

Expression profile analysis of long non-coding RNAs involved in the metformin-inhibited gluconeogenesis of primary mouse hepatocytes

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Abstract. Long non-coding RNAs (lncRNAs) have been demonstrated to regulate metabolic tissue development and function, including adipogenesis, hepatic lipid metabolism, islet function and energy balance. However, the role of lncRNAs in gluconeogenesis remains completely unknown. Metformin reduces glucose output mainly via the inhibition of gluconeogenesis. In the present study, the lncRNA expression profile of primary mouse hepatocytes exposed to cyclic adenosine monophosphate (cAMP), a gluconeogenic stimulus, with or without metformin was analyzed by microarray. Among the 22,016 lncRNAs that were identified, 456 were upregulated and 409 were downregulated by cAMP (fold-change ≥ 2.0). Furthermore, the cAMP-induced upregulation of 189 lncRNAs and downregulation of 167 lncRNAs was attenuated by metformin. The expression levels of eight lncRNAs were validated by reverse transcription-quantitative polymerase chain reaction, and the results were consistent with those of the microarray analysis. Among them, two lncRNAs NR_027710 and ENSMUST00000138573,

were identified to have an association with two protein coding genes, namely peroxisome proliferator-activated receptor- γ coactivator-1 α , a critical transcriptional coactivator in gluconeogenesis, and G protein-coupled receptor 155, respectively. The two protein coding genes exhibited similar expression patterns to their associated lncRNAs. The findings of the present study suggest that lncRNAs are potentially involved in the regulation of gluconeogenesis.

Introduction

The incidence of diabetes mellitus is increasing, and this condition is becoming one of the major causes of morbidity and mortality across the globe (1). Blood glucose levels are maintained within a narrow range in healthy individuals by the liver through the opposing actions of insulin and glucagon (2). Hepatic gluconeogenesis, which is inhibited by insulin and stimulated by glucagon, serves an essential role in the maintenance of a normal blood glucose level during fasting. Gluconeogenesis becomes unrestrained in diabetes due to either deficient insulin secretion in type 1 diabetes mellitus or deficient insulin action in type 2 diabetes mellitus, which contributes to hyperglycemia (3). Therefore, elucidation of the molecular mechanism involved in the regulation of hepatic gluconeogenesis is likely to provide new avenues for the treatment of type 2 diabetes mellitus.

Long non-coding RNAs (lncRNAs) are defined as transcripts of >200 nucleotides (nt) that do not encode protein. According to their position relative to nearby coding genes, lncRNAs can generally be classified into four categories, namely sense overlap, antisense overlap, bidirectional and intergenic noncoding RNAs (4). It has been suggested that lncRNAs serve a pivotal role in physiology and disease (5,6). Morán *et al* (7) uncovered hundreds of islet lncRNAs by strand-specific analysis, some of which were dysregulated in type 2 diabetes or mapped to genetic loci underlying diabetes susceptibility. In adipose tissue, numerous lncRNAs have been identified to regulate adipogenesis (8). In muscle cells, H19 lncRNA has been indicated to mediate the regulation of glucose metabolism (9). However, little is known about the role of lncRNAs in hepatic gluconeogenesis.

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Abbreviations: lncRNA, long non-coding RNA; cAMP, cyclic adenosine monophosphate; PGC-1 α , peroxisome proliferator-activated receptor- γ coactivator-1 α ; Gpr155, G protein-coupled receptor 155; 8-br-cAMP, 8-bromoadenosine 3',5'-cyclic monophosphate; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; PEPCK, phosphoenolpyruvate carboxykinase; CREB, cAMP-response element binding protein

Key words: long non-coding RNAs, gluconeogenesis, microarray, metformin, peroxisome proliferator-activated receptor- γ coactivator-1 α

In the fasting state, the increased secretion of the catabolic hormone glucagon stimulates gluconeogenesis by triggering the cyclic adenosine monophosphate (cAMP)/protein kinase A pathway and promoting the transcription of gluconeogenic genes (10). Metformin is currently the first drug of choice for the treatment of type 2 diabetes mellitus (11). It has been demonstrated that metformin reduces glucose output mainly via the inhibition of gluconeogenesis (12). However, the exact mechanism remains unclear (13-15). To identify whether lncRNAs are involved in the metformin-mediated inhibition of gluconeogenesis, a systematic analysis of the lncRNA expression profile in cAMP-stimulated primary mouse hepatocytes was performed in the present study. The cAMP-induced changes in lncRNA expression that were attenuated by metformin were identified. Among them, the expression levels of eight lncRNAs were validated by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The aim of the present study was to identify the potential role of lncRNAs in the regulation of gluconeogenesis.

Materials and methods

Materials. A total of 48 C57Bl/6 mice (age, 8-12 weeks old; weight, 18-20 g) were purchased from Shanghai Slack Experimental Center (Shanghai, China) and were housed in a specific pathogen free (SPF) environment (24-26°C; relative humidity 50-60%) with a 12-h light/dark cycle and free access to food and water. Dulbecco's modified Eagle's medium (DMEM) and Hank's balanced salt solution (HBSS) were obtained from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Hepatocyte medium was purchased from ScienCell Research Laboratories, Inc. (Carlsbad, CA, USA). Sodium pyruvate, sodium lactate, dexamethasone, bovine serum albumin (BSA), 8-bromoadenosine 3',5'-cyclic monophosphate (8-br-cAMP) and metformin were acquired from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). All the primers used in RT-qPCR were synthesized by Shanghai Sangon Biological Engineering Technology and Services Co., Ltd. (Shanghai, China).

Primary mouse hepatocyte isolation and culture. All experiments were supervised and approved by the laboratory ethics committee of Ruijin Hospital affiliated with Shanghai Jiaotong University School of Medicine (Shanghai, China). Hepatocytes were isolated from 8-12 week old male C57Bl/6 mice. Briefly, following anesthesia, mouse livers were perfused with 10 ml 1X HBSS without calcium, followed by perfusion with 0.05% collagenase IV in calcium-containing HBSS in a recirculating manner. The liver was then detached and filtered through a 70 μ m nylon mesh and hepatocytes were sedimented by centrifugation at 200 \times g for 5 min at 4°C. The hepatocytes were plated onto 6-well plates and grown in hepatocyte medium supplemented with BSA, penicillin/streptomycin and hepatocyte growth factor. The cultures were maintained at 37°C in humidified atmosphere (95% air and 5% CO₂). After 24 h, the cells had become attached to the plates and the medium was replaced with low glucose DMEM containing 0.25% BSA with dexamethasone (100 nM) for prestimulation for 12-16 h. The cells were then incubated with 100 μ M 8-br-cAMP (a permeable analogue of cAMP) in

the presence or absence of 2 mM metformin in glucose-free DMEM containing gluconeogenic substrates (10 mM sodium lactate and 1 mM sodium pyruvate) for 8 h.

RNA extraction and quality control. Total RNA was extracted from primary mice hepatocytes using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The RNA purity and concentration was evaluated with a NanoDrop-2000 spectrophotometer (Thermo Fisher Scientific, Inc.). The optical density (OD) absorbance ratio at 260 and 280 nm (A_{260}/A_{280} ratio) was \sim 2.0 and the OD A_{260}/A_{230} ratio was \sim 1.8. RNA integrity was determined by standard denaturing agarose gel electrophoresis, which revealed that the samples were good quality.

Microarray analysis. ArrayStar mouse lncRNA microarray version 2.0 (ArrayStar Inc., Rockville, MD, USA) is designed for the global profiling of mouse lncRNAs in hepatocytes isolated from 3 mice (i.e., 3 groups at 3 replicates). The sample preparations and microarray hybridization were performed based on the manufacturer's protocols. Briefly, 1 μ g total RNA from each sample was amplified and transcribed into fluorescent cRNA using Agilent's Quick Amp Labeling kits (Agilent Technologies, Inc., Santa Clara, CA, USA) according to the manufacturer's protocol. The labeled cRNAs were hybridized onto the mouse 8x60K lncRNA array. After washing, the arrays were scanned using the Agilent G2505C Microarray Scanner System, and Agilent Feature Extraction software was used to analyze the acquired array images (Agilent Technologies, Inc.). Quantile normalization and subsequent data processing were performed using the GeneSpring GX v12.0 software package (Agilent Technologies, Inc.). The threshold set for significantly differential genes was an absolute log value of fold-change \geq 2 and a P-value \leq 0.05. The microarray analysis was performed by KangChen Bio-tech Inc. Corporations (Shanghai, China).

RT-qPCR. Following total RNA extraction, as described above, cDNA was synthesized by reverse transcription using Superscript II RT reagent kit (Promega Corporation, Madison, WI, USA) according to the manufacturer's protocol. RT-qPCR was performed with the Roche LightCycle[®] 480 instrument (Roche Diagnostics, Basel, Switzerland) using SYBR Premix Ex Taq (Takara Bio, Inc., Otsu, Japan) in a final volume of 10 μ l. The PCR conditions were as follows: Denaturation at 95°C for 10 sec, 40 cycles at 95°C for 5 sec and 60°C for 31 sec. A melting curve was constructed in the temperature range of 60 to 95°C at the end of the amplification. The sequences of the primers used are shown in Table I. Relative gene expression levels were quantified based on the cycle threshold (Cq) values and normalized to the internal control gene β -actin, which received a value of 1. The relative gene expression was calculated using the $2^{-\Delta\Delta Cq}$ method (16).

Statistical analysis. All values are presented as mean \pm standard deviation from at least three independent experiments, and $P \leq 0.05$ was considered to indicate a statistically significant difference. Differential expression levels of genes were compared using ANOVA for multiple groups or Student's t-test for two groups. Statistical analysis was performed by SPSS 18.0 software (IBM, Armonk, NY, USA).

Table I. Sequence of the primers used for reverse transcription-quantitative polymerase chain reaction.

Gene	Forward sequence (5' to 3')	Reverse sequence (5' to 3')
PEPCK	GTGCTGGAGTGGATGTTTCGG	CTGGCTGATTCTCTGTTTCAGG
AK133602	GACCACTCTGACCCCTATCC	TAACCAGCCATTACCCCTC
ENSMUST00000138573	CCTCAGCACAGAAACCACC	AGCCTGTCCAATATGCGT
ENSMUST00000129953	TTCAGCCTACAATCCCAAG	GTGGAATACACCCCGAAGT
uc009njr.1	TTTGTCGCTCATCCACGCT	GCAAATGGTCCCGCAAATA
AA591058	GACCACCAGGCAGCACTTC	ACCACACACACCCACACACAC
NR_027710	TGCCATATCTTCCAGTGACC	TAAGGTTTCGCTCAATAGTCTTG
NR_030715	TGGGGTGGAGAAGACAACA	AGCACAACCACAAGCATCTAC
ENSMUST00000145208	TCTCAACTCTATGCCGCCTT	GCACACTTCCGCTTCACCT
Gpr155	GTTTTTCTGTGCCGTGTTTAACT	GGGGGATTCTCTGTCTGCT
PGC-1 α	ATACCGCAAAGAGCAGGAGAAG	CTCAAGAGCAGCGAAAGCGTCACAG
β -actin	GGCTGTATTCCCCTCCATCG	CCAGTTGGTAACAATGCCATGT

PEPCK, phosphoenolpyruvate carboxykinase; Gpr155, G protein-coupled receptor 155; PGC-1 α , peroxisome proliferator-activated receptor- γ coactivator-1 α .

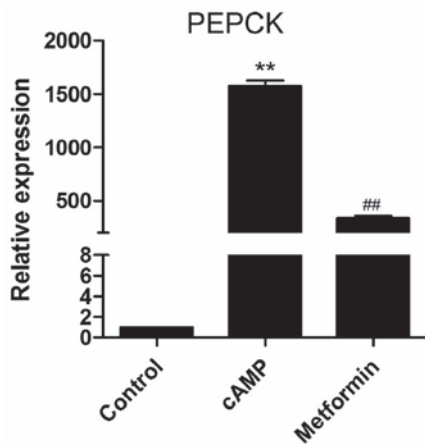


Figure 1. Metformin significantly inhibits cAMP-stimulated PEPCK expression. Primary mouse hepatocytes were incubated with 100 μ M 8-br-cAMP in the presence or absence of 2 mM metformin for 8 h. PEPCK mRNA expression was detected by reverse transcription-quantitative polymerase chain reaction. Results are representative of three independent experiments. **P<0.01 vs. control group; ##P<0.01 vs. cAMP group. cAMP, cyclic adenosine monophosphate; PEPCK, phosphoenolpyruvate carboxykinase; 8-br-cAMP, 8-bromoadenosine 3',5'-cyclic monophosphate.

Results

Phosphoenolpyruvate carboxykinase (PEPCK) expression levels under different conditions. Gluconeogenesis is tightly controlled through the transcriptional regulation of PEPCK, the rate-limiting enzyme of hepatic gluconeogenesis (17). Metformin has been reported to suppress PEPCK expression in primary hepatocytes (14). Prior to microarray analysis, the effect of metformin on PEPCK mRNA expression in the primary mouse hepatocytes was investigated. Following the incubation of primary mouse hepatocytes with 100 μ M 8-br-cAMP for 8 h, PEPCK mRNA expression was significantly increased, and this increase was significantly suppressed by 2 mM metformin (Fig. 1). This indicates that the experimental

conditions are suitable for studying the mechanism of gluconeogenesis. Therefore, the microarray analysis of the lncRNAs expression profile was performed using the same conditions.

Profile of the microarray data. A total of 22,016 lncRNAs were detected in the primary mouse hepatocytes using ArrayStar Mouse LncRNA Microarray version 2.0. The lncRNAs represented in this microarray have been sourced from authoritative databases, including RefSeq, UCSC Knowngenes and Ensembls. Scatter plots were used to assess the variation in the expression of lncRNAs between the cAMP group and the control group (Fig. 2A) or between the metformin group and the cAMP group (Fig. 2B). By setting a threshold for differential expression at fold-change >2.0-fold, 865 differentially expressed lncRNAs (456 upregulated and 409 downregulated lncRNAs) were identified in the cAMP group vs. the control group. Compared with the cAMP group, 4,580 differentially expressed lncRNAs were identified, of which 2,114 lncRNAs were upregulated and 2,466 lncRNAs were downregulated in the metformin group. Among the cAMP-upregulated lncRNAs, 189 were downregulated by metformin. Among the cAMP-downregulated lncRNAs, 167 were upregulated by metformin. Heatmaps demonstrate the lncRNA expression profiles of the groups, and the expression values of the 30 most strongly cAMP-upregulated and cAMP-downregulated lncRNAs (Fig. 2C and D and Table II) reversed by metformin are presented.

Expression signatures of metformin-reversed lncRNAs. It is likely that these metformin-reversed lncRNAs serve a critical role in the regulation of gluconeogenesis. Therefore, some general signatures of these lncRNAs, such as their sources, classification, length distribution and chromosome distribution, were investigated. A pie chart was constructed to show the number of metformin-reversed lncRNAs collected from different databases (Fig. 3A). There were 78 antisense overlap, 25 bidirectional, 214 intergenic and 39 sense overlap lncRNAs among these lncRNAs (Fig. 3B), which were mainly between 400 and 3,600 nt in length (Fig. 3C). Chromosome

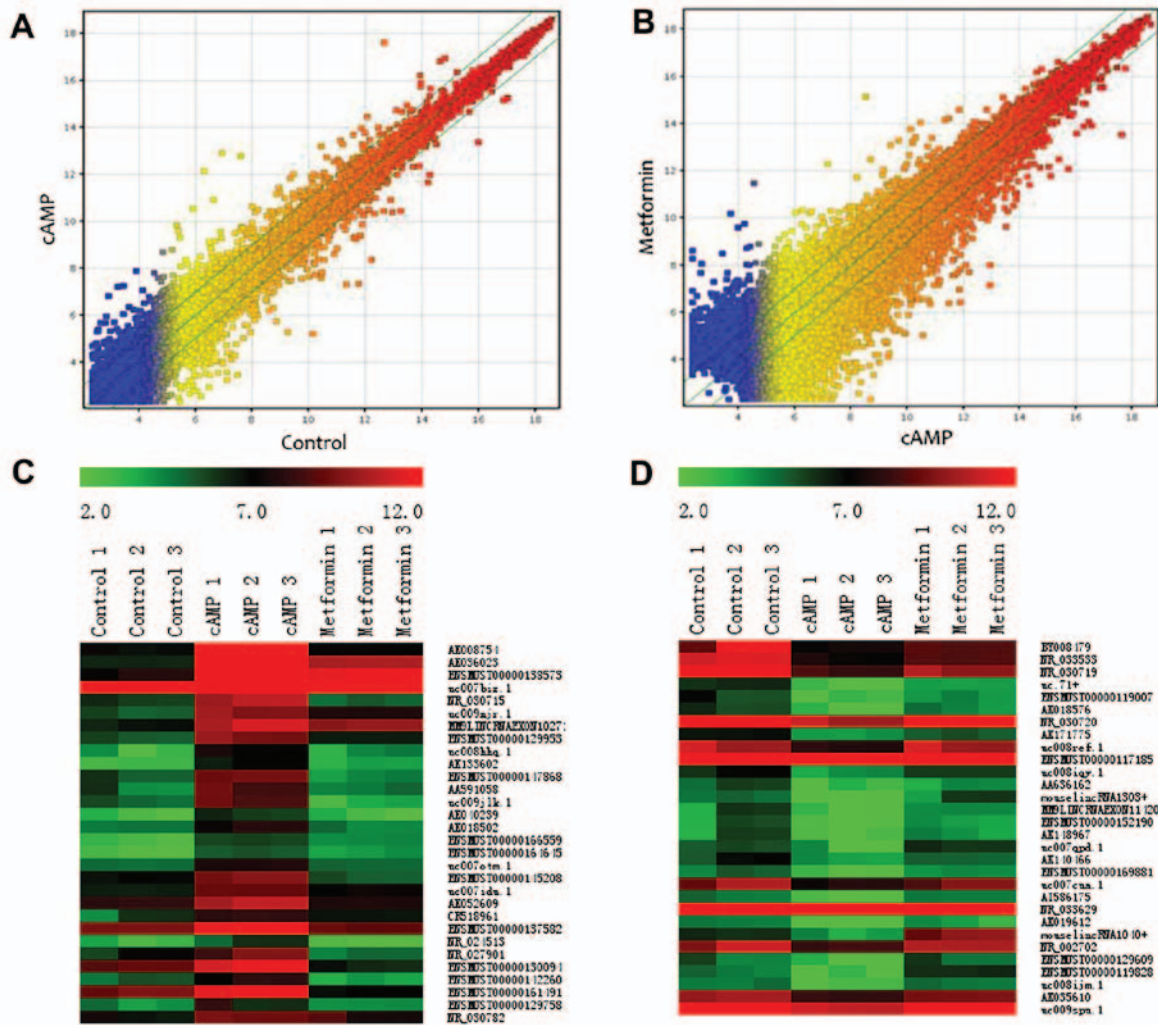


Figure 2. Comparison of lncRNA expression profiles in primary mouse hepatocytes of the three groups. The differential expression profiles of lncRNAs were analyzed by microarray. Scatter plots are presented to show the lncRNA expression variation between (A) the cAMP group and the control group and (B) the metformin group and the cAMP group. The values on the x- and y-axes in the scatter-plot are the normalized signal values of each group (\log_2 scaled). The lncRNAs above the top green line and below the bottom green line in the scatter plots indicate a >2-fold-change of lncRNA expression. The heatmaps show (C) the top 30 cAMP-upregulated lncRNAs and (D) the top 30 cAMP-downregulated lncRNAs reversed by metformin. Red indicates a relatively high expression and green indicates a relatively low expression. lncRNA, long non-coding RNA; cAMP, cyclic adenosine monophosphate.

distribution analysis demonstrated that these metformin-reversed lncRNAs were located on a number of different chromosomes (Fig. 3D and E).

RT-qPCR validation. Among the identified metformin-reversed lncRNAs, 8 cAMP-stimulated and metformin-inhibited lncRNAs (AK133602, ENSMUST00000138573, ENSMUST00000129953, uc009njr.1, AA591058, NR_027710, NR_030715 and ENSMUST00000145208) were selected for analysis by RT-qPCR in order to verify the microarray data. The expression levels of all 8 cAMP-stimulated lncRNAs were significantly decreased in the presence of metformin as shown by RT-qPCR (Fig. 4), consistent with the results of the microarray analysis.

Associated coding gene expression. Ponjavic *et al* (18) emphasized the importance of lncRNA and its adjacent protein-coding gene pairs when investigating the function of lncRNAs. Therefore, using the UCSC genome browser (<http://genome.ucsc.edu/>) and NONCODE database ([\[noncode.org\]\(http://www.noncode.org\)\), the sequences of the eight validated lncRNAs and their associated coding genes were obtained \(data not shown\). The lncRNA ENSMUST00000138573 is a 614-nt antisense overlapping lncRNA associated with the G protein-coupled receptor 155 \(Gpr155\) gene. lncRNA NR_027710 is a sense overlapping lncRNA, which is located near the PGC-1 \$\alpha\$ gene. Notably, RT-qPCR analysis demonstrated that Gpr155 and PGC-1 \$\alpha\$ displayed a similar expression pattern to their associated lncRNAs under the same treatment conditions \(Fig. 5A and B\). Therefore, it is possible that the two lncRNAs modulate gluconeogenesis through their associated protein-coding genes.](http://www.</p>
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Discussion

Excessive glucose output via gluconeogenesis is a critical pathological factor contributing to hyperglycemia in type 2 diabetes mellitus, during which the liver synthesizes glucose from non-carbohydrate precursors, including pyruvate and lactate (19,20). Under the fasting state, increased pancreatic

Table II. Top 30 cAMP-upregulated lncRNAs and cAMP-downregulated lncRNAs reversed by metformin.

Seqname	Fold-change	Regulation (cAMP vs. control)	Fold-change	Regulation (metformin vs. cAMP)
AK008754	63.58867	Up	55.04594	Down
AK036023	57.2507	Up	3.325115	Down
ENSMUST00000138573	37.43524	Up	2.130461	Down
uc007biz.1	31.35165	Up	17.08355	Down
NR_030715	25.00611	Up	45.54781	Down
uc009njr.1	22.53223	Up	4.457007	Down
MM9LINCRNAEXON10271 ⁻	19.73751	Up	2.071386	Down
ENSMUST00000129953	18.7799	Up	8.698158	Down
uc008hhq.1	15.70811	Up	8.776502	Down
AK133602	15.02128	Up	8.871666	Down
ENSMUST00000147868	14.96649	Up	32.93101	Down
AA591058	14.75416	Up	16.43587	Down
uc009jlk.1	12.59451	Up	43.2916	Down
AK040239	8.804657	Up	8.449728	Down
AK018502	7.718255	Up	8.491624	Down
ENSMUST00000166559	7.339848	Up	2.552899	Down
ENSMUST00000164645	6.906824	Up	2.483507	Down
uc007otm.1	6.888684	Up	6.263191	Down
ENSMUST00000145208	6.529995	Up	19.36338	Down
uc007idu.1	6.296531	Up	3.856799	Down
AK052609	6.237578	Up	7.895747	Down
CR518961	6.112049	Up	2.601747	Down
ENSMUST00000137582	6.028889	Up	8.330698	Down
NR_024513	6.003099	Up	10.10774	Down
NR_027901	5.708479	Up	20.74246	Down
ENSMUST00000130094	5.705397	Up	33.7096	Down
ENSMUST00000142260	5.699138	Up	15.27239	Down
ENSMUST00000161491	5.59761	Up	29.0643	Down
ENSMUST00000129758	5.463225	Up	5.843725	Down
NR_030782	5.312138	Up	2.623824	Down
BY008479	21.01573	Down	2.650546	Up
NR_033533	15.91963	Down	2.650648	Up
NR_030719	14.39216	Down	3.259029	Up
uc.71+	12.23815	Down	2.515803	Up
ENSMUST00000119007	8.960969	Down	2.669164	Up
AK018576	8.671295	Down	3.36866	Up
NR_030720	6.8741	Down	4.131914	Up
AK171775	6.527647	Down	2.402267	Up
uc008ref.1	6.215065	Down	5.873268	Up
ENSMUST00000117185	6.061	Down	3.369349	Up
uc008iqy.1	6.043397	Down	3.388012	Up
AA636162	5.986786	Down	2.320948	Up
mouselincRNA1303 ⁺	5.755683	Down	7.49714	Up
MM9LINCRNAEXON11420 ⁺	5.640478	Down	3.268149	Up
ENSMUST00000152190	5.534303	Down	2.962099	Up
AK148967	5.531486	Down	3.036806	Up
uc007qpd.1	5.508539	Down	8.222444	Up
AK140466	5.259056	Down	2.131494	Up
ENSMUST00000169881	5.179452	Down	3.134108	Up
uc007cua.1	5.058929	Down	3.124969	Up

Table II. Continued.

Seqname	Fold-change	Regulation (cAMP vs. control)	Fold-change	Regulation (metformin vs. cAMP)
AI586175	4.876382	Down	4.435117	Up
NR_033629	4.820504	Down	2.005511	Up
AK019612	4.674555	Down	2.644345	Up
mouselincRNA1040+	4.510026	Down	49.62078	Up
NR_002702	4.507347	Down	4.266528	Up
ENSMUST00000129609	4.367185	Down	5.31341	Up
ENSMUST00000119828	4.262482	Down	11.6708	Up
uc008ijm.1	4.213697	Down	4.486794	Up
AK035610	4.009485	Down	2.407165	Up
uc009spu.1	3.959393	Down	2.946273	Up

The table lists only some of the results for lncRNAs with an up- or downregulation in expression in the cAMP group compared with the control group and in the metformin group compared with the cAMP group. Seqname, lncRNA name; fold-change, absolute fold-change between the compared groups; up, upregulation; down, downregulation; cAMP, cyclic adenosine monophosphate; lncRNA, long non-coding RNA.

hormone glucagon interacts with the glucagon receptor and activates adenylate cyclase, thus leading to an elevation of intracellular cAMP (10). Elevated cAMP then triggers gluconeogenesis via activation of the transcriptional factor cAMP-response element binding protein (CREB) (10, 21). It is well known that the activity of the gluconeogenic pathway is controlled by the gene expression of several key enzymes, including PEPCK. A large body of evidence from animal studies and diabetic patients has demonstrated that metformin lowers blood glucose levels by inhibiting gluconeogenesis (12-14). The present research team have also observed that metformin decreases glucose production in primary mouse hepatocytes (data not shown). In the present study, cAMP-induced PEPCK mRNA expression was demonstrated to be suppressed by metformin, consistent with previous studies (13,14).

lncRNAs were initially considered to be transcriptional 'noise' of the mammalian genome; however, there is considerable evidence that lncRNAs serve key roles in various biological processes via the regulation of gene expression at the level of chromatin remodeling, transcriptional control and post-transcriptional processing (22). lncRNAs have also been implicated in numerous human diseases, including Alzheimer's disease (23), cardiovascular diseases (24), diabetes (25) and various cancers (26). There are many studies demonstrating that lncRNAs are involved in the differentiation and homeostasis of metabolic tissues, including islets, skeletal muscle and adipose tissues, as summarized in a recent review (27). Particularly in islets, there is growing evidence that lncRNAs are involved in the determination of β -cell identity and participate in the misregulation of gene expression during type 2 diabetes (7,28). However, to the best of our knowledge, the effect of lncRNA on hepatic gluconeogenesis has not been elucidated. In the present study, a hepatocyte model with cAMP and metformin treatment was used to better understand the potential role of lncRNAs in the hepatic gluconeogenesis process.

Using microarray analysis, 865 and 4,580 differentially expressed lncRNAs were identified in the cAMP group vs.

the control group and the metformin group vs. the cAMP group, respectively. Among the cAMP-regulated lncRNAs, 356 were reversed by metformin treatment, and the expression of 189 AMP-upregulated lncRNAs was decreased by metformin. Previous studies have demonstrated that metformin suppresses the cAMP-stimulated expression of genes involved in gluconeogenesis (14,29). By analyzing the expression signatures of the metformin-reversed lncRNAs, it was found that the majority were long intergenic noncoding RNAs. Therefore, it is possible that these lncRNAs regulate the expression of neighbouring protein-coding genes. A set of 8 cAMP-upregulated and metformin-downregulated lncRNAs was selected with which to validate the results of the microarray using RT-qPCR. The results suggest that metformin may decrease gluconeogenesis by globally altering hepatic lncRNA expression.

Although the precise roles of these lncRNAs in gluconeogenesis remain unknown, lncRNAs have been demonstrated to regulate neighbouring gene expression through cis- and trans-mechanisms (5). Notably, a strong co-expression of lncRNA NR_027710 and PGC-1 α was validated by the RT-qPCR analysis in the present study, as shown in Figs. 4F and 5A. This implies a potential role of NR_027710 in the regulation of PGC-1 α expression. Hormonal signaling regulates hepatic gluconeogenesis by triggering a cascade of transcriptional events involving various transcriptional activators and coactivators, including PGC-1 α , CREB and transducer of regulated CREB activity 2 (30-32). lncRNA NR_027710 has been found to be transcribed from the natural-sense strand of the gene PGC-1 α , which encodes the transcriptional coactivator PGC-1 α . Herzig *et al* (30) demonstrated that PGC-1 α is induced by CREB to trigger the expression of gluconeogenic genes. The results of the present study and previous studies (14,17) demonstrated that 8-br-cAMP caused a significant increase in the PGC-1 α transcript, which was suppressed by metformin. According to the bioinformatic analysis, ENSMUST00000138573, a 614-nt

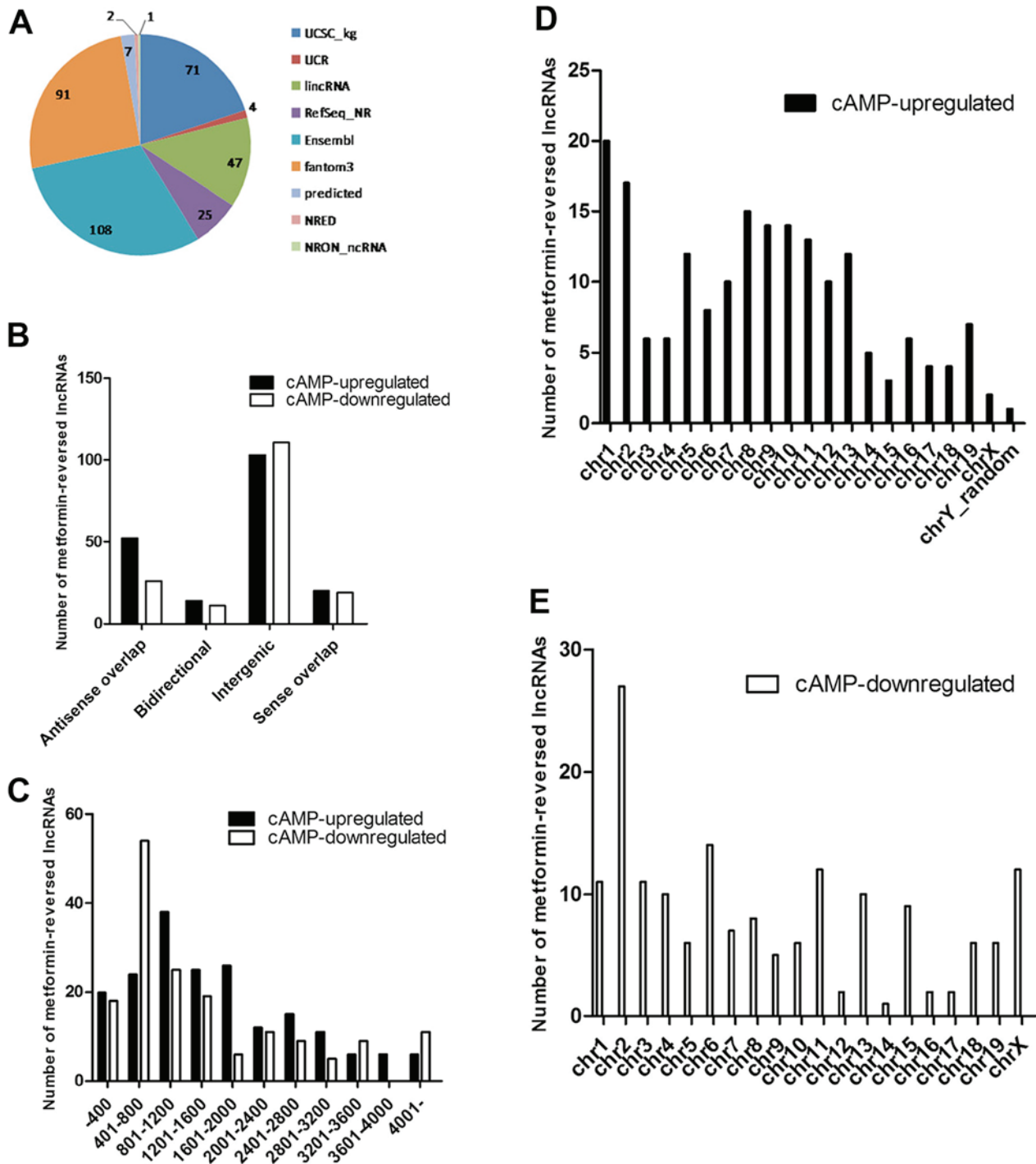


Figure 3. Expression signatures of metformin-reversed lncRNAs regulated by cAMP. (A) The numbers of metformin-reversed lncRNAs from various authoritative databases. (B) Classification of reversed lncRNAs demonstrated that these lncRNAs were mainly intergenic. (C) The majority of the metformin-reversed lncRNAs were between 400 and 1,200 nucleotides in length. Analysis of chromosome distribution showed the numbers of (D) cAMP-upregulated and metformin-downregulated lncRNAs and (E) cAMP-downregulated and metformin-upregulated lncRNAs located in different chromosomes. lncRNA, long non-coding RNA; cAMP, cyclic adenosine monophosphate.

lncRNA exhibits a natural antisense association with the coding gene *Gpr155* in chromosome 2. In the present study, the expression level of *Gpr155* displayed a similar pattern of change to that of lncRNA ENSMUST00000138573. Kobayashi *et al* (33) revealed that *Gpr155* was one of four candidate genes for type 2 diabetes by performing exome sequencing analysis. It has been shown that lncRNAs act as transcriptional cofactors to modulate the transcription

of adjacent protein coding genes (34), which suggests that lncRNA NR_027710 and lncRNA ENSMUST00000138573 may affect the expression of *PGC-1 α* and *Gpr155* through an unknown mechanism. These two lncRNA-mRNA gene pairs highlight the importance of lncRNAs in the modulation of glucose homeostasis.

In conclusion, to the best of our knowledge, the present study is the first to reveal the role of lncRNA in gluconeogen-

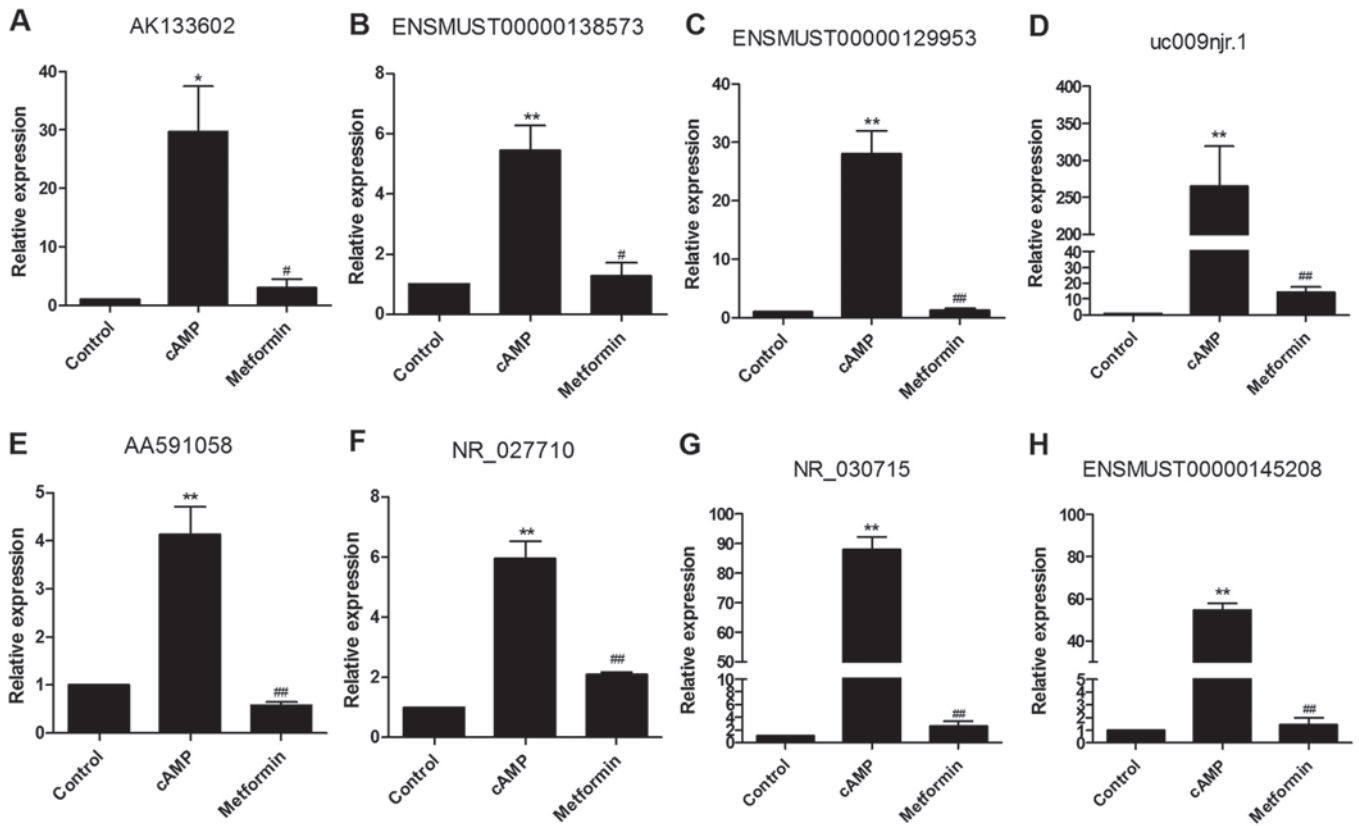


Figure 4. Validation results of eight cAMP-upregulated and metformin-downregulated lncRNAs by RT-qPCR are consistent with those of the microarray. Primary mouse hepatocytes were incubated with 100 μ M 8-br-cAMP in the presence or absence of 2 mM metformin for 8 h. RT-qPCR demonstrated that cAMP-stimulated (A) AK133602, (B) ENSMUST00000138573, (C) ENSMUST00000129953, (D) uc009njr.1, (E) AA591058, (F) NR_027710, (G) NR_030715 and (H) ENSMUST00000145208 were attenuated by metformin. All results were obtained from three independent experiments. * $P < 0.05$ and ** $P < 0.01$ vs. control group; # $P < 0.05$ and ## $P < 0.01$ vs. cAMP group. cAMP, cyclic adenosine monophosphate; lncRNA, long non-coding RNA; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

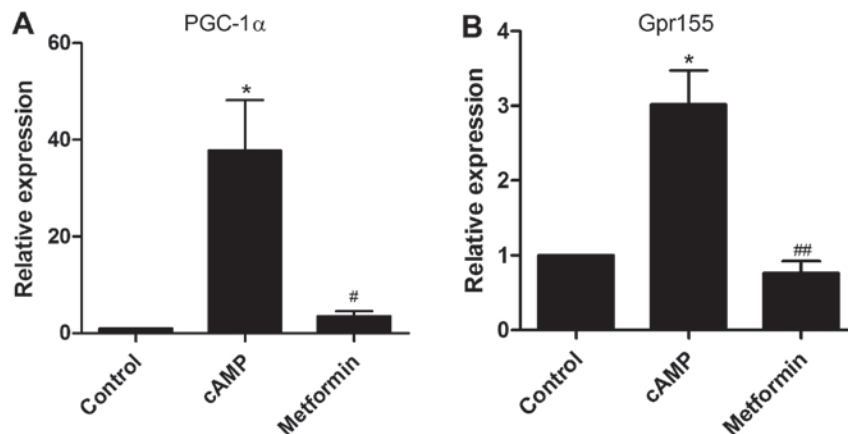


Figure 5. Expression levels of PGC-1 α and Gpr155 are increased by cAMP and are further decreased by metformin treatment. Primary mouse hepatocytes were incubated with 100 μ M 8-br-cAMP in the presence or absence of 2 mM metformin for 8 h. (A) PGC-1 α and (B) Gpr155 mRNA expression was detected by reverse transcription-quantitative polymerase chain reaction. The experiment was repeated three times with similar results. * $P < 0.05$ vs. control group; # $P < 0.05$ and ## $P < 0.01$ vs. cAMP group. PGC-1 α , peroxisome proliferator-activated receptor- γ coactivator-1 α ; Gpr155, G protein-coupled receptor 155; cAMP, cyclic adenosine monophosphate.

esis by conducting a global microarray analysis. It is likely that metformin inhibits hepatic gluconeogenesis by regulating lncRNA expression. In addition, two lncRNAs NR_027710 and ENSMUST00000138573 are associated with PGC-1 α and Gpr155, respectively. The two pairs of lncRNAs and coding

protein genes exhibit a similar expression pattern, suggesting that lncRNAs may exert their functions through interactions with coding transcripts and proteins in gluconeogenesis. The present study provides a new perspective for understanding the glucose-lowering mechanism of metformin. However, the

exact regulatory mechanisms of the specific lncRNAs involved in gluconeogenesis require further investigation, potentially via loss and gain of function studies.

Acknowledgements

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