

Profiling the lncRNA-miRNA-mRNA Interaction Network in the Submandibular Gland of Diabetic Mice

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Abstract

Background: Hyposalivation is one of the common symptoms of diabetes. Although long non-coding RNAs (lncRNAs) have recently been reported to play important roles in the pathogenesis of diabetes, the role of lncRNAs in diabetes-induced hyposalivation remains unknown.

Methods: The present study aimed to explore the function of lncRNA-microRNA-mRNA regulatory network in the submandibular gland (SMGs) under the context of diabetes. lncRNA expression profile of the SMGs was analyzed using microarray technology. Differentially expressed lncRNAs were confirmed using real-time quantitative PCR. Bioinformatics analyses were performed, and Coding-non-coding gene co-expression (CNC) and competing endogenous RNA (ceRNA) networks were constructed to explore the potential mechanisms of diabetes-induced hyposalivation.

Results: A total of 1,273 differentially expressed lncRNAs (536 up-regulated and 737 downregulated) were identified in the SMGs tissues of db/db mice. CNC and ceRNA network analyses were performed based on five differentially expressed lncRNAs validated by real-time quantitative PCR. Gene Ontology analysis of target genes of CNC network revealed that “calcium ion binding” was a highly enriched molecular function. Kyoto Encyclopedia of Genes and Genomes pathway analysis of target genes of ceRNA network revealed that the “mammalian target of rapamycin signaling pathway” was significantly enriched.

Conclusions: On the whole, the findings of the present study may provide insight into the possible mechanism of diabetes-induced hyposalivation.

Background

Diabetes is a group of metabolic diseases characterized by abnormal insulin secretion and/or insulin resistance. In addition to causing long-term damage to various organs, diabetes can also change the function of salivary glands and may cause changes in the composition and volume of salivary secretion, thus affecting oral homeostasis [1]. Some studies have found that the salivary flow of diabetic patients is significantly decreased compared with that of non-diabetic individuals [2, 3]. Clinical research has revealed that 92.5% of elderly patients with type 2 diabetes suffers from decreased salivary flow rate [4]. Saliva plays a key role in digestion, taste, cleaning, the hydration of the oral mucosa and tooth protection, and is essential for maintaining the dynamic balance of the oral environment [5]. Damage to the salivary glands is typically manifested by a reduction in salivary flow, which can be converted into negative associated symptoms, such dry mouth, taste disorders, difficulty in swallowing and chewing, and an increased risk of caries, and dry mouth also aggravates other accompanying symptoms such as periodontal disease and indigestion. These factors ultimately have a negative impact on quality of life of affected individuals [6, 7]. A number of studies have demonstrated that the incidence rate of oral fungal and bacterial infection, lichen planus and caries are high in patients with diabetes [2, 3, 8]. In our previous study, atrophic acini, and the decreased stimulated salivary flow of submandibular glands (SMGs) were observed in db/db mice [9]. However, the mechanism by which diabetes induces SMGs damage is not clear.

Long non-coding RNAs (lncRNAs) are a class of RNA molecules that are longer than 200 nucleotides in length. In recent decades, lncRNAs have been identified as important epigenetic factors that regulate various human diseases, such as cancer, neurodegenerative diseases and cardiovascular diseases [10–14]. Recent studies demonstrated that lncRNAs are associated with the incidence and development of type 2 diabetes. The downregulation of lncRNA ANRIL has been shown to improve the cardiac function index and inflammatory factor expression in diabetic rats, as well as to enhance the pathological state of myocardial tissue and myocardial remodeling, and reduced the area of myocardial collagen deposition [15]. lncRNA NONRATT021972 has been found to be upregulated in diabetic rat nervous system cells, suggesting that it may participate in the pathophysiological processes associated with sympathetic neurons in diabetes [16]. The downregulation of lncRNA SOX2OT overlapping transcript has been shown to regulate the NF-E2-related factor 2/heme oxygenase 1 signaling pathway and exert protective effect against high glucose-induced damage to retinal ganglion cells in vivo and in vitro [17]. Based on these findings, it was hypothesized that lncRNAs may play a role in the represented a potential cause of diabetes-induced reductions in salivary secretion. Competing endogenous RNA (ceRNA) can regulate each other at post-transcription level by competing for common microRNAs (miRNAs) [18]. lncRNA maternally expressed 3 has been shown to function as a ceRNA of miR-214 to promote hepatic insulin resistance by facilitating the expression of activating transcription factor 4 [19]. Protein-coding mRNA DKK1 and PTEN serve as ceRNA, exhibiting crosstalk and affecting the expression of each other via competition for miRNA binding [20]. In the exploration of lncRNAs, the identification of a target gene and its association with diabetes-related xerostomia may aid in the understanding of pathology of diabetes-related xerostomia and may provide further information for the treatment of this disorder.

To further understand the function of lncRNA in the impairment of salivary function in diabetes, the present study characterized the expression profiles of lncRNAs in SMGs tissues of db/db and db/m mice using microarray. The establishment of Coding-non-coding gene co-expression (CNC) and ceRNA networks based on lncRNAs may help to explore the potential functions of lncRNAs in diabetes-induced SMGs dysfunction.

Methods

Animal models

Male db/db and db/m mice (16 weeks old) were purchased from Changzhou Cavens Laboratory Animal (Changzhou, China). The db/db mice were used as the experimental group (n = 9/group). db/db mice are leptin receptor knockout mice, and are commonly used in models of spontaneous type 2 diabetes mellitus [21, 22]. The db/m mice were used as the control group (n = 9/group). All experimental procedures were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals (NIH Publication no. 85 – 23; revised in 1996). All mice were maintained in a controlled environment at room temperature with a relative humidity of ~ 60% and a 12-h light/dark cycle, and fed normal food and water. After being subjected to euthanasia with CO₂ (20% of the cage volume displaced by CO₂ per min), the SMGs were removed immediately from the animals, frozen in liquid nitrogen for 1 min and then stored at -80°C.

RNA extraction and microarray analysis

A total of four mice were randomly selected from each group. Total RNA was extracted from the SMGs tissues using TRIzol® LS reagent (Invitrogen; Thermo Fisher Scientific, Inc.), and was used for lncRNA microarray analysis (KangChen Biotech). The concentration and purity of the RNA were measured using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Inc.), and the quantity and integrity of the RNA was examined using denaturing agarose gel electrophoresis. Microarray hybridization was performed using the Quick Amp Labeling kit, One-Color (Agilent Technologies, Inc.) based on the manufacturer's standard protocols. Agilent Feature Extraction v11.0.1.1 software (Agilent Technologies, Inc.) was used to analyze the acquired array images. Quantile normalization and subsequent data processing were performed using the GeneSpring GX v12.1 software package (Agilent Technologies, Inc.). All microarray hybridization and analyses were performed by KangChen Biotech. The accession number of microarray data was GSE141411, which is currently private and is scheduled to be released on December 03, 2022.

Real-time quantitative PCR (RT-qPCR)

The 11 most significantly differentially expressed lncRNAs were selected for validation by RT-qPCR. Briefly, total RNA was extracted from the two groups (n = 6–9/group) of SMGs tissues using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Total RNA (1 µg) was reverse transcribed into cDNA using 5X All-In-One RT MasterMix (Applied Biological Materials Inc.) according to the manufacturer's instructions. qPCR was performed on a ViiA™ 7 Real-Time PCR system (Thermo Fisher Scientific, Inc.) using a 2X SYBR-Green qPCR Master mix (Bimake). The RT-qPCR primers were synthesized by Sangon Biotech. The $2^{-\Delta\Delta Cq}$ method was used for analysis. The corresponding primers used are listed in Table 1.

Table 1
The Primers of differentially expressed lncRNAs

| lncRNA | Forward primer | Reverse primer |
|--------------------|---------------------------|--------------------------|
| ENSMUST00000137025 | TGCACAACATATGTGAGGAGCATGG | GGAGGCTCAGGCCATTCTTCATTC |
| ENSMUST00000163495 | GCAATGAGGAGAGTGGAGCAACC | GTCTGCCAAGAGTCCGAAGTAACC |
| ENSMUST00000139794 | GACTGGCAGGAAGAACGGATGAC | GGCACACCTGGTTCCATCTTAGAC |
| NR_040589 | TCCCCTTCTCTCGTTCTCTC | CTGTTGCTCGCCTTCCTGCTG |
| AK021108 | GGTGGCTGTGAAGACGCTGAAG | TTGACAATGTGCTCGTGCTGGAG |
| ENSMUST00000120706 | AGGTGGCTGGTTTCTCTGGA | AGCAGCTACTCTTGCCTGGA |
| NR_045306 | GCCTGCCTGCCTCCTCTCTC | TTCTGAGCCTGGGTGTCTCTGG |
| ENSMUST00000142612 | TTGAGCATGTGCCAGACAGAAGTG | TCTCCATCGTGTGCTTCAAGTG |
| ENSMUST00000069768 | ACGGACACCTCTCCTGCCTTG | CTTCTGAAGCTCGCGGAGAATC |
| ENSMUST00000156336 | ATGAGCGACTGAGCAGGACTACC | GGCTGGCGTCTCACCTCTAG |
| ENSMUST00000141103 | AACTGAGCAGAGACAGCGTGTG | TCATGGTCTTGTGCTGAGATGACG |

Bioinformatics analyses

Volcano plot filtering was used to identify differentially expressed lncRNAs based on the threshold defined as fold change > 2.0 (unpaired t-test $P < 0.05$). The differentially expressed lncRNAs between db/db and db/m mice were displayed by hierarchical clustering. Gene Ontology (GO) analysis (<http://www.geneontology.org/>) and Kyoto Encyclopedia of Gene and Genomes (KEGG) analysis (<http://www.genome.jp/kegg/>) were used to explore the roles of the target genes of differentially expressed lncRNAs.

CNC and ceRNA networks

The normalized signal intensities of differentially expressed mRNAs from whole-genome expression profiling (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE141411>) and differentially expressed lncRNA validated by RT-qPCR were used to construct the CNC networks. The lncRNA-mRNA pairs were identified based on a Pearson's correlation coefficient of > 0.95 . The CNC network was constructed using Cytoscape software (The Cytoscape Consortium). Subsequently, the lncRNA-miRNA-mRNA ceRNA networks were constructed based on the microarray data. The lncRNA-miRNA interactions and miRNA-mRNA pairs were predicted using Arraystar's homemade miRNA target prediction software, and TargetScan and miRanda [23–26].

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.0 software (GraphPad Software, Inc.). The data of relative expression levels of lncRNAs are expressed as the means \pm standard error. The unpaired t-test was used to determine whether the two sets of data were statistically significant, and $P < 0.05$ was considered to indicate a statistically significant difference in gene expression difference between the two groups.

Results

Expression profiling of lncRNAs in the SMGs in db/db mice

The stimulated salivary flow rate of the SMGs in db/db mice was significantly decreased by 67.2% following pilocarpine stimulation for 10 min, and the SMGs of the db/db mice exhibited marked acinar enlargement and ductal atrophy [27]. To study the function of lncRNAs in the dysfunction of the SMGs, type 2 diabetes db/db model mice were used as the experimental group, and db/m mice were used as the control group to examine lncRNA expression profiles of the SMGs tissues during type 2 diabetes using microarray analysis. The body weight of the db/db mice was 33.68 ± 0.71 g, while the body weight of the db/m mice was 57.23 ± 0.34 g.

In comparison with the db/m mice, 1,273 differentially expressed lncRNAs were identified in the SMGs tissues from db/db mice, including 536 upregulated and 737 downregulated lncRNAs (fold change > 2.0 , $P < 0.05$). The top 10 upregulated and downregulated lncRNAs are presented in Table 2. A volcano plot of the lncRNA expression profiles is illustrated in Fig. 1A. The heatmaps of the 60 most significantly differentially expressed lncRNAs (30 upregulated and 30 downregulated) is displayed in Fig. 1B.

Table 2
Top 10 upregulated and downregulated lncRNA in microarray

| Probe Name | Regulation | Seqname | Gene Symbol | RNA length | Chrome | Fold Change | P-value |
|---------------|------------|--------------------|------------------|------------|--------|-------------|-------------|
| ASMM10P027186 | up | ENSMUST00000137025 | Mup9 | 787 | chr4 | 118.8211935 | 3.21678E-05 |
| ASMM10P055456 | up | ENSMUST00000169784 | Mup-ps4 | 474 | chr4 | 109.195563 | 8.37692E-05 |
| ASMM10P003383 | up | ENSMUST00000163495 | Tg | 4952 | chr15 | 68.820901 | 2.01088E-06 |
| ASMM10P029469 | up | ENSMUST00000120341 | Gm12897 | 250 | chr4 | 35.7783047 | 5.78685E-05 |
| ASMM10P055944 | up | TCONS_00006898 | TCONS_00006898 | 75 | chr12 | 34.9270569 | 2.16253E-06 |
| ASMM10P058117 | up | AI642987 | humanlincRNA1158 | 226 | chr2 | 33.6200933 | 5.09835E-06 |
| ASMM10P027204 | up | ENSMUST00000121132 | Mup-ps20 | 389 | chr4 | 32.1768747 | 0.000137217 |
| ASMM10UP787 | up | uc.86+ | uc.86 | 340 | chr2 | 28.8579697 | 2.12019E-05 |
| ASMM10P002133 | up | ENSMUST00000139794 | 1700013G23Rik | 446 | chr11 | 25.8974187 | 8.39099E-05 |
| ASMM10P010990 | up | AK078011 | AK078011 | 742 | chr14 | 22.7760404 | 6.19189E-06 |
| ASMM10P026501 | down | NR_040589 | 6330410L21Rik | 3869 | chr3 | 19.1022874 | 0.000141403 |
| ASMM10P020728 | down | NR_040496 | Gad1os | 905 | chr2 | 13.125109 | 0.000521638 |
| ASMM10P002832 | down | ENSMUST00000151559 | Uqcr10 | 510 | chr11 | 12.6015112 | 0.036962683 |
| ASMM10P048643 | down | ENSMUST00000122825 | Myo15 | 2285 | chr11 | 11.9126698 | 0.000174835 |
| ASMM10P010250 | down | AK021108 | AK021108 | 489 | chr13 | 11.7795425 | 0.00555039 |
| ASMM10P036325 | down | ENSMUST00000120706 | Gm11606 | 617 | chr1 | 11.6265157 | 0.005532558 |
| ASMM10P019099 | down | NR_045306 | 4931403E22Rik | 668 | chr19 | 11.4948991 | 0.013329559 |
| ASMM10P025520 | down | AK076356 | AK076356 | 2681 | chr3 | 10.2321104 | 0.000895843 |
| ASMM10P020729 | down | ENSMUST00000150702 | Gm16292 | 608 | chr2 | 9.5576314 | 0.000103482 |
| ASMM10P019262 | down | TCONS_00016118 | XLOC_012418 | 330 | chr19 | 9.1857411 | 0.004675328 |

Validation of lncRNA microarray results

Total RNA was extracted to perform RT-qPCR (n = 6–9/group). A total of 11 lncRNAs (NR-040589, ENSMUST00000142612, ENSMUST00000139794, ENSMUST00000163495, ENSMUST00000137025, AK021108, NR-045306, ENSMUST00000156336, ENSMUST0000014113, ENSMUST00000120706 and ENSMUST0000069768) were selected from the microarray results for validation by RT-qPCR. The findings for five of these lncRNAs, including two downregulated (NR_040589 and ENSMUST00000142612) and three upregulated (ENSMUST00000139794, ENSMUST00000163495, and ENSMUST00000137025) lncRNAs, were consistent with those of the microarray analysis (Fig. 2).

CNC network analysis

In order to identify the regulatory association of mRNAs and the five lncRNAs validated by RT-qPCR in diabetic SMGs, the CNC network of these five lncRNAs along with the differentially expressed mRNAs from the GEO database (GSE141411) was constructed (Fig. 3). There were 615 lncRNA-mRNA connection pairs in the network, including 369 positive connection pairs and 246 negative pairs. The top 10 positive and negative interaction pairs according to the Pearson's correlation coefficient are presented in Table 3. These close interaction pairs may be involved in the regulation of saliva secretion from the SMGs in db/db mice. GO analysis based on the target genes of the CNC network revealed that the most enriched biological process was "Cellular response to hormone stimulus" and the most enriched cellular component was "Extracellular region", while the most enriched molecular function was "Calcium ion binding", as shown in Fig. 4A. KEGG pathway analysis revealed that the main enriched pathways were "Cysteine and methionine metabolism", "Phosphatidylinositol signaling system" and "Vitamin digestion and absorption", as shown in Fig. 4B. These enriched GO terms and pathways may participate in diabetes-induced hyposalivation.

Table 3
Top ten positive and negative correlation pairs of lncRNA-mRNA

| mRNA | lncRNA | Pearson's correlation coefficient | Correlation type | P-value | False discovery rate |
|---------------|--------------------|-----------------------------------|------------------|----------|----------------------|
| Mup11 | ENSMUST00000163495 | 0.999661 | + | 9.75E-11 | 7.06E-06 |
| Mup1 | ENSMUST00000163495 | 0.998694 | + | 5.57E-09 | 0.000202 |
| Mup14 | ENSMUST00000163495 | 0.998433 | + | 9.60E-09 | 0.000232 |
| Mup20 | ENSMUST00000163495 | 0.998044 | + | 1.87E-08 | 0.000338 |
| Mup8 | ENSMUST00000163495 | 0.997129 | + | 5.90E-08 | 0.00078 |
| Mup7 | ENSMUST00000163495 | 0.997041 | + | 6.46E-08 | 0.00078 |
| Defb29 | ENSMUST00000139794 | 0.9964 | + | 1.16E-07 | 0.001203 |
| Kcnj16 | NR_040589 | 0.995913 | + | 1.70E-07 | 0.00154 |
| Mup5 | ENSMUST00000163495 | 0.995466 | + | 2.32E-07 | 0.001869 |
| 1600014C23Rik | NR_040589 | 0.995075 | + | 2.98E-07 | 0.002063 |
| Acin1 | ENSMUST00000139794 | -0.95011 | - | 0.000299 | 0.035206 |
| Zfp36 | NR_040589 | -0.95025 | - | 0.000297 | 0.035084 |
| Mup7 | NR_040589 | -0.9503 | - | 0.000296 | 0.035024 |
| Utp14a | ENSMUST00000142612 | -0.95034 | - | 0.000295 | 0.035024 |
| Scgb2b27 | NR_040589 | -0.95037 | - | 0.000294 | 0.035024 |
| Synm | ENSMUST00000163495 | -0.95042 | - | 0.000293 | 0.035024 |
| H2-M3 | ENSMUST00000139794 | -0.9505 | - | 0.000292 | 0.034963 |
| Ppp1r14a | ENSMUST00000137025 | -0.95051 | - | 0.000292 | 0.034963 |
| Ube2cbp | ENSMUST00000142612 | -0.95073 | - | 0.000288 | 0.034859 |
| Ppp1r14d | ENSMUST00000137025 | -0.95078 | - | 0.000287 | 0.034859 |

CeRNA network analysis

Recent research has highlighted the importance of ceRNAs, which communicate with and regulate each other by competing for binding to shared miRNAs and exert an effect on post-transcriptional regulators [28]. The ceRNA mechanism has been reported to participate in the development of diabetic complications. In the present study, to classify the ceRNA function in the hyposalivation of type 2 diabetes, a ceRNA network was constructed based on five lncRNAs validated by RT-qPCR and differentially expressed mRNAs from microarray analysis (Fig. 5). In total, 37 ceRNAs were found in the ceRNA network. GO analysis based on the target genes of the ceRNA analysis results revealed that the most enriched biological process was "Localization", the most enriched cellular component was "Cell part" and the most enriched molecular function was "Protein binding" (Fig. 6A). KEGG pathway analysis revealed that the main pathways included "Rap1 signaling pathway", "Renal cell carcinoma" and the "mammalian target of rapamycin (mTOR) signaling pathway" (Fig. 6B). These results revealed that biological processes and regulatory pathways may play vital roles in the secretion of SMGs in diabetes.

Discussion

Saliva is mainly secreted from three major salivary glands namely the SMGs, parotid glands and sublingual glands. In humans, approximately 60% of resting saliva and 40% of stimulated saliva is secreted from the SMGs [29]. The SMGs are an important for maintaining saliva secretion and oral health [30]. Notably, the SMGs are more sensitive to changes of physiological and metabolic changes in the body. Therefore, damage to SMGs is related to a variety of diseases. For example, the destruction of SMGs acinar tissue leads to a decrease in saliva secretion in patients with Sjogren's syndrome [31]. However, studies focusing on diabetes-induced damage to the SMG are very limited. Therefore, the present study collected SMG tissues of db/db mice and db/m mice for microarray analysis in order to investigate the underlying mechanisms of lncRNAs in diabetes-induced hyposalivation. Compared with db/m mice, 536 upregulated and 737 downregulated lncRNAs were identified; these differentially expressed lncRNAs may play an important role in diabetes-induced hyposalivation.

The present study performed RT-qPCR validation on 11 lncRNAs with the most significantly dysregulated expression levels. From this validation, the results of five lncRNAs were consistent with those of high-throughput sequencing. We previously determined the whole genome expression profile of SMG tissues in db/db mice and found that 1,146 mRNAs exhibited a significantly dysregulated expression; of these, 606 mRNAs were upregulated and 540 mRNAs were downregulated [32]. lncRNAs are regarded as the primary section of the CNC network, particularly the ceRNA network. However, the functional roles and regulatory mechanisms of the CNC and ceRNA networks in diabetes-induced hyposalivation remain unknown. Therefore, the present study used these five lncRNAs to perform CNC and ceRNA network analyses in combination with 1,146 dysregulated mRNAs.

GO analysis of mRNA obtained from the CNC network of five lncRNAs showed that "Calcium ion binding" is highly enriched in the molecular function. The mobilization of Ca^{2+} plays an important role in salivary secretion, the activation of muscarinic cholinergic receptors rapidly triggers the release of intracellular Ca^{2+} from the endoplasmic reticulum and subsequently the influx of Ca^{2+} from the extracellular medium, resulting in a sustained increase in intracellular Ca^{2+} [33, 34]. The increased intracellular Ca^{2+} induces the transport of aquaporin 5, leading to the formation of water pores and thus promoting a rapid increase in transcellular water permeability [35]. In addition, a study found that adiponectin can also induce salivary secretion of the db/db mouse by activating adenosine monophosphate-activated protein kinase and the Ca^{2+} signaling pathway played an important role in this process [36]. In the human and rabbit SMGs, the activation of muscarinic cholinergic receptors and transient receptor potential vanilloid subtype 1 increased salivary secretion via increased intracellular Ca^{2+} [37, 38]. In patients with epiphora following human transplanted epiphora SMG transplantation, the elevated intracellular Ca^{2+} mobilization induced by muscarinic acetylcholine receptors activation contributed to hypersecretion [37]. In spontaneously hypertensive rats, the damaged Ca^{2+} response to carbachol was confirmed in acinar cells of spontaneously hypertensive rats, which may also be related to the reduced salivary secretion caused by hypertension [39]. These studies indicate that the increased intracellular Ca^{2+} derived from extracellular and intracellular Ca^{2+} plays an important role in the salivary secretion of SMGs. In the present study, the results from microarray analysis showed that significantly altered "calcium ion binding", suggesting that the salivary secretion from the SMGs may be also affected by the Ca^{2+} signaling pathway during diabetes, and this process may be regulated by these five lncRNAs. However, the regulatory mechanisms of the related lncRNA-mRNA interaction require further research.

lncRNAs regulate mRNAs via various mechanisms, one of which is ceRNA-mediated changes in the expression of downstream molecules regulated by miRNAs. Therefore, the present study performed ceRNA network analysis on five significantly altered lncRNAs and 1,146 dysregulated mRNAs to identify the related pathways regulated by the miRNA pathway. In addition, the obtained mRNAs of the ceRNA network were analyzed by GO and KEGG analyses. KEGG analysis revealed that the "mTOR signaling pathway" was significantly enriched, suggesting that these five lncRNAs may affect the downstream "mTOR signaling pathway" through ceRNAs. The phosphatidylinositol 3-kinase (PI3K)/protein-serine-threonine kinase (Akt)/mTOR pathway is an intracellular signaling pathway that plays a key role in regulating cell cycle-mediated processes, including cellular quiescence and cell proliferation [40], as well as various disease such as epithelial ovarian cancer [41]. It has been demonstrated that the "mTOR signaling pathway" plays an important role in the pathophysiological process of diabetes. In MC3T3-E1 cells, high glucose levels have been shown to increase the production of reactive oxygen species and induced autophagy by inhibiting the Akt/mTOR pathway [42]. The mTOR/PI3K/Akt pathway is involved in the regulation of autophagy in diabetic kidney disease [43]. The PI3K/Akt/mTOR pathway has also been shown to be significantly downregulated in the brains of rats with streptozotocin-induced type 2 diabetes; this may explain the neurodegeneration commonly observed in diabetes [44]. In addition, the mTOR pathway is associated with the process of salivary secretion. Bleomycin has been shown to induce the epithelial-to-mesenchymal transformation of human SMG cells via the Akt/mTOR pathway [45]. In our previous studies, it was found that autophagy induced aquaporin 5 degradation through the PI3K/Akt/mTOR pathway signaling pathway, resulting in a decreased salivary secretion from the SMG in db/db mice [9]. These studies suggest that the "mTOR signaling pathway" may play an important role in SMGs injury caused by diabetes. The five lncRNAs we verified may involve in the regulation of the "mTOR signaling pathway" through the ceRNA mechanism via miRNA sponging, but further research is needed.

Currently, it is difficult to rapidly and efficiently study the association between lncRNAs and diseases by relying only on traditional biological experiments. With the development of high-throughput sequencing technology and bioinformatics, the application of digital techniques and large data in the medical field is becoming increasingly extensive. It is very effective for the diagnosis, treatment of diseases and the improvement of the quality of life of patients. *Liu et al* [46] found eight lncRNAs, including WDFY3-antisense (AS)2, cancer susceptibility 8 and UGDH-AS1, which were associated with the overall survival of the patients with esophageal cancer using RNA-sequencing and bioinformatics analysis; their study provided a new direction for the search for novel molecular therapeutic targets and prognostic markers for esophageal cancer. By constructing a lncRNA-miRNA-mRNA ceRNA network and analyzing the ceRNAs in the development of cervical squamous cell carcinoma, *Song et al* [47] found two novel lncRNAs, ADAMTS9-AS2 and MEG3, which may play important roles in the pathogenesis of cervical squamous cell carcinoma. lincRNA-p21 has been identified as a potential novel prognostic biomarker for the prognosis of diffuse large B cell lymphoma, and has been shown to regulate cell proliferation and the cell cycle in vitro [48]. The application of bioinformatics technology and large data may be effective for the identification of potential lncRNA functions and lncRNA-disease associations, hence

decreasing the time and cost of biological experiments. In the present study, microarray analysis was performed using SMG tissues, followed by bioinformatics analysis. This may aid in the rapid and efficient identification of lncRNAs associated with hyposalivation in patients with diabetes.

Conclusions

In conclusion, by performing CNC and ceRNA network analyses, the results of the present study revealed that these five differentially expressed lncRNAs (NR_040589, ENSMUST00000142612, ENSMUST00000139794, ENSMUST00000163495 and ENSMUST00000137025) may play an important role in diabetes-induced SMG dysfunction via “calcium ion binding” and the “mTOR signaling pathway”, which potentially served as the downstream pathways regulated by the five lncRNAs. The findings presented herein enhance the current understanding of the roles of lncRNAs in salivary secretion in diabetic mice. These findings may also provide valuable information for further research, and precise therapeutic targets for diabetes-induced hyposalivation, helping to improve the quality of life of diabetic patients.

Abbreviations

lncRNAs: Long non-coding RNAs

miRNA: MicroRNA

SMGs: Submandibular glands

RT-qPCR: Real-time quantitative polymerase chain reaction;

mTOR: Mammalian target of rapamycin;

GO: Gene Ontology;

KEGG: Kyoto Encyclopedia of Genes and Genomes;

CNC: Coding-non-coding gene co-expression;

CeRNA: Competing endogenous RNA.

PI3K: Phosphatidylinositol 3-kinase;

Akt: Protein-serine-threonine kinase

Declarations

Ethics approval and consent to participate

All animal protocols were approved by the Laboratory Animal Welfare Ethics Branch and Biomedicine Ethics Committee on Peking University (approval no. LA2015071). All animal experiments performed at the animal department of Peking University Health Science Center (Beijing, China).

Availability of data and materials

The datasets generated and/or analyzed during the current study are available at the GEO database repository (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE141411>). “GSE141411” is currently private and is scheduled to be released on December 03, 2022.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

XS, RX and LW have made substantial contributions to the conception and design of the work. HL, YZ, XC and LL have made substantial contributions to the acquisition and analysis of the work. LL, XS and HL have made substantial contributions to the interpretation of data of the work. XS and HL have drafted the work. RX have substantively revised the manuscript. All authors have approved the submitted version and agreed both to be personally accountable for the author's own contributions and to ensure that questions related to the accuracy or integrity of any part of the work.

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No.

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Figures

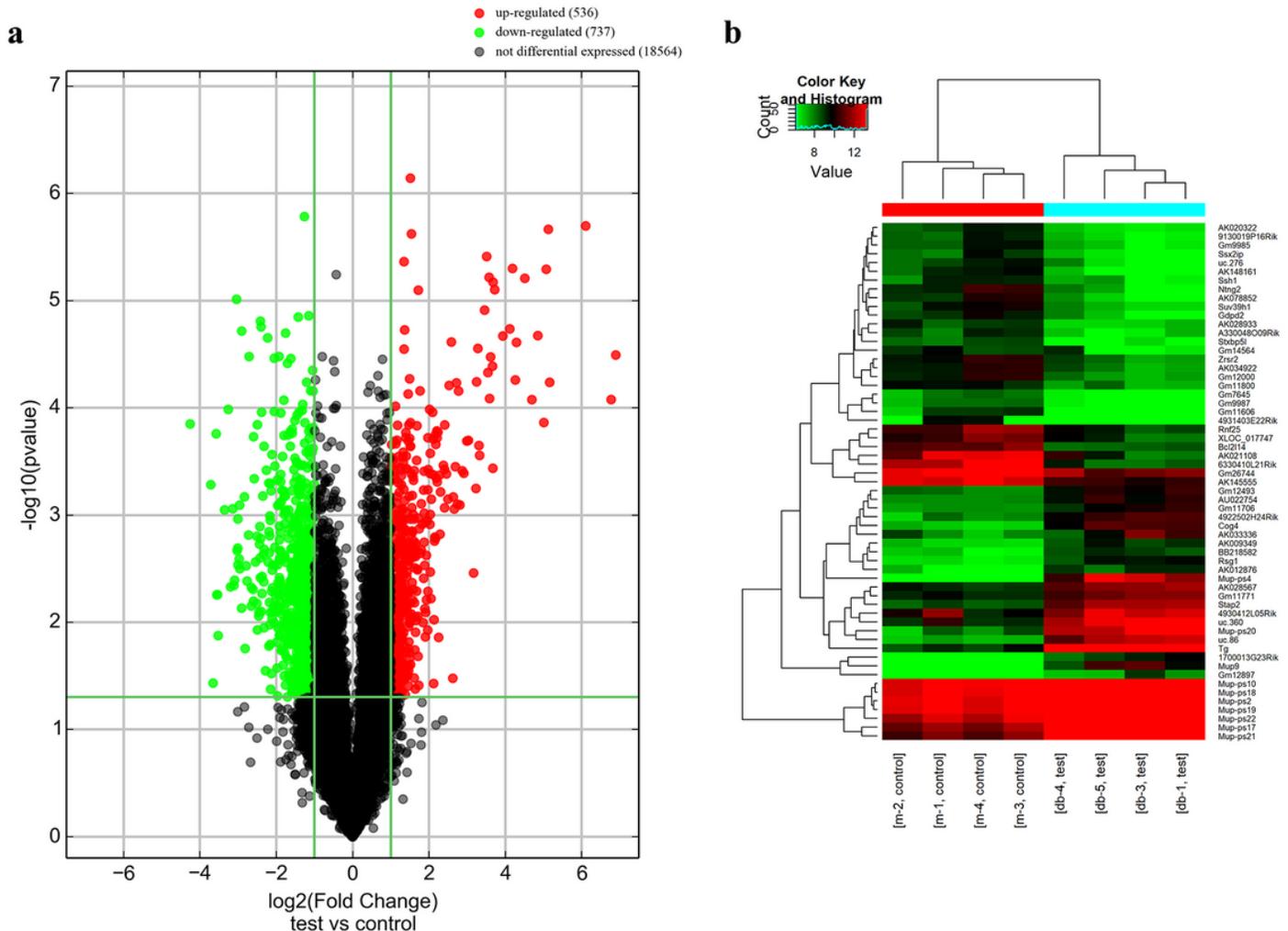


Figure 1

Expression profiling changes of significantly differentially expressed lncRNAs in db/db and db/m mice. (A) Volcano plots presenting differences in the expression of lncRNAs between db/db mice and db/m mice. (B) Heatmaps showing the expression profiles of the top 60 mRNAs with the most distinct fold change in expression between the db/db mice and db/m mice. lncRNAs, long non-coding RNAs.

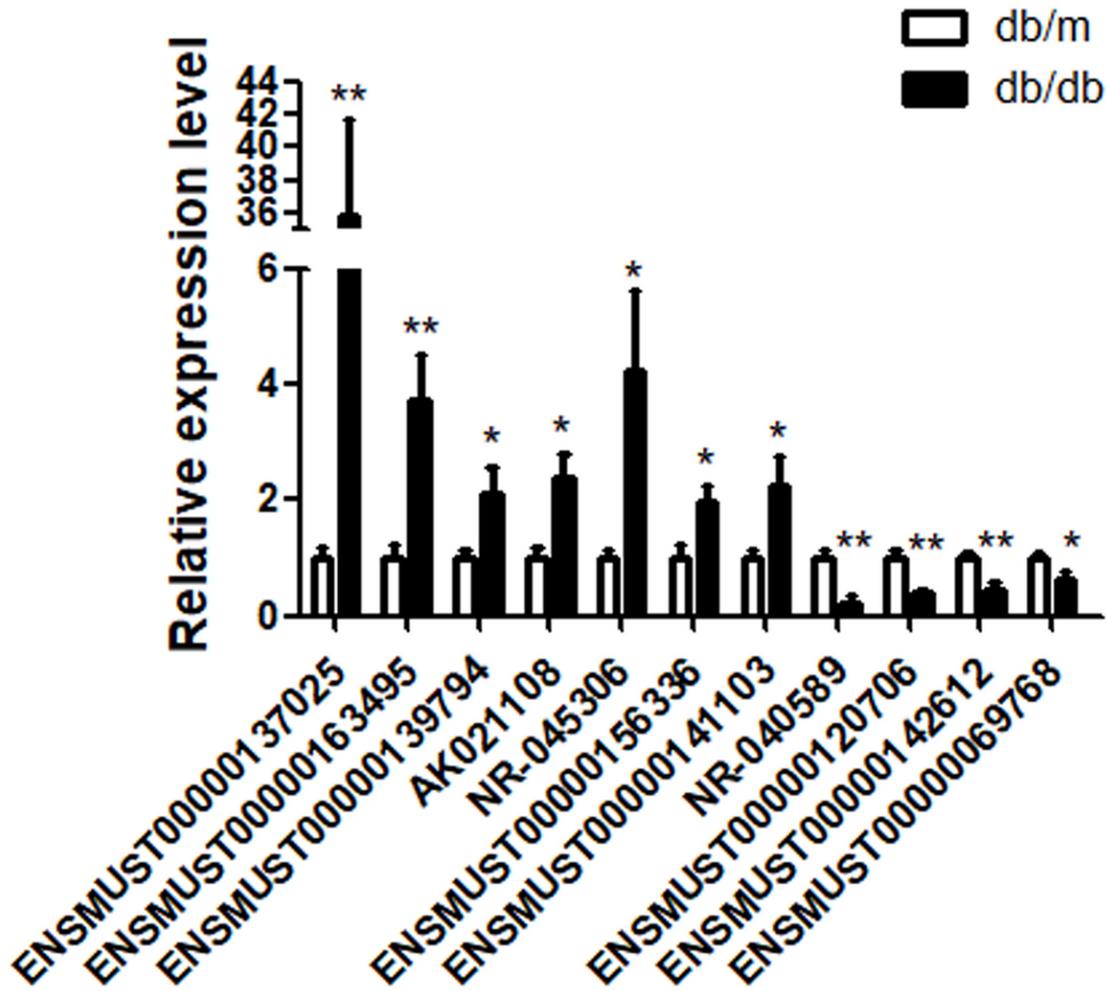


Figure 2

Validation of lncRNA expression. Relative expression levels of selected lncRNAs as detected by RT-qPCR. β -actin was used as a housekeeping gene for normalizing changes in specific gene expression. * $P < 0.05$ and ** $P < 0.01$ vs. db/m mice, $n = 6-9$ /group. RT-qPCR, reverse transcription-quantitative PCR; lncRNAs, long non-coding RNAs.

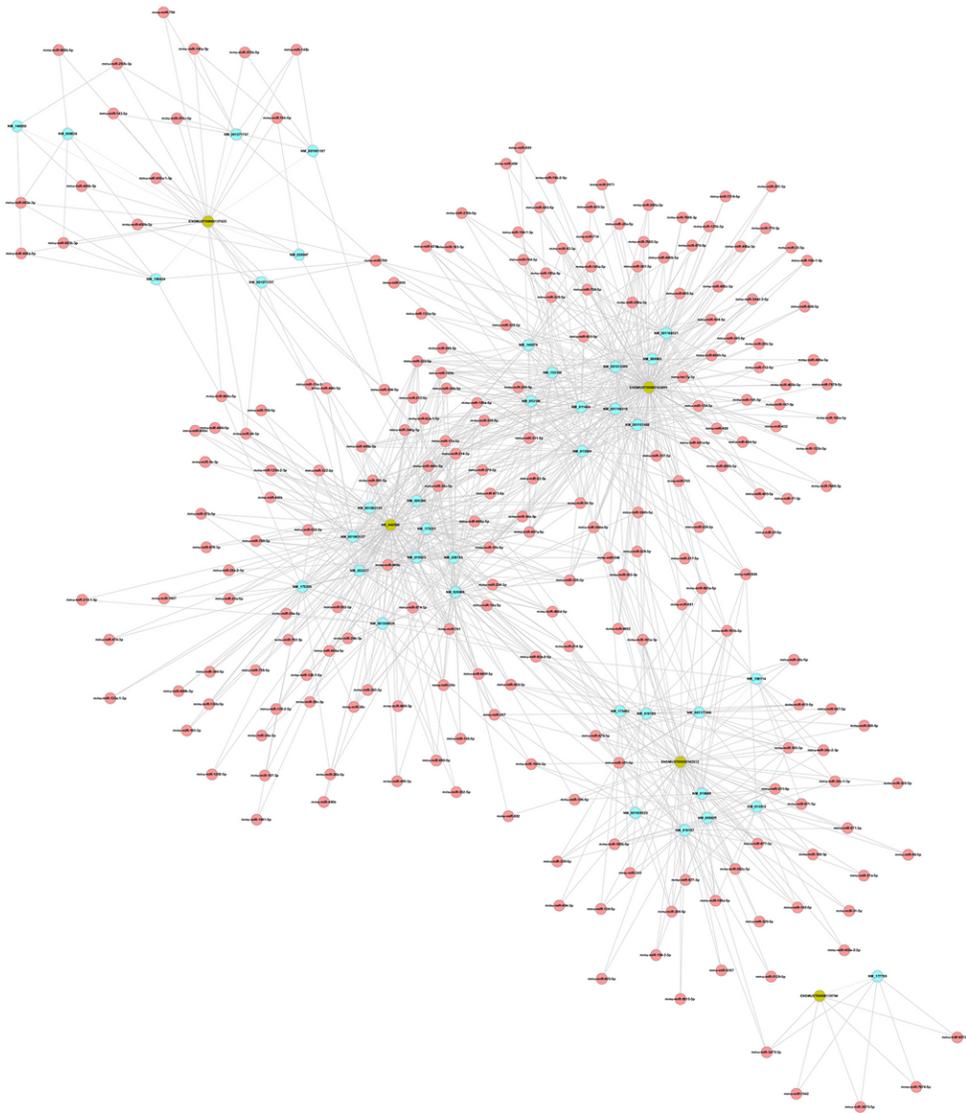


Figure 5

ceRNA network. Red circles represent miRNAs, blue circles represent mRNAs and green circles represent lncRNAs. ceRNA, competing endogenous RNA; lncRNAs, long non-coding RNAs.

