# LncRNA *Malat1* regulates iPSC-derived β-cell differentiation by targeting the miR-15b-5p/*Ihh* axis

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**Research**

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# Abstract

**Background:** Differentiation of induced pluripotent stem cell (iPSC)-derived β-like cells is a novel strategy for treatment of type 1 diabetes. Elucidation of the regulatory mechanisms of long noncoding RNAs (lncRNAs) in β-like cells derived from iPSCs is important for understanding the development of the pancreas and pancreatic β-cells and may improve the quality of β-like cells for stem cell therapy.

**Methods:** β-like cells were derived from iPSCs in a three-step protocol. RNA sequencing and bioinformatics analysis were carried out to screen the differentially expressed lncRNAs and identify the putative target genes separately. LncRNA Malat1 was chosen for further research. Series of loss and gain of functions experiments were performed to study the biological function of this lncRNA. Quantitative real-time PCR (qRT-PCR), Western blot analysis and immunofluorescence (IF) staining were carried out to separately detect the functions of pancreatic β-cells at the mRNA and protein levels. Cytoplasmic and nuclear RNA fractionation and fluorescence in situ hybridization (FISH) were used to determine the subcellar location of lncRNA Malat1 in β-like cells. Flow cytometry and enzyme-linked immunosorbent assays (ELISAs) were performed to examine the differentiation and insulin secretion of β-like cells after stimulation with different glucose concentrations. Structural interactions between lncRNA Malat1 and miR-15b-5p and between miR-15b-5p and Ihh were detected by dual luciferase reporter assays (LRAs).

**Results:** We found that the expression of lncRNA Malat1 declined during differentiation, and overexpression of this lncRNA notably 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 bioinformatics prediction, mechanistic analysis and downstream experiments.

**Conclusion:** This study established an unreported regulatory network of *lncRNA Malat1* and the miR-15b-5p/*Ihh* axis during the differentiation of iPSCs into β-like cells. In addition to acting as an oncogene promoting tumorigenesis, *lncRNA Malat1* may be an effective and novel target for treatment of diabetes 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 transplantation, methods involving β-cells derived from embryonic stem cells (ESCs)/induced pluripotent stem cells (iPSCs) are unlimited 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 signalling inhibitors and activators or gene reprogramming[6]. Although important progress has been made in differentiation protocols *in vitro*, the 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.

Noncoding ribonucleic acids (ncRNAs) are not translated or do not encode proteins through standard mechanisms and are considered nonfunctional RNAs[8]. As research has progressed, ncRNAs such as microRNAs (miRNAs), long noncoding RNAs (lncRNAs) and circular RNAs (circRNAs) have been found to participate in cellular signalling and regulation of various processes[9-11]. In recent years, as transcripts have been shown to exceed 200 bp, lncRNAs have been proven to control gene regulation, organ differentiation and both developmental and pathological processes[12-14]. Moreover, studies have indicated that lncRNAs participate in pancreatic and islet development, β-cell formation and function and T cell differentiation *in vitro*[15-18]. Given the above, identification of functional lncRNAs in the β-cell differentiation process and exploration of their mechanisms in detail are conducive to optimizing β-cell differentiation protocols and elucidating 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-cell differentiation *in vitro*. The lncRNA metastasis-associated lung adenocarcinoma transcript 1 (*Malat1*) was highly expressed in iPSCs and decreased over time during β-cell formation. Functional experiments and RNA-seq indicated that *Malat1* overexpression affected pancreatic progenitor differentiation by upregulating *Indian hedgehog* (*Ihh*), a *Hedgehog* pathway ligand with low reported levels in developing and mature pancreatic tissues[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 *Malat1* overexpression in β-cells could suppress the insulinogenic ability of the pancreas; this finding 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 the $GFP^+$ 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-seq and data analysis

Total RNA was isolated at four time points (miPSCs; 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).

RNA sequencing and data analysis were performed as previously described[21].

## LncRNA–mRNA network and Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses

We constructed a lncRNA–mRNA network to ensure interactions between lncRNAs and mRNAs based on the normalized signal intensity of specific expression in both types of RNA. After calculating Pearson's correlation coefficient (PCC), we built the network from significantly correlated pairs. Moreover, GO and KEGG analyses were applied to analyse the main functions of the 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). As described in our previous study[21, 22], first-strand complementary DNA (cDNA) of lncRNAs, mRNAs and miRNAs was synthesized, and their relative expression levels were calculated with the $2^{-\Delta\Delta Ct}$ method. As an internal normalized control, U6 was used for miRNAs and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used 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)

After all cells were harvested as single cells, we carried out FCM as described in detail in our previous study[21]. Cells were analysed using a BD LSRFortessa X-20 Flow Cytometer (BD Biosciences, Franklin Lakes, NJ, USA), and the results were analysed using FlowJo software (FlowJo LLC [BD Biosciences], Ashland, OR, USA). 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 to 24-well plates and cultured them for 12 h. After preincubation in Krebs–Ringer bicarbonate (KRB) buffer without glucose for 2 h, the cells were stimulated with KRB buffer 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 the insulin content using an ultrasensitive mouse insulin assay kit (Mercodia AB, Uppsala, Sweden) per the manufacturer's instructions.

## Immunofluorescence (IF) staining

iPSC-derived β-like cells cultured on glass coverslips and samples were fixed with 4% paraformaldehyde and then washed. IF staining of iPSC-derived β-like cells and samples was performed as described in detail in our previous study[22]. Images were acquired with a Leica DFC7000 T imaging system (Leica, Wetzlar, Germany). The 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) (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)

Western blotting was performed as described previously[21]. Primary and HRP-conjugated secondary antibodies (all from Abcam) were anti-*Ihh* 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 the manufacturer's instructions. Then, we analysed cytoplasmic and nuclear RNAs via qRT-PCR as described above.

### Fluorescence in situ hybridization (FISH)

This assay was performed with a FISH Kit (RiboBio, Guangzhou China) per the 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 lentiviruses overexpressing lncRNA *Malat1* and determined the lentivirus titre. On day 4 of step 3, we seeded iPSC-derived β-like cells in six-well culture dishes and infected them with lentivirus–cytomegalovirus (CMV)–lncRNA *Malat1* at a multiplicity of infection (MOI) of 20 or lentivirus–CMV–negative control at an MOI of 40. Seventy-two hours after the cells reached 40–50% confluence, we examined the lncRNA *Malat1* expression via qRT-PCR.

### Dual luciferase reporter assay (LRA)

We performed LRAs 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 cotransfected the 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 the manufacturer's instructions.

### *In vivo* implantation of alginate hydrogel

Alginate hydrogel was constructed as described in our previous study[23]. C57BL/6J mice were obtained from the animal centre 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 in alginate hydrogel. Next, we injected alginate hydrogel containing β-like cells ($1\times10^6$ cells/1 mL hydrogel) into the mouse renal capsules (n = 6). All operations were performed under sterile conditions, and the mice were sanitized using iodophor. All mice were carefully monitored and well cared for after the procedure.

### Statistical analysis

We analysed 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 ± standard error of the mean (SEM). $P < 0.05$ was considered statistically significant.

## Results

### LncRNA *Malat1* was downregulated during pancreatic β-cell differentiation

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

To select potentially important lncRNAs that might regulate iPSC-driven β-cell differentiation, we performed RNA-seq to identify 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 analyse mRNAs and lncRNAs. LncRNAs with a >2-fold difference in expression (FDR < 0.05) were selected (Fig. 2b). Of these, more than 100 lncRNAs were downregulated, exhibiting four coincident expression patterns in the series test of cluster (STC) analysis results: profiles 1, 2, 4 and 5 ($P < 0.05$) (Fig. 2c). After prescreening, we selected profile 2, which contained 33 transcripts with stable expression across all three stages, for further analysis. Coexpression networks of these lncRNAs and of hundreds of correlated mRNAs were determined; most of them had positive correlations (PCC > 0.99) (Fig. 2e). GO analyses of the mRNAs revealed that profile 2 lncRNAs might

participate in transcriptional regulation, cell proliferation and apoptosis, which are all crucial to stem cell differentiation (Fig. 2d). Interestingly, lncRNA *Malat1* was identified and was downregulated at all four time points, as confirmed 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 the transformation of the stem cell state to a differentiation state. To verify this hypothesis, 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 h of transfection, *NKX6-1*$^{+}$ pancreatic endocrine progenitor cell formation underwent a substantial decrease, from 21.4±0.35% to 11.2±0.56% (n=3) (Fig. 3e). qRT-PCR analysis revealed that *Malat1* overexpression suppressed the transcription of some key regulators of endocrine progenitors, including *Pdx1*, *Nkx6-1*, *Ngn3*, *Gata4/6*, *NeuroD1*, and paired box protein 6 (*Pax6*) (Fig. 3f). Interestingly, the *Hedgehog* family member *Ihh* showed noteworthy upregulation compared with the abovementioned transcription factors (TFs). In addition, IF staining of *Pdx1*, *Nkx6-1*, *Ngn3* and *NeuroD1* indicated that the *Malat1*-transfected cells showed a defect in pancreatic endocrine-lineage formation (Fig. 3c, d). The abovementioned 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 target gene prediction

To determine how *Malat1* regulated β-cell differentiation *in vitro*, we performed mRNA-seq to identify the target genes of *Malat1*. We constructed a *Malat1*-targeting small interfering RNA (siRNA) for transfection of 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 consistent 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 the mRNA-seq data (Fig. 4d). KEGG analysis of the 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* signalling pathways, as well as cell adhesion molecules (CAMs) (Fig. 4c). The mRNA-seq results suggested that silencing *Malat1* might negatively regulate *Ihh*, which would in turn increase the 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 address this issue, we first determined the subcellular location of *Malat1*. FISH and cell fractionation analysis both indicated that lncRNA *Malat1* was mostly located in the cytoplasm (Fig. 5a, 5b), indicating it may regulate *Ihh* expression via a ceRNA network. In addition, bioinformatics prediction analysis showed that lncRNA *Malat1* had a miRNA-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's disease and other pathological and physiological processes[24-27] but not in β-cell differentiation. Therefore, we wondered whether miR-15b-5p could regulate β-cell formation and whether *Ihh* was its downstream target. To validate the relationship between miR-15b-5p and *Ihh*, we used Western blotting to detect the protein level of *Ihh*. The 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 mutant (mut) vectors of the 3'-UTR of *Ihh* (Fig. 6b). LRA results revealed that only the wt 3'-UTR of *Ihh* showed a significant response to miR-15b-5p, displaying a low level of translation after miR-15b-5p overexpression (Fig. 6c). Therefore, our results showed that miR-15b-5p may 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 aimed to elucidated its role in β-cell differentiation *in vitro*. Using *Ihh* as a negative control (NC), we downregulated miR-15b-5p expression at the MPC stage using antagomir and cultured the cells until day 21. The qRT-PCR results 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*), indicating abnormal β-cell maturation (Fig. 6g). Moreover, regardless of the glucose concentration, miR-15b-5p deficiency impaired insulin production and secretion of β-cells (Fig. 6f). In addition, IF staining proved that aberrant low expression of miR-15b-5p weakened the protein levels of *INS*, *Nkx6-1* and *MafA* in β-cells (Fig. 6d, e). 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), suggesting a possible interaction between these two molecules. Then, we constructed a luciferase vector of *Malat1* (Luc–*Malat1*–wt) and a mutated form (Luc–*Malat1*–mut) (Fig. 7a). The results indicated that miR-15b-5p could effectively suppress the luciferase activity of *Malat1* but not of mutated *Malat1*, indicating that the predicted binding sites of *Malat1* is likely to bind to miR-15b-5p specifically (Fig. 7b). However, more research will be needed to clarify whether Ihh is direct target gene of Malat1 during the differentiation.

In addition to structural interactions between lncRNA Malat1 and Ihh, *Malat1* regulation of *Ihh* expression at the protein level needs further validation. As shown by the WB results, *Malat1* deficiency led to downregulation of *Ihh* (Fig. 7c). Moreover, *Malat1* overexpression upregulated the expression of *Ihh* (Fig. 7d). In addition, lncRNA *Malat1* neutralized the inhibitory effect of miR-15b-5p on *Ihh* expression (Fig. 7e). The qRT-PCR results demonstrated that knockdown of *Malat1* led to downregulation of some important TFs involved in differentiation of pancreas, such as *NKX6-1*, *Pdx1*, *Ngn3*, *Pax6*, *NeuroD1*, *Gata6* (Fig.7f).

Rescue experiments indicated that downregulation of *Ihh* expression reversed the negative impact of *Malat1* overexpression on β-cell differentiation (Fig. 7g), indicating 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 glycaemic control of β-cells *in vivo*

To further investigate the influence of high expression of *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[23]. Next, we harvested approximately $1\times10^6$ β-cells from the *Malat1* overexpression and NC groups and transplanted them into STZ-induced diabetic mice (n = 6). Twenty-four days post-transplantation, the fasting blood glucose of the mice with transplanted NC cells gradually returned to baseline (Fig. 8d). In contrast, differentiated cells with high expression of *Malat1* could not radically reverse hyperglycaemia.

Furthermore, 40 days after transplantation, we harvested mouse kidneys with transplanted cells and stained them using IF. The results indicated that the cells overexpressing *Malat1* showed worse maturation *in vivo* after transplantation than normal β-cells, with lower protein levels of *C-peptide Mafa* and *Nkx6.1*, three key markers of pancreatic endocrine cells (Fig. 8c). Therefore, the abovementioned experiments showed that the gradual downregulation of lncRNA *Malat1* was important to β-cell differentiation, while aberrant overexpression affected cell formation *in vitro* and redifferentiation *in vivo*.

## Discussion

PSCs, from which neurons, cardiomyocytes, haematopoietic cells and β-cells can be differentiated *in vitro* and *in vivo*, show promise in regenerative medicine[28-34]. 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 the use of immunosuppressants[35]. As reported, the differentiation of iPSCs into all kinds of somatic cells is influenced by numerous factors, such as extracellular matrix (ECM), TFs, cell signal transduction, ncRNAs and other complex biological processes[36-39]. LncRNAs, a hot area of research in epigenetic regulation, participate in numerous biological and cellular processes, including chromatin remodelling, transcription, metabolism, development, differentiation and tumorigenesis[40-43].

Emerging evidence shows that other molecules, mainly ncRNAs, also play vital regulatory roles in the development of pancreatic and islet cells[17, 44, 45]. NcRNAs such as miRNAs, small nucleolar RNAs (snoRNAs) and lncRNAs, which are not translated or do not encode a protein, were initially thought to lack biological functions[46, 47]. Moreover, cell therapy is considered as a potential therapeutic alternative for diabetes[48-50]. Islet or pancreatic cell transplantation can restore glucose homeostasis by replenishing β-cells[51, 52]. We adopted a three-step differentiation protocol imitating pancreatic development *in vivo* to identify differentiation-associated lncRNAs during iPSC-induced β-like cell differentiation in this study[2]. Our data indicated that among hundreds of differentially expressed lncRNAs we discovered using this protocol, lncRNA *Malat1* might play an important role in β-like cell differentiation (Fig. 2). This molecule could reduce the *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[53, 54]. *NGN3* is expressed in all endocrine progenitors, starting with a series of TFs whose expression regulates endocrine cell differentiation, including *Nkx2.2*, *NeuroD1*, *NKX6-1*, *PAX4*, *PAX6* and *ISL*[55-58].

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[59]. Studies have shown that deletion of *Ihh* leads to the formation of annular pancreas, suggesting that loss of *Ihh* from the gut endoderm around the pancreatic bud causes excessive growth of the pancreas[60]. As expected, miR-15b-5p knockdown reduced the quantity and quality of β-cells after 21 days of culture. Additionally, these polyhormonal insulin-expressing cells in the experimental group could not release enough insulin to respond to high-concentration glucose (Fig. 6). *Malat1* functioned as a ceRNA to block miR-15b-5p and influenced the expression of *Ihh* at the protein level *in vitro* (Fig. 7). This finding indicated that this cytoplasmic lncRNA (Fig. 5) might have a regulatory relationship with the *Hedgehog* signalling 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 lncRNA *Malat1* knockdown was indispensable for the 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 the formation and development of the pancreas at the early stage and might affect the expression of the *Hedgehog* signalling pathway during differentiation. These findings indicated that *Malat1* acted as a molecular sponge to regulate the expression and function of *Ihh* by directly and

competitively binding to miR-15b-5p via its 3'-UTR, thereby mediating the 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: noncoding RNA; lncRNA: long noncoding RNA; circRNA: circular RNA; Ihh: Indian hedgehog; EBCs: embryonic body cells; MPCs: multilineage precursor stem cells; RNA-seq: RNA-sequencing; miRNA: microRNA; DEmiRNAs: 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


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### Availability of data and materials

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

### Author's contributions

YW, XX and JL drafted the study, designed the experiments, monitored the project progression, analysed the data, interpreted the data and wrote the manuscript. JC, HW, JQ, BW and ZX performed the animal experiments, collected and analysed the data and helped write the manuscript. QS, ZW and SJ provided

financial support, designed the research, carried out data analysis and wrote the manuscript. All authors read and approved the final manuscript.

### Ethics approval

All animal experiments were performed 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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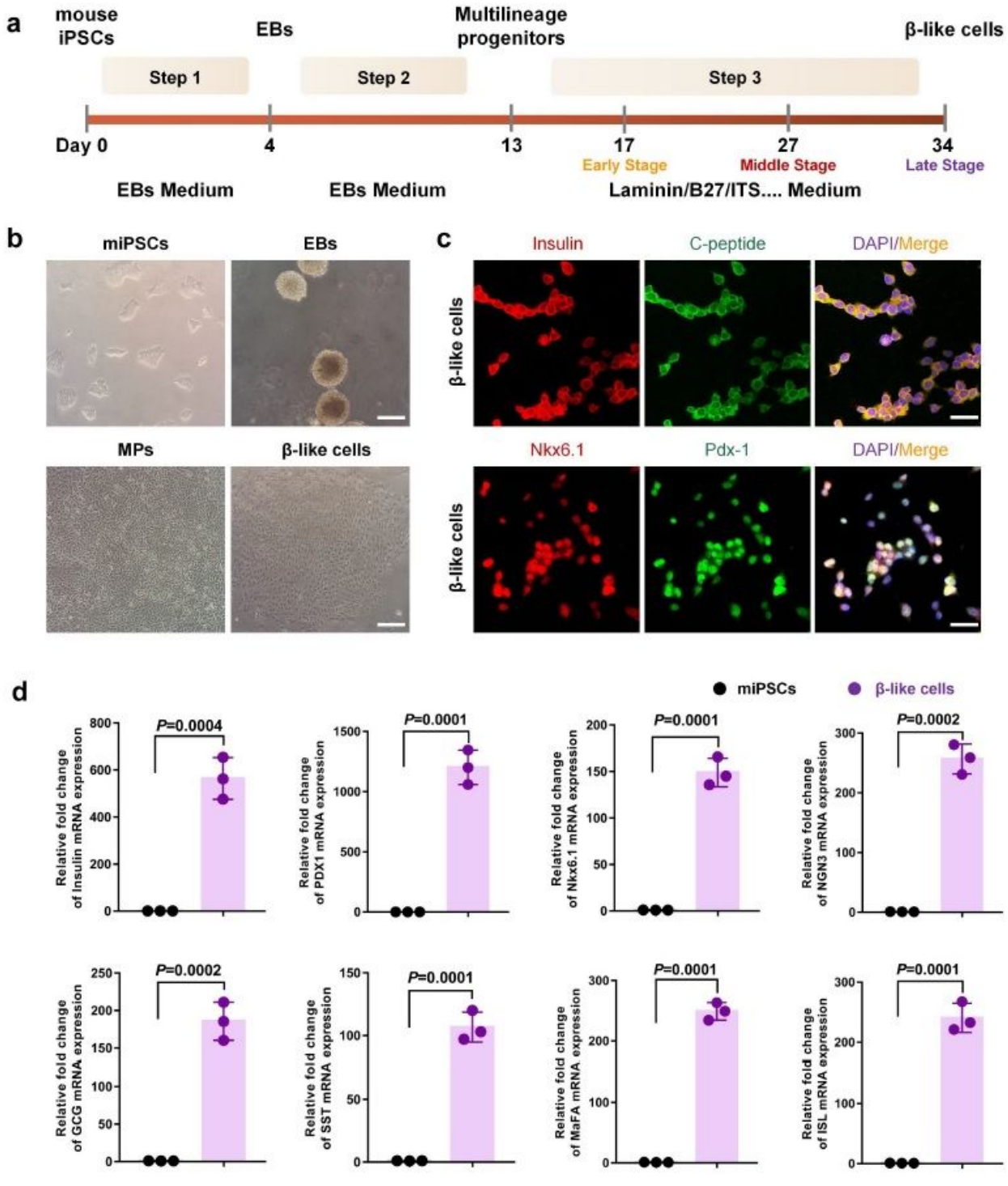
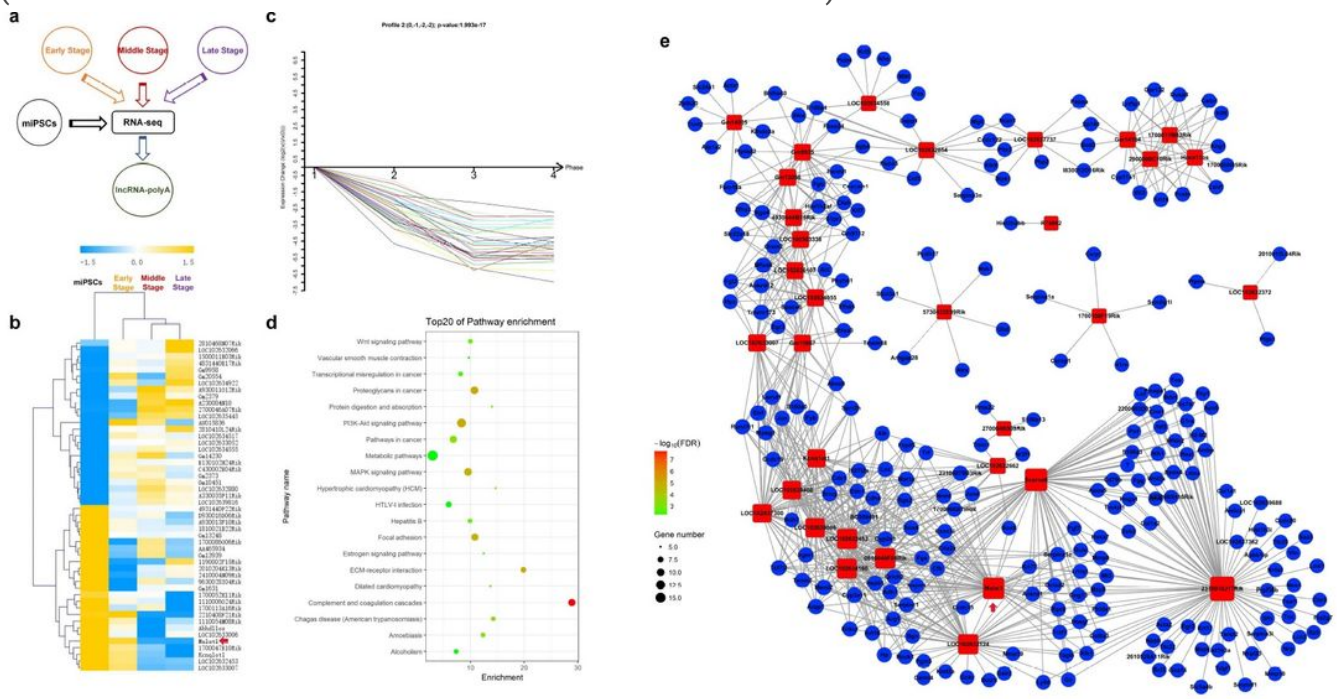
# Figures




## Figure 1

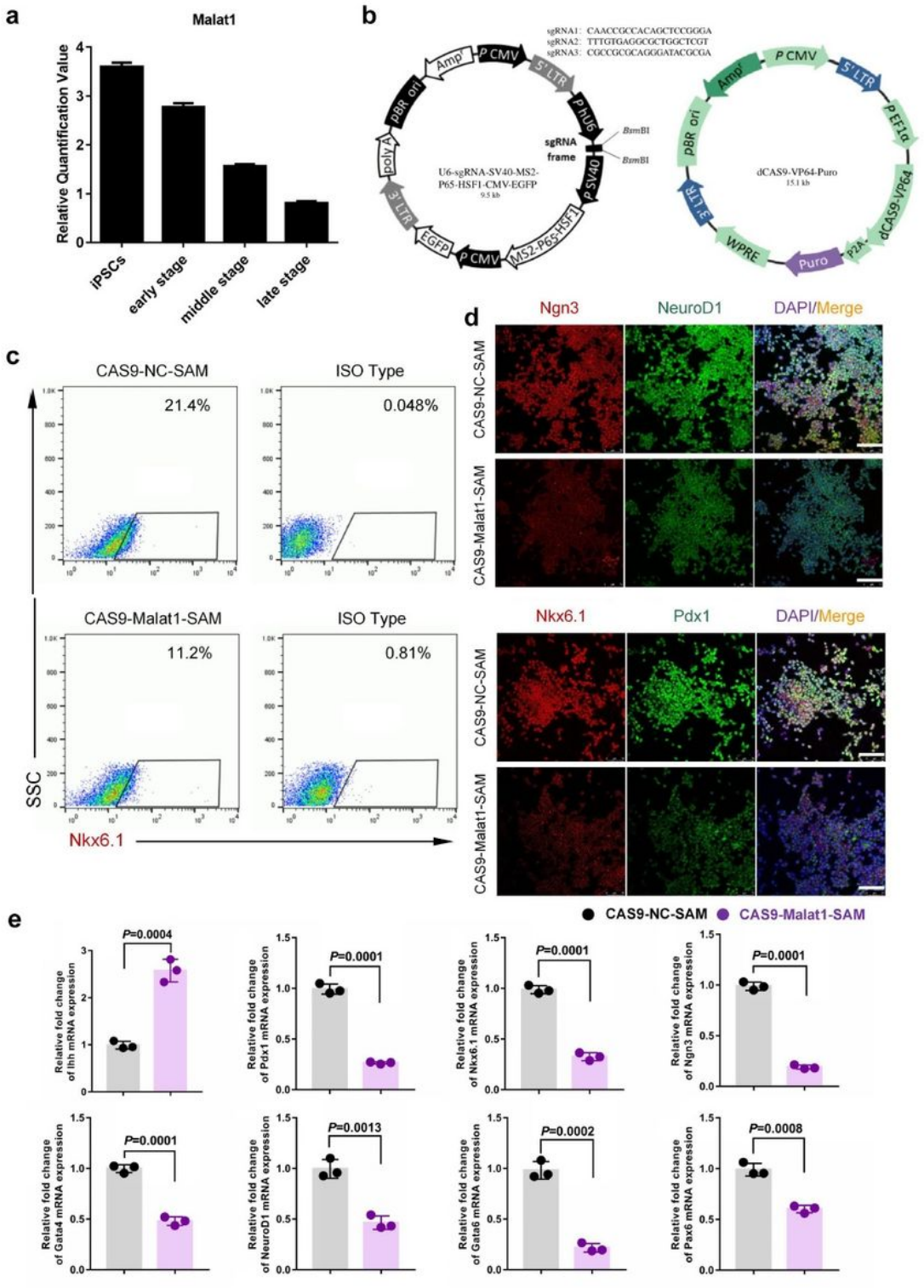
Overview of 3-step differentiation protocol. a Flow diagram of three-step differentiation protocol. EBs, Embryoid Bodies; MPs, Multilineage Progenitors. b Morphologies of differentiating iPSCs into β-like cells at 4 stages during differentiation. Scale bar: 20 μm. c Co-immunostaining of insulin with 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□0.0001 and P=0.0001 were described as P=0.0001).




## Figure 2

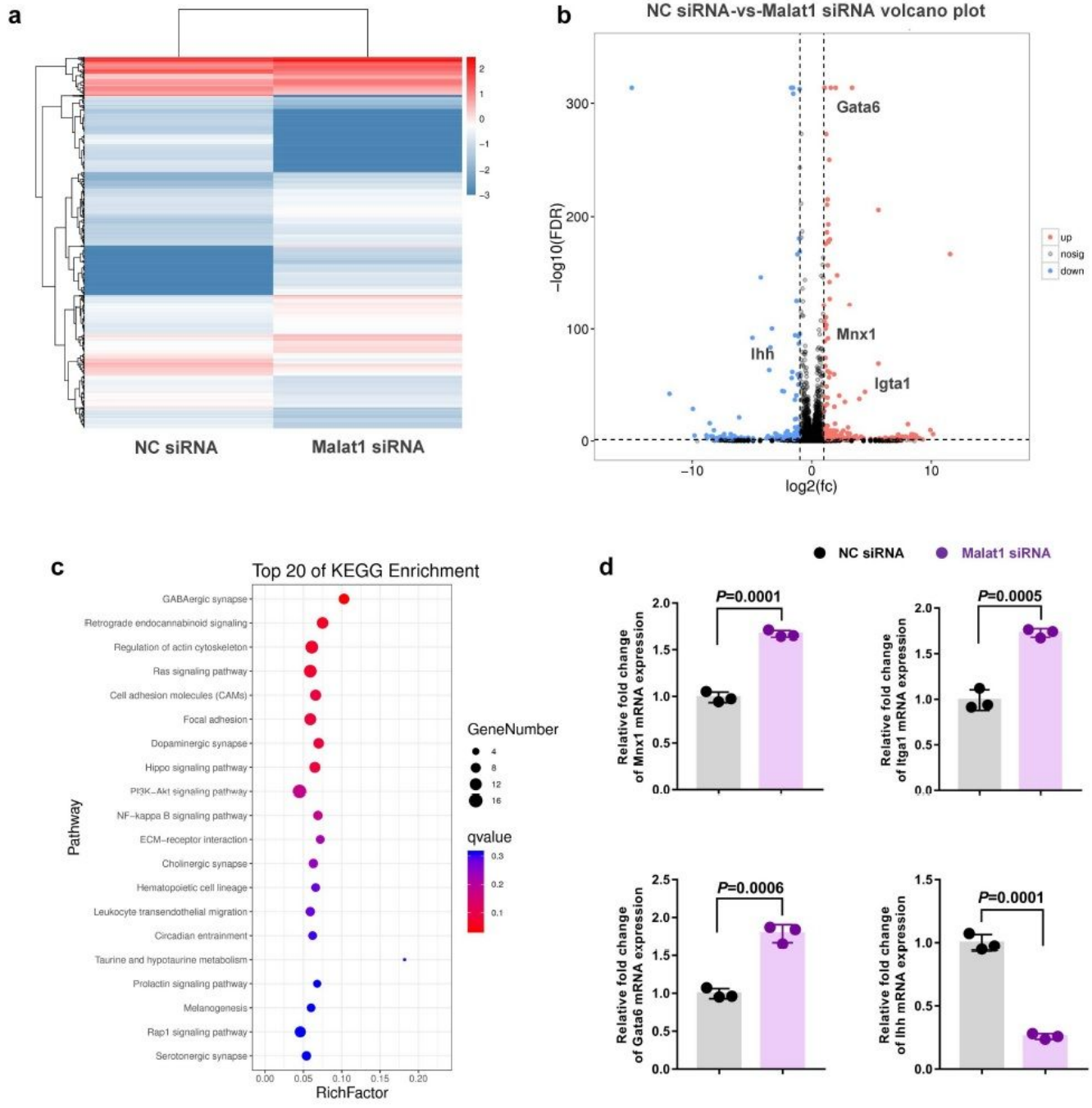
Differentially expressed lncRNAs during iPSC-derived β-like cell differentiation. a Flow diagram exhibiting the RNA-sequencing for total RNA isolated from iPSC-derived β-like cells in 4 time points during the differentiation. (iPSCs, Early stage: EBs, Middle stage :MPs, late stage:β-like cells.) b Heatmap 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

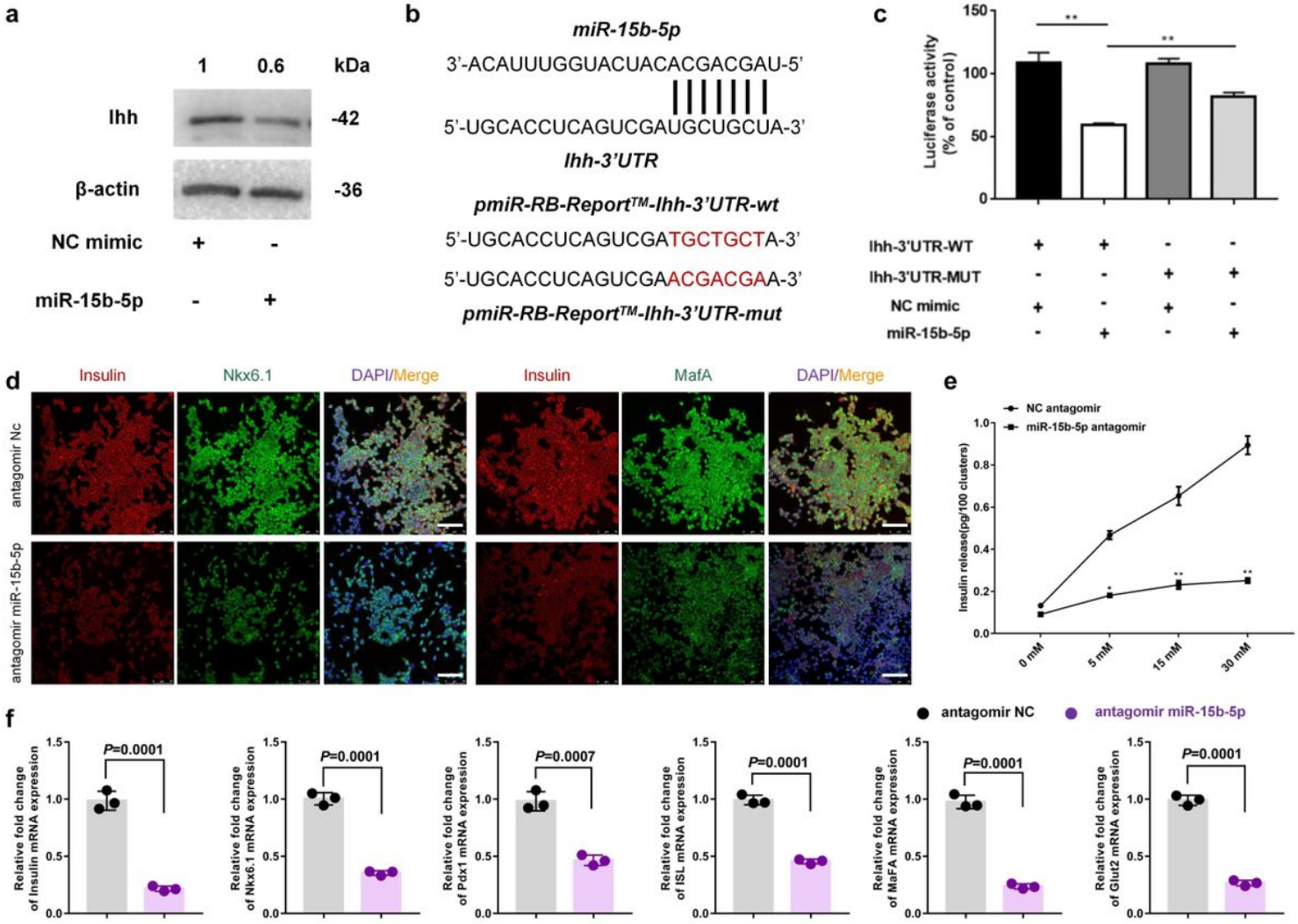
with NeuroD1 and Nkx6.1 with Pdx1 of β-like cells. Scale bar: 75 μm. e Quantitative RT-PCR analysis for the expression level of Ihh and some important transcription factors (Pdx1, Nkx6.1, Ngn3, Gata4, NeuroD1, Gata6, Pax6) of differentiation of pancreas at early stage. GAPDH was used as the internal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




## Figure 4

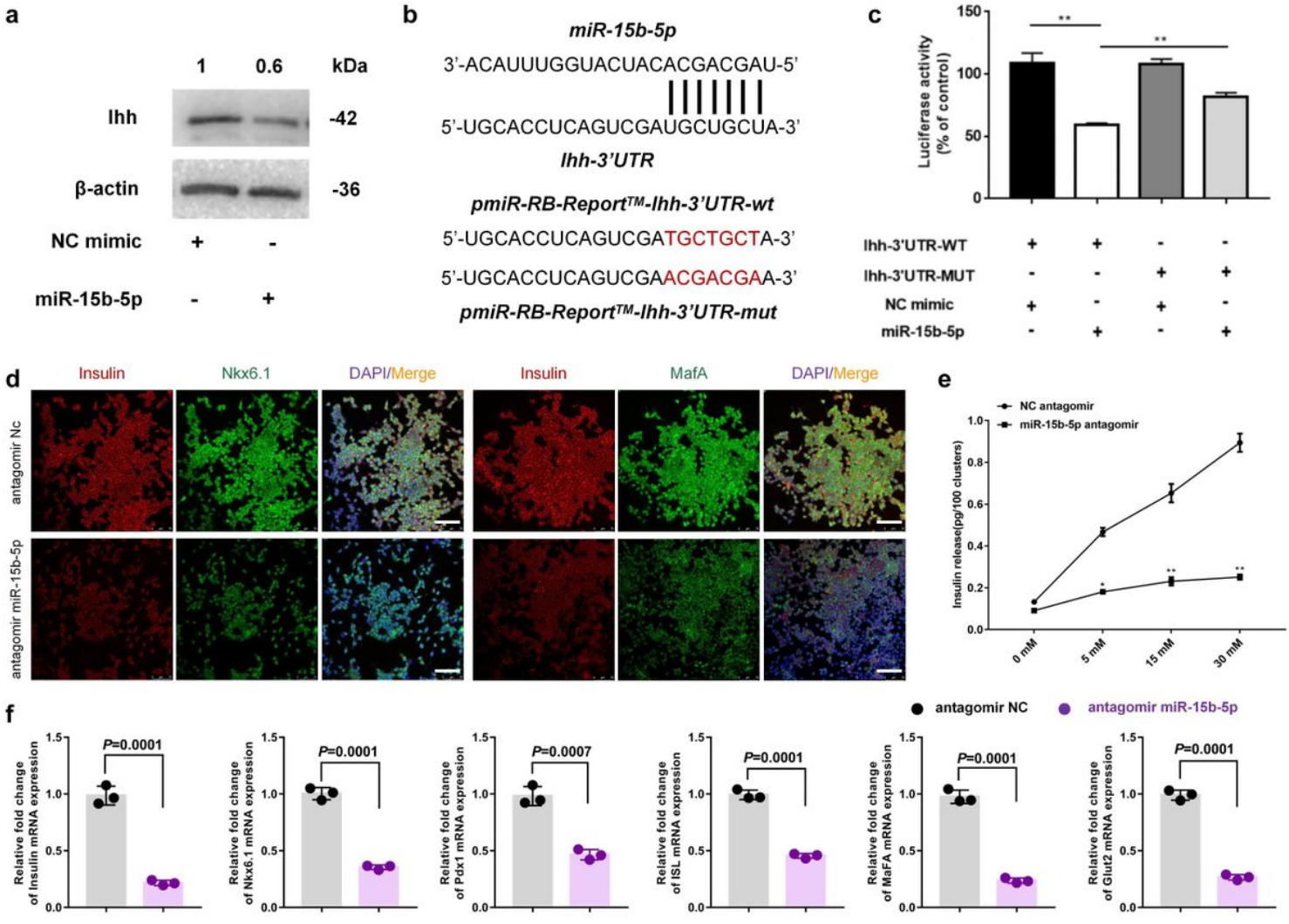
Predication of target genes of lncRNA Malat1. a Heatmap shows selected DEGs (differentially expressed genes). (fold change≥2 and P value < 0.05). b A volcano plot of the DEGs between lncRNA Malat1

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□0.0001 and P=0.0001 were described as P=0.0001).



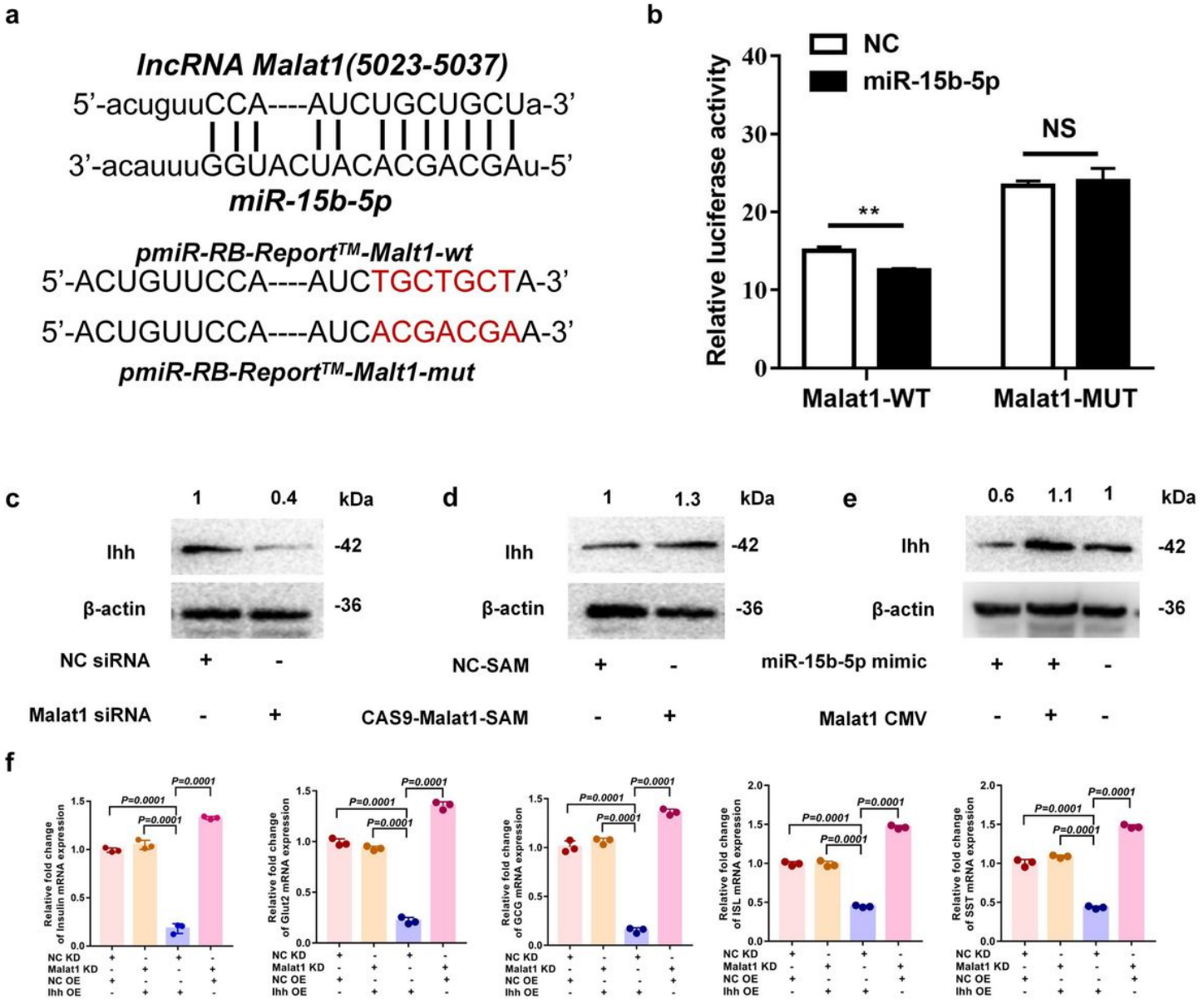

## Figure 5

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



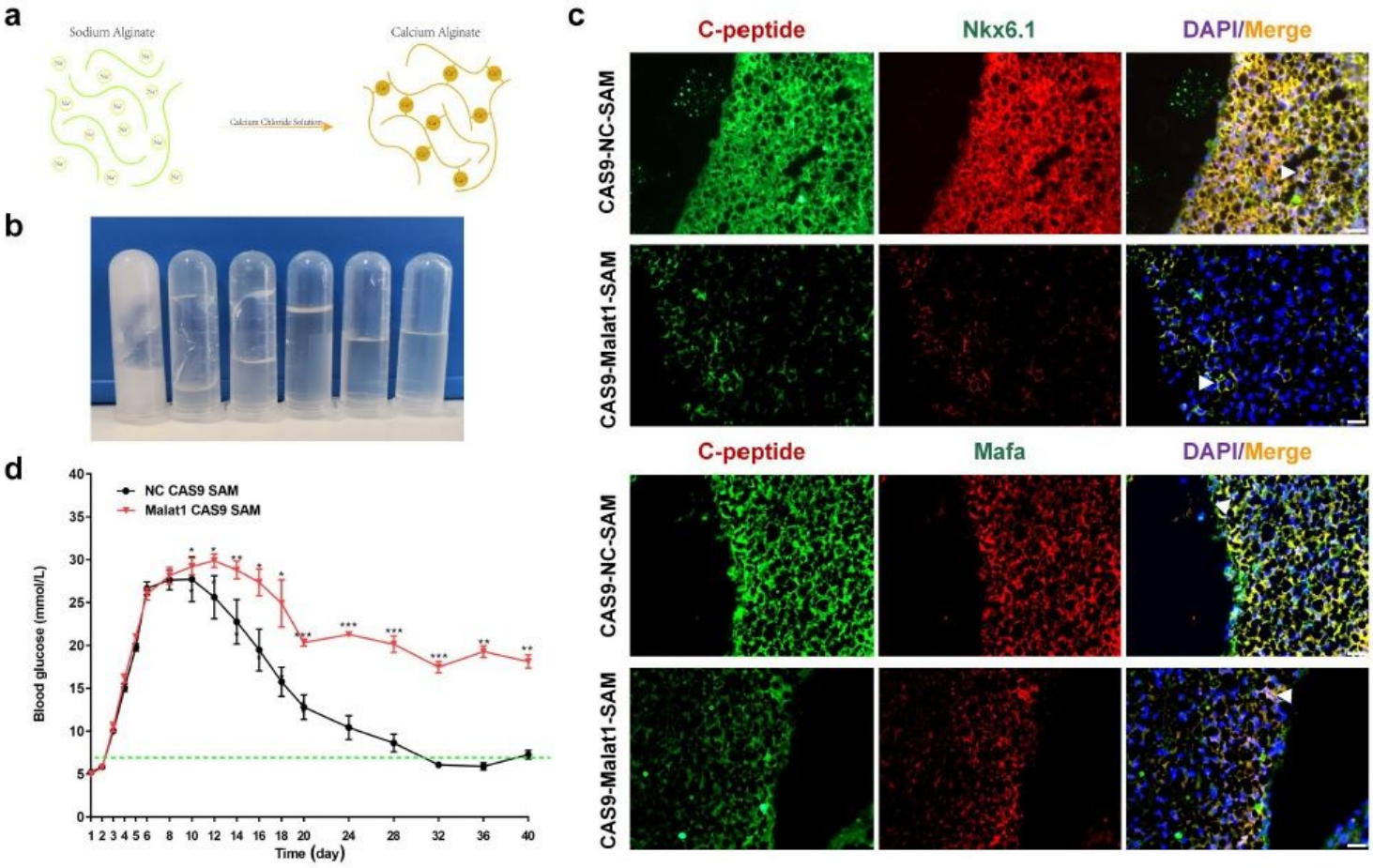

## Figure 6

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 ± 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 μm. 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□0.0001 and P=0.0001 were described as P=0.0001).




## Figure 7

lncRNA Malat1 regulated expression of Ihh by competitively binding miR-15b-5p possibly. a Predicted miR-15b-5p targeting sequence in 3'UTR of lncRNA Malat1 (lncRNA Malat1 3'UTR-wt) and mutant form of lncRNA Malat1 3'UTR constructed (lncRNA Malat1 3'UTR-mut). b Dual-luciferase reporter assay for determination the influence of miR-15b-5p on lncRNA Malat1 3'UTR-wt activity in iPSCs-derived β-like cells. Luciferase activity was analyzed and shown as mean ± s. e.m. n = 3. **P < 0.01, Student's t-test. c-e Western blotting analysis for the effects of lncRNA Malat1 knockdown, lncRNA Malat1 overexpression and miR-15b-5p and lncRNA Malat1 overexpression on the expression of Ihh protein. β-Actin was used as the loading control. f Quantitative RT-PCR analysis for the expression level of some important markers and transcription factors (Pdx1, Nkx6.1, Insulin, ISL, MaFA, Glut2) of pancreatic islet functional genes and key transcription factors. GAPDH was used as the internal control. Error bars showed SEM (n = 3). P values are from (two-sided) dependent t-test (Both P□0.0001 and P=0.0001 were described as P=0.0001).




## Figure 8

LncRNA Malat1-overexpressing iPSC-derived β-like cells were unable to reverse diabetes in vivo. a Diagram of the transformation of sodium alginate hydrogel into calcium alginate hydrogel after incubation with calcium chloride sodium. b Images of alginate hydrogel with different concentrations of calcium. c Coimmunostaining of C-peptide with Nkx6.1 and C-peptide with Mafa of whole grafts. Scale bar: 50 μm. d Fasting blood glucose levels post-transplantation. Data are presented as the mean ± s.e.m. n =6. *P < 0.05, **P < 0.01, ***P < 0.001, Student's t-test.