LncRNA *MALAT1* Regulates iPSC-derived B-cell Differentiation by Targeting the miR-15b-5p/*Ihh* Axis

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Research

Keywords: β -like cells, induced pluripotent stem cells (iPSCs), lncRNA Malat1, miR-15b-5p, *Ihh*, differentiation

Posted Date: October 14th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-89983/v1>

Abstract

Background: iPSCs-derived β -like cell differentiation provides a novel strategy for type 1 diabetes treatment. Clarifying the regulatory mechanisms of lncRNAs in β -like cells derived from induced pluripotent stem cells (iPSCs) is not only significant for understanding the development of pancreas or pancreatic β cells, but also helpful for improving the quality of β -like cells for stem cell therapy.

Methods: β -like cells derived from iPSCs followed a three-step protocol. RNA-sequencing was carried out to screen the differentially expressed lncRNAs which was probably involved in the differentiation of pancreatic β cells. Bioinformatics was performed to analyze the putative target genes of significantly differentially expressed lncRNAs. lncRNA Malat1 was chosen for further research. Lentivirus vector, siRNA vector, antagomir vector and mimic vector were constructed for overexpression of lncRNA Malat1, knockdown of lncRNA Malat1, knockdown of miR-15b-5p and overexpression of miR-15b-5p respectively. Quantitative Real-Time PCR (qRT-PCR), Western Blot and Immunofluorescence (IF) staining were carried out to detect the functions of pancreatic β cells at mRNA and protein level separately. Cytoplasmic and nuclear RNA fractionation and Fluorescence in situ hybridization (FISH) were to ventilate the subcellar location of lncRNA Malat1 in β -like cells. Flow cytometry and ELISA were performed to examine differentiation efficiency and function of insulin secretion from β -like cells after being stimulated with different concentrations of glucose. Structural interactions between lncRNA Malat1 and miR-15b-5p and between miR-15b-5p and *Ihh* were detected by Dual luciferase reporter assay (LRA).

Results: We found that expression of lncRNA Malat1 was on the decline during the differentiation and overexpression of this lncRNA obviously impaired the differentiation and maturation of β -like cells derived from iPSCs *in vitro* and *in vivo*. Localized to the cytoplasm, *lncRNA Malat1* could function as a competing endogenous RNA (ceRNA) of miR-15b-5p to regulate the expression of *Ihh* according to the bioinformatic prediction, mechanistic analysis and downstream experiments.

Conclusion: This study built an unreported regulatory network of *lncRNA Malat1* and miR-15b-5p/*Ihh* axis during differentiation of iPSCs into β -like cells. Except for acting as a proverbial oncogene promoting tumorigenesis, *lncRNA Malat1* may provide effective and novel molecule for diabetes cell therapy in the future.

Background

Stem cell therapy, a promising alternative treatment for diabetes, has been explored for decades[1–3]. Compared with islet and whole-pancreas transplantations, β cells derived from embryonic stem cells (ESCs)/induced pluripotent stem cells (iPSCs) are without limitations in terms of donor acquisition and immunosuppressive-agent application[4, 5]. Existing technologies are based on stepwise differentiation that simulates pancreatic β -cell development *in vivo*, using either several signaling inhibitors and activators or gene reprogramming[6]. Although brilliant progress has been made in differentiation

protocols *in vitro*, acquisition efficiency and cell function also need improvement[7]. Therefore, understanding the sophisticated mechanisms of ESC/iPSC-derived β -cell differentiation *in vitro* is critical.

Non-coding ribonucleic acids (ncRNAs) are not translated or encoded for proteins in traditional view and are considered nonfunctional RNAs[8]. As research has progressed, ncRNAs such as microRNAs (miRNAs), long non-coding RNAs (lncRNAs) and circular RNAs (circRNAs) have been found to participate in cellular signaling and process regulation[9–11]. In recent years, as transcripts have exceeded 200 bp, lncRNAs have been proven to control gene regulation, organ differentiation and both developmental and pathological processes[12–14]. Meanwhile, studies indicate that lncRNAs participate in pancreatic and islet development, β -cell formation and function and T cell differentiation *in vitro*[15–18]. Given the above, seeking functional lncRNAs in the β cells differentiation process and exploring their mechanism in detail are conducive to optimize such β cells differentiation protocols and speculate the process of islet β -cell development *in vivo*.

Herein, we established a three-step iPSC-derived β -cell differentiation protocol to identify crucial lncRNAs and clarify their molecular mechanisms. RNA sequencing (RNA-seq) was performed at four time points: the iPSC stage and the early, middle and late stages of β cells differentiation *in vitro*. The lncRNA metastasis-associated lung adenocarcinoma transcript 1 (*MALAT1*) is highly expressed in iPSCs and decreases over time during β -cell formation. Functional experiments and RNA-seq indicated that unconventionally overexpressed *MALAT1* affected pancreatic-progenitor differentiation by upregulating *Indian hedgehog* (*Ihh*), a *Hedgehog* pathway ligand with reported low expression levels in developing and mature pancreatic tissue[19, 20]. Furthermore, using bioinformatics analysis and biochemical techniques, we demonstrated that *MALAT1* could act as a competing endogenous RNA (ceRNA) of miR-15b-5p to regulate *Ihh* expression. To supplement this finding, we performed an *in vivo* transplantation experiment, which proved that overexpressed *MALAT1* in β cells could suppress the pancreas's insulinogenic ability; this was consistent with the results of *in vitro* differentiation.

Materials And Methods

Cell culture and differentiation

We obtained mouse green fluorescent protein (GFP)–iPSCs from Innovative Cellular Therapeutics, Ltd. (Shanghai, China) and cultured them in feeders under mouse ESC (mESC) culture conditions. These mouse iPSCs (miPSCs) were induced to differentiate into β -like cells using a three-step protocol[2]. In brief, we obtained embryoid bodies (EBs) from GFP⁺ iPSCs in step 1, differentiated them into multilineage progenitor cells (MPCs) in step 2 and induced the MPCs to differentiate into β -like cells using β -cell–selective differentiation medium in step 3.

RNA sequencing and data analysis

Total RNA was isolated at four time points (miPSC; EBs at day 0, stage 1; MPCs at day 7, stage 2; β -like cells at day 21, stage 3) during differentiation using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). In

brief, we acquired raw RNA-seq samples by sequencing polyadenylated (poly[A]⁺) fractions. The sequencing library of each RNA sample was compiled using an Ion Total RNA-Seq Kit v2.0 (Life Technologies, Gaithersburg, MD, USA) per manufacturer's instructions. RNA-seq was performed by NovelBio Corp. Laboratory (Shanghai, China) using the Ion Proton instrument, resulting in an average of ~14 million reads/sample and an average sequence length of 150 bp. We filtered raw reads for poor-quality sequencing adaptors, poly(A) and bases using FAST-QC software (Version 0.11.9) (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>) to evaluate such factors as quality distribution of nucleotides, position-specific sequencing quality, GC content and the proportion of polymerase chain reaction (PCR) duplication. Then, using MapSplice (<http://www.netlab.uky.edu/p/bioinfo/MapSplice2>) and HTSeq (<https://github.com/simon-anders/htseq>), we carried out clean-read alignment and gene-level read count estimation, respectively, against the mouse reference genome (GRCm38).

lncRNA–mRNA network and GO and KEGG analyses

We constructed a lncRNA–mRNA network to assure interactions between lncRNA and mRNA based on normalized signal intensity of specific expression in both types of RNA. After calculating Pearson's correlation coefficient (PCC), we built the network from significant correlated pairs. Moreover, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses were applied to analyze the main function of differentially expressed genes (DEGs). Then, we used the false discovery rate (FDR) to correct the *P*-value, as described in detail in our previous study.

RNA extraction and quantitative reverse-transcription polymerase chain reaction (qRT-PCR) analysis

Total RNA was isolated using a RNeasy Mini Kit (QIAGEN, Düsseldorf, Germany). We used a HiScript III 1st Strand cDNA Synthesis Kit (+ genomic deoxyribonucleic acid [gDNA] wiper) (Vazyme Biotech Co., Ltd., Nanjing, China) to synthesize first-strand complementary DNA (cDNA) of lncRNAs and mRNAs, and miRNA 1st Strand cDNA Synthesis Kits (via stem-loop) (Vazyme Biotech Co., Ltd., Nanjing, China) to synthesize first-strand cDNA of miRNAs, per manufacturer's instructions. Relative expression levels of lncRNAs, mRNAs and miRNAs were calculated via the $2^{-\Delta\Delta C_t}$ method. As internal normalized control, we used U6 for miRNAs and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) for lncRNAs and mRNAs. The qRT-PCR primer sequences for lncRNAs, mRNAs, miRNAs, U6 and GAPDH were designed and synthesized by RiboBio (Guangzhou, China). We performed all experiments independently and in triplicate.

Flow cytometry (FCM)

All cells were harvested as single cells. First, we incubated the cells in Reagent 1: Fixation (Beckman Coulter, Brea, CA, USA) for 15 min and washed them once. Next, cells were incubated in Reagent 2: Permeabilization (Beckman Coulter) for 15 min and washed once. Then, we resuspended them in phosphate-buffered saline (PBS) with primary antibody and incubated them for 30 min. After one more wash, cells were analyzed using a BD LSRFortessa X-20 Flow Cytometer (BD Biosciences, Franklin Lakes,

NJ, USA), and results were analyzed using FlowJo software (FlowJo LLC [BD Biosciences], Ashland, OR, USA). We performed all procedures at room temperature (RT). Primary antibodies included Alexa Fluor 647 mouse anti–natural killer 6 homeobox 1 (*NKX6-1*) and Alexa Fluor 647 mouse immunoglobulin G 1k (IgG1k) isotype control (BD Biosciences).

Glucose-stimulated insulin secretion

We transferred β -like cells into 24-well plates and cultured them for 12 h. After pre-incubation in Krebs–Ringer Bicarbonate (KRB) buffer without glucose for 2 h, cells were stimulated with KRB containing various concentrations of glucose (0, 5, 15 and 30 mM) for 2.5 h. Then, we collected the supernatants. We performed an enzyme-linked immunosorbent assay (ELISA) to assess insulin content using an ultrasensitive mouse insulin assay kit (Mercodia AB, Uppsala, Sweden) per manufacturer's instructions.

Immunofluorescence (IF) staining

After culturing them on glass coverslips for 48 h, we fixed iPSC-derived β -like cells with 4% paraformaldehyde for 15 min and washed them three times with PBS. Then, cells were permeabilized with 0.3% (v/v) Triton X-100 for 10 min, blocked with 5% donkey serum and incubated with primary antibodies at 4° C for 12 h. We stained the cells with corresponding fluorescent secondary antibodies for 2.5 h and 4',6-diamidino-2-phenylindole (DAPI) (Solarbio, Beijing, China) for 12 min at RT. After washing cells twice with PBS, we acquired images using a Leica TCS SP8 confocal imaging system (Leica, Wetzlar, Germany). Primary antibodies were anti-insulin (*INS*) antibody, anti–pancreas/duodenum homeobox protein 1 (anti-*PDX1*) antibody, anti–C-peptide antibody, anti-*MafA* antibody (all four from Abcam, Cambridge, UK), anti–neurogenic differentiation 1 (anti-*NeuroD1*) antibody (Proteintech, Wuhan, China), anti–neurogenin 3 (anti-*NGN3*) antibody (Santa Cruz Biotechnology, Dallas, TX, USA) and *NKX6-1* (D804R) rabbit monoclonal antibody (mAb) and glucagon (*GCG*) antibody (both from Cell Signaling Technology [CST], Danvers, MA, USA). Secondary antibodies (all from Abcam) included donkey anti-rabbit (Alexa Fluor 555), goat anti–guinea pig, goat anti-rat and goat anti-mouse (all three were Alexa Fluor 647) antibodies.

Western blot (WB)

We harvested the cells and then lysed them on ice using radioimmunoprecipitation assay (RIPA) buffer (high) (Solarbio, Beijing, China) for 30 min. Protein concentrations were detected using a Bicinchoninic Acid (BCA) Protein Assay (Thermo Fisher Scientific, Waltham, MA, USA). We separated proteins via sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred them to polyvinylidene difluoride (PVDF) membranes (MilliporeSigma, Burlington, MA, USA), and probed them with primary antibody at 4° C overnight. After three washes with tris-buffered saline + Polysorbate 20 (TBST), we incubated the membranes with horseradish peroxidase (HRP)–conjugated secondary antibodies and visualized signals via enhanced chemiluminescence (ECL). Primary and HRP-conjugated secondary antibodies (all from Abcam) were anti-*lhh* antibody, anti- β -actin antibody and goat anti-rabbit HRP antibody.

Cytoplasmic and nuclear RNA fractionation

Cytoplasmic and nuclear RNAs of mouse MPCs were partitioned using a PARIS Kit (Life Technologies) per manufacturer's instructions. Then, we analyzed cytoplasmic and nuclear RNAs via qRT-PCR as described above.

Fluorescence *in situ* hybridization (FISH)

This assay was performed with a FISH Kit (RiboBio, China) per manufacturer's instructions. LncRNA *MALAT1*-cy3 FISH probes were designed and synthesized by RiboBio. We used mouse U6 and mouse 18S FISH probes as nuclear and cytoplasmic controls, respectively. Images were acquired using the Leica TCS SP8 confocal imaging system.

LncRNA *MALAT1* lentiviral transduction

Genechem (Shanghai, China) produced lentivirus overexpressing lncRNA *MALAT1* and also determined the lentivirus titer. At day 4 of step 3, we seeded iPSC-derived β -like cells into six-well culture dishes and infected them with 20 multiplicity of infection (Moi) lentivirus-cytomegalovirus (CMV)-lncRNA *MALAT1* or 40 Moi lentivirus-CMV-negative control. Seventy-two hours after cells reached 40–50% confluence, we examined lncRNA *MALAT1* expression via qRT-PCR.

Dual luciferase reporter assay (LRA)

We performed a LRA to detect interactions between lncRNA *MALAT1* and miR-15b-5p and between *Ihh* and miR-15b-5p. Wild-type lncRNA *MALAT1* (WT-3'-untranslated region [UTR]-lncRNA *MALAT1*), mutant lncRNA *MALAT1* (MUT-3'UTR-lncRNA *MALAT1*), wild-type *Ihh* (WT-3'UTR-*Ihh*) and mutant *Ihh* (MUT-3'UTR-*Ihh*) were cloned into the reporter vector for miR-15b-5p targeting. We co-transfected WT-3'UTR or MUT-3'UTR vectors with miR-15b-5p or miRNA mimic control. Firefly and Renilla luciferase activities were demonstrated using a Dual Luciferase Assay (Promega Corp., Fitchburg, WI, USA) 2 days post-transfection per manufacturer's instructions.

In vivo implantation of alginate hydrogel

Alginate hydrogel was constructed as described in our previous study[21]. C57BL/6J mice were from the animal center of Nantong University. All animal experiments were carried out according to the Institutional Animal Care guidelines and were approved by the Animal Ethics Committee of the Medical School of Nantong University. We induced diabetes in mice by intraperitoneal injection of streptozotocin (STZ), as reported previously[2]. At day 21, all groups of β -like cells were digested and resuspended with alginate hydrogel. Next, we injected alginate hydrogel containing β -like cells (1×10^6 cells/1 mL hydrogel) into the mice's renal capsules ($n = 6$). All operations were performed under sterile conditions, and mice were sanitized using iodophor. All mice were carefully monitored and well cared for after the procedure.

Statistical analysis

We analyzed all between-group results using Student's *t* test and GraphPad Prism software version 8.0 (GraphPad Software, Inc., San Diego, CA, USA). All error bars represent the mean $\pm$ standard error of mean (SEM). $P < 0.05$ was considered statistically significant.

Results

LncRNA *MALAT1* was a downregulated lncRNA in pancreatic β -cell differentiation

We obtained pancreatic β cells from mouse GFP⁺ iPSCs using three-step protocol (Fig. 1a). First, iPSCs were separated into single cells and then cultured into EBs in a low-adsorption dish. Next, we induced the EBs into multilineage progenitors (MPs) for further differentiation. Finally, after 21 days' induction, β cells differentiated to display tennis racket morphology and formed numerous clusters (Fig. 1b). After 34 days' differentiation, the final cell cytoplasm expressed two distinctive markers of pancreatic β cells, insulin and C-peptide protein (Fig. 1c). Moreover, compared with the miPSCs, β -like cells express insulin and mature beta-cell markers at mRNA level, such as Pdx1, Nkx6.1, Mafa, ISL, Ngn3, GCG, SST and Glut2 (Fig. 1d). As our previous study, this three-step protocol has been proven effective and stable.

To select potentially important lncRNAs that might regulate iPSC-driven β -differentiation, RNA-seq was performed to seek differentially expressed lncRNAs at four sequential time points (Fig. 2a). We harvested iPSCs, as well as β cells in the early (day 4 of step 3), middle (day 14 of step 3) and late (day 21 of step 3) stages, and used poly(A) to analyze mRNAs and lncRNAs. LncRNAs with >2-fold differential expression (FDR < 0.05) were picked up (Fig. 2b). Of these, more than 100 lncRNAs were downregulated, exhibiting four coincident expression patterns in series test of cluster (STC) analysis results: profiles 1, 2, 4 and 5 ($P < 0.05$) (Fig. 2c). After pre-screening, we selected profile 2, which contained 33 transcripts with stable expression across all three stages, for further analysis. Co-expression networks of these lncRNAs and of hundreds correlative mRNAs were exhibited; most of them had positive correlations (PCC > 0.99) (Fig. 2e). GO analyses of the mRNAs revealed that profile 2 lncRNAs might participate in transcription regulation, cell proliferation and apoptosis, all processes that are crucial to stem cell differentiation (Fig. 2d). Interestingly, lncRNA *MALAT1* was one of them and was downregulated at all four time points, as validated by qRT-PCR (Fig. 3a). As mentioned above, bioinformatics analysis indicated that *MALAT1* might play an important role in iPSC-derived β -cell differentiation.

Aberrant overexpression of *MALAT1* disturbed pancreatic β -cell differentiation

LncRNA *MALAT1* was highly expressed in iPSCs but was gradually downregulated during step 3 differentiation. Therefore, we speculated that the relatively low expression of *MALAT1* contributed to transformation of stem cell state to differentiation state. To verify this conjecture, we used clustered regularly interspaced short palindromic repeats (CRISPR)–associated protein 9 (*CAS9*)–*MALAT1* synergistic activation mediator (SAM) to transfect MPCs and maintain a stable high expression level of *MALAT1* early in the differentiation process (Fig. 3b). After 72 hours of transfection, *NKX6-1*⁺ pancreatic endocrine-progenitor cell formation underwent an obvious decrease, from $21.4 \pm 0.35\%$ to $11.2 \pm 0.56\%$

n=3 (Fig. 3c). Analysis by qRT-PCR revealed that *MALAT1* overexpression suppressed transcription of some key regulators of endocrine progenitors, including *PDX1*, *NKX6-1*, *NGN3*, *GATA4/6*, *NeuroD1*, and paired box protein 6 (*PAX6*) (Fig. 3e). Interestingly, *Hedgehog* family member *Ihh* showed noteworthy upregulation compared with the aforementioned transcription factors (TFs). In addition, IF staining of *PDX1*, *NKX6-1*, *NGN3* and *NeuroD1* indicated that *MALAT1*-transfected cells showed a defect in pancreatic endocrine-lineage formation (Fig. 3d). The above-described findings demonstrated that abnormal upregulation of *MALAT1* could affect *NKX6-1*⁺ pancreatic endocrine-progenitor differentiation and that a time-ordered decline in *MALAT1* expression might be necessary for β -cell transformation.

Subcellular location of lncRNA *MALAT1* in MPCs and its possible target gene prediction

To understand how *MALAT1* regulated β -cells differentiation *in vitro*, we performed mRNA-seq to find the target genes of *MALAT1*. We constructed a *MALAT1*-targeting small interfering RNA (siRNA), with which we transfected MPCs. Hierarchical-clustering data revealed that 296 genes were downregulated and 364 genes were upregulated within 72 h after *MALAT1*-siRNA transfection (Fig. 4a). Intriguingly, as shown in the volcano plot, the expression level of *Ihh* decreased, while those of *GATA6*, motor neuron and pancreas homeobox 1 (*MNX1*) and integrin subunit alpha 1 (*IGTA1*) increased, which was in keeping with the results of *MALAT1* overexpression (Fig. 4b). As reported, loss of *Ihh* can promote pancreatic endocrine-lineage development and lead to the malformation known as annular pancreas in humans. To validate the veracity of the abovementioned DEGs, we performed qRT-PCR, and the results correlated well with mRNA-seq data (Fig. 4d). KEGG analysis of DEGs revealed that *MALAT1* influenced several important pathways in cell differentiation and survival, such as the phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)-protein kinase B (*Akt*) and *Ras* signaling pathways, as well as cell adhesion molecules (CAMs) (Fig. 4c). Results of mRNA-seq suggested that silencing *MALAT1* might negatively regulate *Ihh*, which would in turn increase expression of some key TFs and promote endocrine-progenitor differentiation.

If *Ihh* is the target gene of lncRNA *MALAT1*, what is the detailed molecular mechanism of this regulatory relationship? To obtain the answer, one must first discover the subcellular location of *MALAT1*. FISH and cell fractionation analysis both indicated that lncRNA *MALAT1* was mostly located in cytoplasm (Fig. 5a, 5b), implying that it might regulate *Ihh* expression via a ceRNA network. In addition, bioinformatics prediction analysis showed that lncRNA *MALAT1* had a miR-15b-5p targeting site and that *Ihh* might be a downstream target of miR-15b-5p.

miR-15b-5p regulated β -cell differentiation by targeting *Ihh*

MiR-15b-5p has been reported as an important regulator in lung cancer, breast cancer, Alzheimer disease and other pathological and physiological processes[22-25], but not in β -cell differentiation. Therefore, we wondered whether miR-15b-5p could regulate β -cell formation and if *Ihh* were its downstream target. To validate the relationship between miR-15b-5p and *Ihh*, we used Western blotting to detect the protein level of *Ihh*. Results showed that *Ihh* was obviously negatively regulated by overexpressed miR-15b-5p (Fig. 6a). Next, in accordance with the putative binding sites between miR-15b-5p and *Ihh*, we constructed wild-

type (wt) and mutation (mut) vectors of the 3'-UTR of *Ihh* (Fig. 6b). LRA results revealed that only the wt 3'-UTR of *Ihh* showed a statistically significant response to miR-15b-5p, displaying a low translation level after miR-15b-5p overexpression (Fig. 6c). Therefore, our results showed that miR-15b-5p could specifically target the 3'-UTR of *Ihh* and regulate its translation.

As miR-15b-5p has not been previously reported to be involved in β -cell differentiation and function, we attempted to understand the role it might play in β -cell differentiation *in vitro*. Using *Ihh* as negative control (NC), we downregulated miR-15b-5p expression at the MPC stage using antagomir and cultured the cells until day 21. Data from qRT-PCR demonstrated that although this was not true for *Ihh*, the absence of miR-15b-5p led to the downregulation of genes such as *INS*, solute carrier family 2 member 2 (*GLUT2*), insulin gene enhancer protein (*ISL*), *PDX1*, *NKX6-1*, *GCG* and somatostatin (*SST*), representing damage to β cells maturation. (Fig. 6f). Meanwhile, whether the glucose used for stimulation was at a low or a high concentration, miR-15b-5p deficiency impaired insulin production and secretion of β cells (Fig. 6e). In addition, IF staining proved that aberrantly low expression of miR-15b-5p apparently weakened the protein levels of *INS*, *NKX6-1* and *MafA* in β cells (Fig. 6d). Therefore, stable expression of miR-15b-5p was necessary for β -cell differentiation *in vitro*, and its absence was detrimental to cell function and maturation.

MALAT1 regulated β -cell differentiation via the miR-15b-5p–*Ihh* axis

Using bioinformatics to compare sequences, we found that *MALAT1* contained binding sites for miR-15b-5p (5023–5037), which meant a possible interaction between the two. Then, we constructed a luciferase vector of *MALAT1* (Luc–*MALAT1*–wt) and a mutated form (Luc–*MALAT1*–mut) thereof (Fig. 7a). Results indicated that miR-15b-5p could effectively suppress the luciferase activity of *MALAT1* but not of mutated *MALAT1*, meaning that the predicted binding sites of *MALAT1* could specifically bind to miR-15b-5p (Fig. 7b).

Except for structural interactions between lncRNA Malat1 and *Ihh*, whether *MALAT1* could regulate *Ihh* expression at the protein level needed further validation. As WB results showed, *MALAT1* deficiency led to downregulation of *Ihh* (Fig. 7c). Meanwhile, *MALAT1* overexpression could upregulate the expression of *Ihh* (Fig. 7d). In addition, lncRNA *MALAT1* could neutralize miR-15b-5p's inhibitory effect on *Ihh* expression (Fig. 7e). Furthermore, rescue experiments indicated that downregulation of *Ihh* expression reversed the negative impact of *MALAT1* overexpression on β -cell differentiation (Fig. 7f), which meant that *Ihh* might be a functional target of *MALAT1*. Taken together, these findings confirmed that *MALAT1* could regulate *Ihh* expression by competitively binding miR-15b-5p, thereby influencing iPSC-derived β -cell differentiation.

Aberrant overexpression of *MALAT1* influenced the glycemic-control ability of β cells *in vivo*

To further investigate the influence of highly expressed *MALAT1*, we transplanted β like cells transfected with lentivirus into the renal capsules of diabetic mice using alginate hydrogel (Fig. 8a, 8b) in accordance with a protocol reported in a previous study[21]. Next, we harvested β cells from the *MALAT1*

overexpression and NC groups about 1×10^6 before we transplanted them into STZ-diabetic mice ($n = 6$). After 24 days post-transplantation, the fasting blood glucose of mice into which we had transplanted NC cells gradually returned to baseline (Fig. 8c). In contrast, the differentiated cells with high expression of *MALAT1* could not radically reverse hyperglycemia.

Furthermore, 40 days after transplantation, we harvested mice kidneys with transplanted cells and stained them using IF. Results indicated that cells overexpressing *MALAT1* showed worse maturation *in vivo* transplantation compared with normal β cells, having lower protein levels of C-peptide and glucagon, two key markers of pancreatic endocrine cells (Fig. 8d). Therefore, the abovementioned experiments showed that the gradual downregulation of lncRNA *MALAT1* was important to β -cells differentiation, while aberrant overexpression thereof affected cell formation *in vitro* and redifferentiation *in vivo*.

Discussion

PSCs (Pluripotent stem cells), from which neurons, cardiomyocytes, hematopoietic cells and β cells can be differentiated *in vitro* and *in vivo*, show promise as a tool of regenerative medicine[26-32]. Additionally, iPSCs can be reprogrammed from somatic cells with specific TFs such as Pituitary–Octamer–Unc-86 (POU) class 5 homeobox 4 (*OCT4*), *Nanog*, sex-determining region Y–related high-mobility group (HMG) box 12 (*SOX12*) and Krüppel-like factor 4 (*KLF4*) without the limitations of embryonic damage and use of immunosuppressants[33]. As reported, differentiation of iPSCs into all kinds of somatic cells is influenced by numerous factors, such as extracellular matrix (ECM), TFs, cell signaling transduction, ncRNAs and other complex biological process[34-37]. LncRNAs, a hot area of research in epigenetic regulation, participate in numerous biological and cellular processes, including chromatin remodeling, transcription, metabolism, development, differentiation and tumorigenesis[38-41].

Emerging evidence shows that other molecules, mainly ncRNAs, also play vital regulatory roles in the development of pancreatic and islet cells[17, 42, 43]. NcRNAs such as miRNAs, small nucleolar RNAs (snoRNAs) and lncRNAs, commonly described as which is not translated or not encodes for a protein, were initially thought to be without biological functions[44, 45]. Meanwhile, cell therapy is considered to be a potential therapeutic alternative for diabetes[46-48]. Islet or pancreatic-cells transplantation can restore glucose homeostasis by replenishing β cells[49, 50]. We adopted a three-step differentiation protocol imitating pancreatic development *in vivo* to find differentiation-associated lncRNAs during iPSC-induced β -like cell differentiation in this study[2]. Our data indicated that out of hundreds of differentially expressed lncRNAs we discovered using this protocol, lncRNA *MALAT1* might play an important role in β -like cells differentiation (Fig. 2). It could reduce *NKX6-1*⁺ cell count and regulate the expression of some key TFs, including *PDX1*, *NKX6-1*, *NGN3*, *GATA4*, *GATA6*, *NeuroD1* and *PAX6*, in the early stage of pancreatic development (Fig. 3). *PDX1*-expressing epithelial progenitors are essential to the development of the pancreas and intestines, giving rise to pancreatic endocrine, exocrine and ductal cells[51, 52]. *NGN3* is expressed in all endocrine progenitors, starting with the series of TFs whose expression regulates endocrine-cell differentiation and that includes *Nkx2.2*, *NeuroD1*, *NKX6-1*, *PAX4*, *PAX6* and *ISL*[53-56].

Our studies revealed that *MALAT1* likely functioned as a negative regulator in the early period of β -cell differentiation (Fig. 3). Then, bioinformatics prediction indicated that lncRNA *MALAT1* acted as a molecular sponge for miR-15b-5p via its 3'-UTR binding site and regulated the expression of *Ihh*, a direct downstream target (Fig. 4). *Ihh* is localized and expressed in the gut endoderm, which is important for epithelial differentiation[57]. Studies show that deletion of *Ihh* leads to formation of annular pancreas, suggesting that loss of *Ihh* from the gut endoderm around the pancreatic bud causes the pancreas to grow too large[58]. As expected, miR-15b-5p knockdown reduced the quantity and quality of β cells after 21 days' culture. Also, these polyhormonal insulin-expressing cells in the experimental group could not release enough insulin to respond to the stimulus of high-concentration glucose (Fig. 6). *MALAT1* functioned as a ceRNA to block miR-15b-5p and influenced expression of *Ihh* at the protein level *in vitro* (Fig. 7). This indicated that this cytoplasmic lncRNA (Fig. 5) might have a certain regulatory relationship with the *Hedgehog* signaling pathway, which is widely reported to negatively regulate *PDX1* during early pancreatic development. However, the concrete regulatory mechanism between these molecules needs more research. Our *in vivo* experiments showed that knockdown of lncRNA *MALAT1* was indispensable for differentiation of insulin-producing cells, although aberrant overexpression affected cell formation *in vitro* and differentiation *in vivo*.

Conclusion

We found that lncRNA *MALAT1*, a broadly studied lncRNA, played a vital role in the differentiation of iPSC-derived β -like cells, both *in vivo* and *in vitro*. *MALAT1* regulated the expression of a series of TFs indispensable for formation and development of the pancreas at the early stage and might affect expression of the *Hedgehog* signaling pathway in the differentiation process. These findings indicated that *MALAT1* acted as a molecular sponge to regulate the expression and function of *Ihh* by directly and competitively binding its 3'-UTR to miR-15b-5p, thereby mediating differentiation of iPSC-derived β -like cells. The current study therefore provides new evidence that ncRNAs, including lncRNAs and miRNAs, can serve as epigenetic targets for improving mature β -like cell differentiation efficiency and cell functions.

Abbreviations

MALAT1: metastasis associated lung adenocarcinoma transcript 1; PSCs: Pluripotent stem cells; ESCs: embryonic stem cells; iPSC: Induced Pluripotent Stem Cell; ncRNA: non-coding RNA; lncRNA: long non-coding RNA; cirRNA: circular RNA; *Ihh*: Indian hedgehog; EBCs: Embryonic body cells; MPCs: Multilineage precursor stem cells; RNA-seq: RNA-sequencing; miRNA: microRNA; DE miRNAs: differentially expressed miRNAs; qPCR: Quantitative Real-time Polymerase Chain Reaction; KEGG: Kyoto Encyclopedia of Genes and Genomes; GO: Gene Ontology; FDR: false discovery rate; NC: Negative Control; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; *Nkx6.1*: NK6 homeobox 1; *Pdx1*: pancreatic and duodenal homeobox 1; *Nkx2.2*: NK2 homeobox 2; *NGN3*: Neurogenin3; *Gata6*: GATA binding protein6; *Pax4*: Paired box 4; *Pax6*: Paired box6; *ISL*: ISL LIM homeobox; *Gck*: Glucokinase; *GCG*: Glucagon; *Glut2*: facilitated

glucose transporter, member2; SST: Somatostatin; Mafa: v-maf musculoaponeurotic fibrosarcoma oncogene family, protein A; KRB: Krebs-Ringer Bicarbonate buffer; FISH: Fluorescence in situ hybridization; UTR: Untranslated region; STZ: Streptozotocin.

Declarations

Acknowledgements

The authors thank Yan Huang Ph.D. and Yang Xu for their critical comments on the experimental design and text editing.

Funding

This work was supported by grants obtained from Medical Innovation Team Program of Jiangsu Province (no. CXTDA2017013), Science and technology project of Nantong City (no. MS12019058, JCZ18006), China Postdoctoral Science Foundation funded project (no. 2019M661904–2019M651929), Jiangsu Planned Projects for Postdoctoral Research Funds (no. 2019Z154).

Availability of data and materials

All data generated and/or analyzed during this study are contained in this published article.

Author's contributions

YW, XX and JL drafted the study, designed the experiments and monitored the project progression, the data analysis, the data interpretation and wrote the manuscript. JC, HW, JQ, BW and ZX performed animal experiments, collected and analyzed the data and helped wrote the manuscript. QS, ZW and SJ provided financial support, designed the research, carried out data analysis and wrote manuscript. All authors read and approved the final manuscript.

Ethics approval

All animal experiments were according to Institutional Animal Care guidelines and were approved by the Animal Ethics Committee of the Medical School of Nantong University (Protocol #20180228-004).

Consent for publication

All authors gave consent for publication.

Competing interests

The authors indicate no potential conflicts of interest.

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Figures

Figure 1

Overview of 3-step differentiation protocol. **a** Flow diagram of three-step differentiation protocol. EBs, Embryoid Bodies; MPs, Multilineage Progenitors. **b** Morphologies of differentiating iPSCs into β-like cells at 4 stages during differentiation. Scale bar: 20 μm. **c** Co-immunostaining of insulin with C-peptide and Co-immunostaining of Nkx6.1 with Pdx1 of β-like cells at step 3 on day 21. Scale bar: 75 μm. **d** Quantitative RT-PCR analysis for the expression level of several key transcription factors during

development of pancreatic β cells (insulin, PDX1, Nkx6.1, NGN3, GCG, SST, MaFA, ISL). GAPDH was used as the internal control. Error bars showed SEM (n = 3). P values are from (two-sided) dependent t-test (Both $P \leq 0.0001$ and $P = 0.0001$ were described as $P = 0.0001$).

Figure 2

Differentially expressed lncRNAs during iPSC-derived β -like cell differentiation. a Flow diagram exhibiting the RNA-sequencing for total RNA isolated from iPSC-derived β -like cells in 4 time points during the differentiation. (iPSCs, Early stage: EBs, Middle stage :MPs, late stage: β -like cells.) b Heatmap shows selected differentially expressed lncRNAs (fold change 2 and P value < 0.05). c Series Test of Cluster (STC), profiles 1,2,4 and 5 ($P < 0.05$) were carried out to screen for down-regulated lncRNAs during the differentiation. Differentially expressed pathways were analyzed by Gene Ontology analysis and d KEGG pathway enrichment analysis for target genes. The size of the bubbles represents the number of target genes associated with each pathway. e The regulatory network of lncRNA-target genes. Blue circles represent target genes and red squareness represents some differentially expressed lncRNAs.

Figure 3

Overexpression of lncRNA Malat1 suppresses differentiation of β -like cells. **a** Quantitative RT-PCR analysis for the expression level of lncRNA Malat1 during different stages of the differentiation. GAPDH was used as the internal control. Error bars showed SEM ($n = 3$). **b** Diagram for structural representation of lentivirus to overexpress lncRNA Malat1. **c** Flow cytometry for illustrating expression of insulin in populations of β -like cells. Black text indicates the percentage of insulin. **d** Co-immunostaining of Ngn3

with NeuroD1 and Nkx6.1 with Pdx1 of β -like cells. Scale bar: 75 μ m. e Quantitative RT-PCR analysis for the expression level of Ihh and some important transcription factors (Pdx1, Nkx6.1, Ngn3, Gata4, NeuroD1, Gata6, Pax6) of differentiation of pancreas at early stage. GAPDH was used as the internal control. Error bars showed SEM (n = 3). P values are from (two-sided) dependent t-test (Both $P \leq 0.0001$ and $P = 0.0001$ were described as $P = 0.0001$).

Figure 4

Predication of target genes of lncRNA Malat1. a Heatmap shows selected DEGs (differentially expressed genes). (fold change $\geq$ 2 and P value < 0.05). b A volcano plot of the DEGs between lncRNA Malat1

knockdown (Malat1 siRNA) and control (NC siRNA) β -like cells at stage MPCs. c KEGG pathway enrichment analysis for DEGs. d Quantitative RT-PCR analysis for the expression level of some DEGs (Mnx1, Itga1, Gata6 and Ihh). P values are from (two-sided) dependent t-test (Both $P \leq 0.0001$ and $P = 0.0001$ were described as $P = 0.0001$).

Figure 5

Subcellular localization of lncRNA Malat1. a Fluorescence in situ hybridization (FISH) of lncRNA Malat1, U6 and18S in MPCs. Scale bars, 75 μ m. b Distribution of lncRNA Malat1 in the cytoplasm and nucleus of MPCs.

Figure 6

Knockdown of miR-15b-5p impaired the differentiation of β -like cells. **a** Western blotting analysis for the effects of miR-15b-5p knockdown on the expression of Ihh protein. β -Actin was used as the loading control. **b** Predicted miR-15b-5p targeting sequence in 3'UTR of Ihh (Ihh 3'UTR-wt) and mutant form of Ihh 3'UTR constructed (Ihh 3'UTR-mut). **c** Dual-luciferase reporter assay for determination the influence of miR-15b-5p on Ihh 3'UTR-wt activity in iPSCs-derived β -like cells. Luciferase activity was analyzed and shown as mean $\pm$ s. e. m. $n = 3$. $**P < 0.01$, Student's t-test. **d** Co-immunostaining of Insulin with Nkx6.1 and Insulin with MaFA of β -like cells. Scale bar: 75 μ m. **e** Glucose-stimulated insulin secretion in vitro. The insulin concentration levels were determined after exposure of iPSCs-derived β -like cells on day 21 of 3 step to different glucose concentrations. (0, 5, 15, 30 mM) **f** Quantitative RT-PCR analysis for the expression level of some important markers and transcription factors (Pdx1, Nkx6.1, Insulin, ISL, MaFA, Glut2) of differentiation of pancreas at mature stage after being transfected with antagomir miR-15b-5p. GAPDH was used as the internal control. Error bars showed SEM ($n = 3$). P values are from (two-sided) dependent t-test (Both $P \leq 0.0001$ and $P = 0.0001$ were described as $P = 0.0001$).

Figure 8

lncRNA Malat1 overexpressed iPSCs-derived β like-cells were unable to reverse diabetes in vivo. a Diagram of transformation of sodium alginate hydrogel into calcium alginate hydrogel after being disposed with calcium chloride sodium. b Images of alginate hydrogel with different concentrations of calcium. c Fasting blood glucose levels post-transplantation. Data are presented as mean $\pm$ s. e. m. $n = 6$. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, Student's t-test. e Co-immunostaining of Insulin with Glucagon (GCG) and Insulin with Somatostatin (SST) of whole grafts. Scale bar: 75 μ m.