Expression profiles of long noncoding RNAs in cardiac stem cells under hyperglycemic conditions

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From previous reports, the incidence of ischaemic heart disease and acute myocardial infarction is greater in people with diabetes than in nondiabetic individuals, and has suffered from a higher incidence of mortality [1–3]. Meanwhile, the risk of post-myocardial infarction death is also increased in diabetic patients compared to those without diabetes. Of 157,142 people with an MI in England and Wales between 2003–2006, the relative risk of death or recurrence of MI for those with diabetes in the first 90 days was 1.3 (95%CI: 1.26–1.33) crude rates and 1.16 (95%CI: 1.1–1.2) when controlling for age, gender, heart failure and surgery for MI compared with those without diabetes [4]. However, the mechanisms associated with diabetes-induced low myocardial regeneration and repair capability after myocardial injury remain elusive.

The cardiac stem cells (CSCs), as one subtype of cardiac immature cells in the mammalian heart, play a pivotal role in the maintenance of heart homeostasis and cardiac repair since it possess the ability to differentiate into all constituent cells of cardiac tissue including cardiomyocytes, vascular smooth muscle and endothelial cell [5–7]. It is reported that intracoronary infusion of autologous CSCs is effective in improving LV systolic function and reducing infarct size in patients with heart failure after myocardial infarction [8]. In physiological conditions, a large pool of functionally CSCs in human’s heart can be stimulated to propagate, differentiate, and partially replace the damaged cardiomyocytes due to ischemia or myocardial infarction [9]. Emerging evidences suggested that hyperglycemia could inhibit cardiac stem cell-mediated cardiac repair and angiogenic capacity [10]. However the specific mechanism for the poor myocardial regeneration capability of CSCs under hyperglycemia condition is still unknown.

Recently, long noncoding RNAs (lncRNAs) are implicated in the complex molecular circuitry of cellular processes. They were emerging as important regulators of eukaryotic gene expression, and represent key modulators of cell behavior [11–14]. The scientists performed lncRNA expression profiling of retinas using microarray analysis in a mouse model of streptozotocin (STZ)-induced diabetes. They found that lncRNAs are involved in the pathogenesis of diabetic retinopathy through the modulation of multiple pathogenetic pathways, and represent viable targets for both clinical diagnostic strategies and therapeutic intervention [15,16]. Furthermore, the other study also revealed that differential expression of lncRNAs between sperm samples from diabetic and non-diabetic mice may play an important role in the pathogenesis of diabetes-related low fertility.

Based on the previous studies, we proposed our hypothesis that hyperglycemia could induce different expression patterns of lncRNAs in CSCs, which may contribute to low myocardial regeneration capability of patients with diabetes. We use lncRNA microarray technology to inspect the difference of lncRNAs expression in CSCs between high and normal glucose culture, then apply gene ontology and pathway analysis to identify the expression patterns of lncRNAs on responding to hyperglycemia condition. The present study for the first time demonstrated the role of lncRNAs on low myocardial repair and regeneration capability of CSCs in patients with diabetes.

All animals received humane care in compliance with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH Publication No. 86–23, revised 1996). All procedures and handling of the animals during the study were reviewed and approved by the Ethical Committee of Zhejiang University for Animal Experimentation.

Cardiac stem cells (CSCs) were isolated from one to three-day-old neonatal Sprague–Dawley rats. We cut the thoracic wall open and rapidly isolate the hearts with a scissor. And then rinsed the hearts with ice cold DMEM to remove blood, atria and vascular tissue from the ventricles. The ventricles were transferred into a dish containing fresh ice...
cold medium and washed ventricles with ice cold IMDM for 3 times. After wash, we collected the ventricles to a 5 ml sterile bottle containing 2 ml of ice cold HBSS. We cut the ventricles into small pieces with a scissors. Digest tissue fragments with digestion solution. We added 5 ml IMDM containing 10% FBS in the remaining tissue fragments, gently pipette the remaining tissue fragments in the bottle with 5 ml pipette up and down for 6 times to facilitate cardiomycocytes to come off the tissue fragments and collected supernatant in 10% FBS IMDM on ice. We centrifuged the collected cell suspension for 5 min at 800 rpm and resuspended cell pellet in 5 ml of culture medium. We passed the cell suspension through a 200 μm nylon mesh cell strainer into a new 50 ml tube. We pre-plated the collected cell suspension on cell culture dish 2 times, 45 min once to remove fibroblasts and other type of cells. Finally, the CSCs maintained in IMEM/normal glucose (4500 mg/l; Gibco, Carlsbad, CA, USA) containing 10%FBS and 10,000 U/ml Penicillin/Streptomycin, in 10 cm plastic plates at 37 °C in an incubator with 5% CO₂.

CSCs at P₁ passage were evaluated by flow cytometry using a Becton-Dickinson FACS caliber (San Jose, CA) with 10,000 events collected. The cells were stained with d-ascorbate at control (4500 mg/l) or high (18,000 mg/l) of 72 h in 10% FBS medium.

Three high glucose threatened cell samples were regarded as the experimental group, and three untreated cell samples were regarded as the control group. After culturing with high glucose medium or normal culturing medium for 72 h, total RNA was extracted and purified using mirVana™ miRNA Isolation Kit (Cat#AM1561, Ambion, Austin, TX, US) following the manufacturer’s instructions and checked for a RIN number to inspect RNA integration by an Agilent Bioanalyzer 2100 (Agilent technologies, Santa Clara, CA, US). Prior to extracting, the cells were deplated with DEPC for RNAs-free. RNA quality and quantity were measured by agarose gel electrophoresis and D₂₅₀/D₂₈₀.

Total RNAs extracted from experimental and control groups were amplified and labeled by Low Input Quick Amp Labeling Kit, One-Color (Cat#5190-2305, Agilent technologies, Santa Clara, CA, US), following the manufacturer’s instructions. Labeled cRNAs were purified by RNeasy Mini Kit (Cat#74106, QIAGEN, GmBH, Germany). The labeled cRNAs were hybridized onto a 4 × 44 K rat lncRNA Microarray V5.7 Array that contained about 9780 lncRNA and 19,291 mRNA. Each slide was hybridized with 1.65 μg Cy3-labeled cRNA using Gene Expression Hybridization Kit (Cat#5188-5242, Agilent technologies, Santa Clara, CA, US) in Hybridization Oven (Cat#G2545SA, Agilent technologies, Santa Clara, CA, US), according to the manufacturer’s instructions. After 17 h hybridization, slides were washed in staining dishes (Cat#121, Thermo Shandon, Waltham, MA, US) with Gene Expression Wash Buffer Kit (Cat#5188-5327, Agilent technologies, Santa Clara, CA, US), following the manufacturer’s instructions.

Slides were scanned by Agilent Microarray Scanner (Cat#G2565CA, Agilent technologies, Santa Clara, CA, US) with default settings, Dye channel: Green, Scan resolution = 5 μm, PMT 100%, 10%, 16 bit. Data were extracted with Feature Extraction software 10.7 (Agilent technologies, Santa Clara, CA, US). Raw data were normalized by Quantile algorithm, Gene Spring Software 11.0 (Agilent technologies, Santa Clara, CA, US).

Gene Ontology (GO) analysis included the categories of biological process, cellular component and molecular function were performed on account of Gene Ontology (http://www.geneontology.org). Fold changes and P value acted as the standard to indicate the significance of GO term enrichment (FC ≥ 2, P < 0.01 was considered statistically significant). Pathway analysis was also used to classify differentially expressed genes on account of the Kyoto Encyclopedia of Genes and Genomes (KEGG) database to predict pathways that we were interested in. Fold changes and P value acted as the standard to indicate the significance of GO term enrichment (FC ≥ 2, P < 0.01 was considered statistically significant).

We chose the mRNA related to fibrosis, inflammation and apoptosis from the detected mRNA which changed more than two folds with the P value less than 0.01. We via cis-acting calculated out the lncRNA correlated with the three biological changes.

After treatment with or without high glucose for 72 h, CSCs were rinsed with cold PBS, lysed in 600 μl mirVana Lysis/Binding buffer (Ambion, Inc., Austin, TX, USA) and total RNA was extracted using the mirVana™ miRNA Isolation Kit (Ambion, Inc., Austin, TX, USA). Real-time PCR was run using a total of 5-ng template cDNA for each sample. Each sample was run in duplicate, using ABI FAST SYBR green supermix (Applied Biosystems; Foster City, CA) for multiple genes, including CUST__3812__P2482311958 and Tc712 (CUST__3812__P2482311958, Forward primer: 5’TCCAAGACATTGCACATAGG 3’; Reverse primer: 5’CTTGTTGCTGGCTTTTATTA 3’; Tc712, Forward primer: 5’CCTAGGGCATCGGTGTTGT 3’; Reverse primer: 5’CCAGGAGAGCCCGAGACGGT 3’). The relative mRNA level was presented as units of 2^-ΔCt (ΔCt= Ct(gene of interest) – Ct(gene of reference)).

All statistical analyses were performed using SPSS 19.0 software. All quantitative data were presented as mean ± standard deviation (mean ± SD). Student’s t-test was used to calculate the statistical significance. The association of the two variables was established using the Pearson product-moment correlation coefficient (r). The genes that changed more than two folds (including two folds) and P < 0.01 were considered statistically significant.

CSCs were isolated from one- to three-day-old neonatal Sprague-Dawley rats and maintained in IMEM/normal glucose containing 10% FBS and 10,000 U/ml Penicillin/Streptomycin, in 10 cm plastic plates at 37 °C in an incubator with 5% CO₂. CSCs at P₁ passage were evaluated by flow cytometry using a Becton-Dickinson FACS caliber (San Jose, CA) with 10,000 events collected. The flow cytometry analysis suggested that the CSCs we isolated could express stem cell markers: scal-1 (98.2%), Isl-1 (96.7%), and MDR-1 (94.8%); but were negative for hematopoietic cell markers including CD31 (0.7%) and CD34 (9.2%).

The lncRNA expression profile of CSCs during high glucose induction was obtained using lncRNA microarray. The results of clustering analysis showed that the expression levels of lncRNA between the experimental and control groups were significantly different. After high glucose induction, 173 lncRNAs were up-regulated and 227 lncRNAs were down-regulated (fold change > 2.0, P < 0.01) (Fig. 1A). LncRNA expresses different from each other, which presented in the expression profiles according to heat map (Fig. 1B).

By miRNA microarray analysis, 1526 mRNAs were identified as differentially expressed between the experimental and control groups, as shown in Fig. 1C. Of these mRNAs, 642 were up-regulated and 884 were down-regulated (fold change > 2.0, P < 0.01) (Fig. 1C). mRNA expresses different from each other, which presented in the expression profiles according to heat map (Fig. 1D).

GO analysis of differentially expressed mRNAs showed that the most significant were related to the cellular component, biological processes, and molecular function such as ‘activation of JAK2 kinase activity (GO:0042977),’ ‘activation of Janus kinase activity (GO:0042976),’ ‘adipose tissue development (GO:0060612),’ ‘alpha-beta T cell differentiation (GO:0046632),’ ‘apoptosome (GO:0043293),’ ‘apoptotic cell clearance (GO:0043277),’ ‘apoptotic nuclear changes (GO:00302620),’ ‘apoptotic process (GO:006915),’ ‘fibroblast growth factor binding (GO:0017134),’ ‘fibroblast growth factor receptor binding (GO:0005104),’ ‘fibroblast growth factor-activated receptor activity (GO:0005007),’ ‘fibroblast proliferation (GO:0048144),’ and ‘positive regulation of transforming growth factor beta production (GO:0016363)’ (Figs. 2A). We speculated the corresponding lncRNA and their biological processes by cis-acting such as ‘B cell receptor signaling pathway,’ ‘NF-κappa B signaling pathway,’ ‘PI3K-Akt signaling pathway,’ and ‘Oxidative phosphorylation’ (Figs. 2B). KEGG analysis of differentially expressed mRNAs showed that there were 40 most significant pathways such as ‘G-protein coupled receptor signaling pathway,’ ‘G-protein coupled receptor activity,’ ‘negative regulation of B cell receptor signaling pathway,’ ‘negative regulation of cyclin catalytic process,’ and ‘receptor-mediated endocytosis’ (Figs. 2C).
We speculated 40 lncRNA pathways by cis-acting such as ‘inflammatory response’, and ‘positive regulation of tumor necrosis factor production’ (Figs. 2D). The results of pathway analysis also include the mTOR, type II diabetes mellitus, PI3K-Akt, NF-kappa B, ECM-receptor interaction and VEGF signaling pathways. For example, the seq9112 changed 12.12 folds affecting RAT01062 and RAT27178, then, the two mRNAs regulated the expression of gene cd40 to adjust the NF-kappa B signaling pathway (Figs. 2E, KEGG map04064), the molecule CD40 redacted by gene cd40 is the receptor of CD40L, the combine CD40 and CD40L can trigger a series of inflammation through NF-kappa B signaling pathway as shown in the KEGG map04064.

The seq6745 changed 3.4 folds and seq2562 changed 5.5 folds affecting RAT08008 and RAT07058, then, the two mRNAs regulated the expression of gene Inpp5d to adjust the apoptotic process, but no report was made describing the function of the genes in apoptotic process.

The seq8433 changed 5.5 folds affecting RAT19160 and RAT19161, then, the two mRNAs regulated the expression of gene Fgfr1 to adjust the fibroblast growth factor receptor signaling; and so on.

We were specifically interested in the lncRNA expression profiles which regulate fibrosis, inflammation and apoptosis in cardiac tissues, thus the datasets concerning other functions were excluded from further investigation. We select 341 mRNAs correlating to inflammation, fibrosis and apoptosis from GO analyses such as ‘activation of NF-kappa B-inducing kinase activity (GO:0007250)’, ‘apoptotic signaling pathway (GO:0097190)’, ‘fibroblast growth factor receptor binding (GO:0005104)’, ‘I-kappa B kinase/NF-kappa B signaling (GO:0007249)’, ‘positive regulation of transforming growth factor beta receptor signaling pathway (GO:0030511)’ (Fig. 3A). And then we speculated 56 lncRNAs (Fig. 3B) by cis-acting that correlated to inflammation, fibrosis and apoptosis and expression changes higher.

**Fig. 1.** The statistics of lncRNA between high glucose group and the control group. A) Four hundred lncRNAs were identified as expressed higher than twofold change in high glucose than in normal. Forty-three percent lncRNAs were up-regulated and fifty-seven percent down-regulated in high glucose cells. B) Differences in expression of lncRNA. Heat map present of the expression profile of lncRNA. Each column represents a sample and each row represents a gene. High relative expression is indicated by red and low relative expression by green. C) The statistics of mRNAs between high glucose group and the control group. 1526 mRNAs were identified as expressed higher than twofold change in high glucose than in normal. 42.07% mRNAs were up-regulated and 57.93% down-regulated in high glucose cells. D) Differences in expression of mRNAs. Heat map present of the expression profile of mRNAs. Each column represents a sample and each row represents a gene. High relative expression is indicated by red and low relative expression is indicated by green.
than two-fold with P value less than 0.01. We selected 32 mRNAs correlating to inflammation, fibrosis and apoptosis from KEGG analyses (Fig. 3C). And then we speculated 35 lncRNAs by cis-acting (Fig. 3D) that correlated to inflammation, fibrosis and apoptosis and expression changes higher than two-fold with P value less than 0.01.

We selected the pathways correlating to inflammation, fibrosis and apoptosis and analyzed the numbers of related genes (Fig. 4A). We constructed the lncRNA-mRNA co-expression network to identify interactions among differentially expressed lncRNAs and mRNAs. Several mRNAs were chosen as targets from Fig. 4A. The networks of mRNAs and the pathways correlating to inflammation, fibrosis and apoptosis were contrasted (Fig. 4B). CNC networks of inflammation, fibrosis and apoptosis-related mRNAs and co-regulated lncRNAs were constructed by GO and KEGG analysis and trans-acting indicating that mRNAs and lncRNAs were closely correlated (Fig. 4C).

To validate the microarray assay findings, a random selection of 1 lncRNA and 1 mRNA were examined. Consistent with the microarray data, RT-PCR showed that CUST_3812_PI428311958, and Tcf712 were up-regulated compared with normal cells (Fig. 5).
The discovery that the adult heart contains a pool of CSCs that play a pivotal role in the maintenance of heart homeostasis and cardiac repair follow heart injury has dramatically changed the traditional view of the heart as a postmitotic organ [17,18]. Several investigators have found that CSCs have a great potential to generate new myocardium after myocardial injury [19,20]. Preclinical studies have shown that resident CSCs can be isolated from the adult heart, expanded in vitro, and then injected into the damaged adult heart to stimulate cardiac repair after infarction [21]. However, CSCs dysfunction exists in various kinds of diseases, and may be responsible for the insufficient regeneration of cardiac myocytes and coronary vessels. The previous study demonstrated that hyperglycemia could induce CSC dysfunction and the impairment regenerative capacity [10,22]. The patients with type 2 diabetes mellitus demonstrated worse clinical outcomes after acute myocardial infarction for poor regenerative capacity of CSCs [23]. In the present study, we isolated CSCs from neonatal SD rats and cultured in high glucose IMEM for 72 h. It is very interesting to notice that a total of 400 IncRNAs and 1526 mRNAs were more than 2 times the variation.

![Fig. 3. The genes regulated fibrosis, inflammation and apoptosis express profiles. A) The heat map of going analysis mRNA. B) The heat map of IncRNA by cis-acted from going analysis mRNA. C) The heat map of KEGG analysis mRNAs. D) The heat map of IncRNA by cis-acted from KEGG analysis mRNA.](image-url)
between high glucose and normal glucose cultured CSCs. The present data indicated that hyperglycemia might change the microenvironment and biological behavior of CSCs in patients with diabetes.

In eukaryotic cells, protein-coding RNA (mRNA) only occupies about 2% of the genome, the rest massive number of transcripts was classified to noncoding RNAs (ncRNAs) [24]. These ncRNAs bind to the complementary site on the 3' UTR of targeting mRNAs and block protein translation or modulate mRNA stability on a post-transcriptional level [25]. For the last decade, long noncoding RNAs (lncRNAs) were most intensively studied because it involved the complex molecular circuitry of cellular processes. Due to its length (>200 bp), lncRNAs can fold into secondary or higher orders of structure making it more flexible in targeting proteins or gene sites, and a unique set of lncRNAs that were associated with disease severity and progression [26,27]. In the present study, we found that hyperglycemia could induce special expression patterns of lncRNAs in CSCs. The present data provided compelling

![Fig. 4. Networks of fibrosis, inflammation and apoptosis-related mRNAs and co-regulated IncRNA. A) The numbers of related genes participate in the pathways correlating to inflammation, fibrosis and apoptosis. B) The networks of mRNAs and the pathways correlating to inflammation, fibrosis and apoptosis. Nodes colored in gray represent mRNAs, while nodes colored in brown represent the pathways. C) CNC networks of inflammation, fibrosis and apoptosis-related mRNAs and co-regulated IncRNA. Nodes colored in yellow represent lncRNA, while nodes colored in green represent mRNAs.

![Fig. 5. CUST_3812_P428311958 (A), and Tcf712 (B) were analyzed by RT-PCR. Gene expression was determined by the ΔCt method, results were normalized to β-actin expression. *P < 0.05 versus normal.](image)
evidence that CSCs undergo phenotypic changes in response to environmental influences. The functions of most of these IncRNAs have not been determined, and there is no existing database available housing their functional annotations. We attempted to correlate mRNA and IncRNA expression in order to indirectly determine IncRNA functions based on the study of their mRNA. We systematically analyzed the functions of differentially expressed mRNAs by GO annotation and pathway analysis. We found that the functions associated with these targets were related to many aspects of diabetic progression and were involved in immune responses, fibrosis and cell apoptosis. From the IncRNA-mRNA co-expression network, CSCs presented different IncRNAs and mRNAs under diabetic condition, specifically, a persistent alteration of 40 IncRNA pathways related to the mTOR, PI3K-Akt, NF-kappa B, and ECM-receptor interaction signaling pathways. For example, the seeq9112 changed 12.12 folds affecting RAT01062 and RAT27178, then, the two mRNAs regulated the expression of gene cd40 to adjust the NF-kappa B signaling pathway. The present data demonstrated that high glucose induced CSCs presented special expression patterns of IncRNAs and mRNAs, and related to inflammation, fibrosis and apoptosis, which may explain the impairment of myocardial regeneration and repair capability of CSCs in patients with diabetes.

In conclusion, the present data provides the first evidence for special expression patterns of IncRNAs in CSC under high glucose culture. GO and KEGG pathway analysis suggested that the primary biological processes of these IncRNAs were related to immune responses, fibrosis and cell apoptosis. These special expression patterns of IncRNAs may play a role in low myocardial regeneration and repair capability of CSCs in patients with diabetes. However, most of the IncRNAs in the co-expression network were not annotated. There is still a long way to do further, larger functional in-depth analyses regarding molecular IncRNAs in CSCs under hyperglycemia condition.

Conflict of interest

The authors report no relationships that could be construed as a conflict of interest.

We adhere to the statement of ethical publishing as appears in the International of Cardiology [28].

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Reference