fish liver and is widely involved in the energy metabolism\(^50\). Interestingly, ACP1 was one of DElncRNAs of MSTRG.80946.2 in the co-expression network. Therefore, we further verified the relationship between MSTRG.80946.2 and its targets in BRL cells. It was confirmed that MSTRG.80946.2 did regulate ACP1, TSPY1 and Tsn expression in rats under cold stress.

Figure 12. qRT-PCR validation of high throughput sequencing. Validation of 10 selected DElncRNAs. T-test p-values \(< 0.05\) are considered to be significantly different, \(*\) represents a p-value \(< 0.05\) and \(* *\) represents a p-value \(< 0.01\).

Figure 13. The agarose gel electrophoresis map of RACE product. M: DL2000 DNA Marker. 5′ RACE: 583 bp (include link sequence); 3′ RACE: 383 bp (include link sequence).
In conclusion, this study was the first to systematically identify cold stressed-associated lncRNAs in rats liver and construct lncRNAs DE profiles. lncRNAs played crucial roles in energy metabolism, reproductive performance, growth and development and immunity in rats by regulating mRNAs under cold stress. The MSTRG.80946.2 was verified by network and experiments to be a cold responsive key lncRNA, regulating protein-coding genes ACP1, TSPY1 and Tsn. However, the detailed mechanism of lncRNAs under cold stress still requires further experimental verification.

Figure 14. The subcellular positioning of MSTRG.80946.2 in BRL cell.

Figure 15. qRT-PCR verification of relative expression after ASO-MSTRG.80946.2 silencing. (A) The relative expression of MSTRG.80946.2. (B) The relative expression of ACP1. (C) The relative expression of Tsn. (D) The relative expression of TSPY1. *P < 0.05, **P < 0.01.
**Materials and Methods**

**Ethics statement.** This research strictly followed the principles of animal use in the China Laboratory Animal Science Association. And the study obtained the approval of the Animal Ethics Committee of Heilongjiang Bayi Agricultural University. In order to reduce animal suffering, all surgery was performed under sodium anesthesia with pentobarbital.

**Samples collection, library preparation and high-throughput sequencing.** Our team established an animal cold stress model early on and confirmed that liver is a key target organ for cold stress injury. Six 12 weeks old SPF Wistar male rats were equally divided into two groups, using artificial intelligence climate chamber for cold stress group (4 ± 0.05) °C and normal control group (24 ± 0.05) °C kept for 12 h. The relative humidity was 45 ± 0.10%, and diets or water and other conditions were treated equally. The next day, liver samples were collected from anesthetized rats to extract total RNA. According to manufacturer's description to construct cDNA libraries using SMART Kit (Clontech, USA) [31]. The HiSeq. 2500 plat was used to perform sequencing. Processing raw data for quality control and got Q30 and GC sizes of clean data. High throughput sequencing was performed by Biomarker Technologies Co., Ltd. (Beijing, China).

**Bioinformatics identification of lncRNAs.** Screening transcripts with length ≥200 bp and exon number ≥2. The coverage of each transcript was calculate by cufflinks software, and selected the transcript with reads minimum coverage ≥3. The IncRNAs spliced by two splicing softwares and screened out to obtain new predicted IncRNAs set. We used cuffcompare information to screen different types of IncRNAs such as lincRNA, intronic IncRNA, and anti-sense IncRNA. Four methods for screening transcript coding potential by CNCI, PhyloCSF analysis, CPC and pfam protein domain analysis [32-34].

**Analysis of DElncRNAs.** The expression level of IncRNAs was estimated by FPKM (fragments per kilobase of transcript per million fragments mapped). In the process of differential expression of IncRNAs, Fold Change ≥2 and FDR < 0.01 were used as screening criteria [35]. Hierarchical clustering analysis of DElncRNAs. The cis target genes were predicted to analyze genes in range of 10 kb up- and down-stream of IncRNA on same chromosome. The prediction of trans target genes based on possible co-expression relationship between IncRNA and mRNA [36]. GO and KEGG analysis of DElncRNA target genes by DAVID (https://david.ncifcrf.gov/).

**IncRNA-mRNA networks construction.** DElncRNA-mRNA interaction network was created for Cytoscape visualization [37]. And selected key node DElncRNA and DEMRNA to construct co-expression regulatory network. Furthermore, screening for the hub IncRNA via this network.

**Verification of sequencing results by qRT-PCR experiment.** To further verify the sequencing results, we selected 10 DElncRNAs randomly to qRT-PCR. Follow the 2⁻µµ SYBR Green qPCR Mix Kit, and the reaction system was 25µl. β-actin as an internal reference primer. Primers were designed using Oligo6 online software. Primers were synthesized by Sangon (Shanghai, China). Forward and reverse primer sequences: MSTRG.80946.2: F: 5′-GCACAAGTTCCATCACTTCCGCTGTTT-3’ R: 5′-GGAACGCACCACTGTGGAAC-3’. MSTRG.488.1: F: 5′-AGACCGGCACCTATCCCTCA-3’ R: 5′-AACCTCTGGAGGCTCCGATG-3’. MSTRG.73505.5: F: 5′-CTTCGTCGCTGACTG-3’ R: 5′-CCATAGGCTTCATTGCGTCTT-3’. MSTRG.52070.1: F: 5′-CACGCAAGTCCTGCTGACACGA-3’ R: 5′-TCACACAGTTCACGCAAGGA-3’. MSTRG.4553.16: F: 5′-CCGACCAAGGATCTCTGCAAGC-3’ R: 5′-TGCTGGAATGAATGCTGGAT-3’. MSTRG.7147.72: F: 5′-TGCTGCAACACTTGCTTCT-3’ R: 5′-TCCATGACTGCGATTCCACT-3’. MSTRG.487.14: F: 5′-AGACGTGAGTCTCTGGAAG-3’ R: 5′-TCAGGACCTCTGGAAG-3’. MSTRG.59045.2: F: 5′-TCACACAGTTCCTCGGAGA-3’ R: 5′-GCCACACAGTCTGACAT-3’. MSTRG.35578.84: F: 5′-AGACGTGCGAAGGACACACA-3’ R: 5′-TCACAGACCTCAGGAAG-3’. MSTRG.25020.14: F: 5′-CGGCCAGCGAGAAACGAAAC-3’ R: 5′-TCCTGCTGTCCGCTTCTCT-3’. MSTRG.73505.5: F: 5′-CTTCTGGAGATGCTGAGAG-3’ R: 5′-TGCTGGAATGACCTCGGAG-3’. Results data are expressed as mean ± standard deviation. ANOVA (One-way analysis of variance) was used to check the significance of the mean using Graphpad Prism 7.0.

**Rapid amplification of the cDNA ends.** To identify the full length sequence of IncRNA MSTRG.80946.2, RACE experiments were performed using the SMARTer TM RACE cDNA Amplification Kit (Clontech, USA) according to the manufacturer’s instructions. The general procedure of 5′ RACE and 3′ RACE were as follows: synthesis of first strand cDNA, amplification of target cDNA, cloning and amplification of RACE product, screening and sequencing. The product was subjected to agarose gel electrophoresis. The specific primers sequences are as follows: 5’ RACE: TGCTCACACTCCGAGGCACAG; 5’ RACE: CAGACATTTTGAGTGTGCGCT.

**Cell culture.** BRL cells (normal rat hepatocytes) were purchased from the Chinese Academy of Sciences Cell Resource Library (Beijing, China). The cell culture medium were10% FBS, 10 ml fetal bovine serum (gibco, USA) and penicillin-streptomycin solution 0.1%. The cells were cultured in 37°C, 5% CO₂ incubator (Binder, Germany), updated the solution after 24 h.

**Fluorescence in situ hybridization.** FISH was performed according to fluorescence in situ hybridization kit (RiboBio, Guangzhou, China). BRL cells were washed with PBS and fixed in 4% paraformaldehyde for 15 min. Then, the permeate of 0.3% Triton X-100 was added to stand for 8 min, washed twice with PBS, and kept at 37°C for 30 min. Anti-MSTRG.80946.2, anti-U6 or anti-18S probes were hybridized overnight at 37°C. The next day, DAPI counterstained the cells and imaged using CLSM (confocal laser scanning microscope) (Leica, Germany).
In vitro lncRNA silencing assay. Specific ASO interference sequence targeting lncRNA MSTRG.80946.2 by Ribobio Biotechnology (Guangzhou, China). The ASO plasmids were transfected into BRL cells at 200 nmol for 24 h, and then subjected to qRT-PCR. The ASO sequence are: ASO-MSTRG.80946.2: 5′-TTAATCGTACCCACGCTTGG-3′, ASO-NC: 5′-TTAATGGGAGGCTGCCATG-3′. Transfection was performed using Lipofectamine RNAiMAX (Thermo Scientific, USA).

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**Author contributions**

H.J. and S.L. designed the experiments. C.N. carried out most of the experiment, data analysis, and wrote the main manuscript text, and all authors reviewed the manuscript.

**Competing interests**

The authors declare no competing interests.

**Additional information**

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