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miRNA-30e regulates abnormal differentiation of small intestinal epithelial cells in diabetic mice by downregulating Dll4 expression

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Abstract

Objectives: Depression of the Notch/Hes1 pathway has been reported to play a role in abnormal differentiation of intestinal epithelial cells (IECs) in diabetes mellitus (DM). However, the mechanism by which this pathway influences IEC differentiation has remained unclear. In this study, we have investigated the role of microRNAs (miRNAs) in regulating the Notch/Hes1 pathway in IECs of DM mice.

Materials and methods: Integrated comparative miRNA microarray technology was used to determine the expression profile of miRNAs in IECs of DM mice. After bioinformatic analysis, an miRNA with altered expression levels, miRNA-30e, was identified as a candidate for regulating the Notch pathway in DM. A luciferase reporter assay confirmed that miRNA-30e targeted 3′-UTR of the Notch gene. The role of miRNA-30e in regulating Notch signalling was then explored by up- and downregulating its expression *in vitro* and *in vivo*.

Results: Abnormal differentiation of IECs in DM mice was associated with reduced activity of the Dll4/NICD/Hes1 signal pathway. Based on bioinformatic analyses, increased expression of miRNA-30e was identified as a potential candidate for regulating Notch signalling. miRNA-30e targeted the 3′-UTR of Dll4 and downregulated Dll4 expression in primary IECs and IEC-6 cells. Exogenous miRNA-30e reduced activity of the Dll4/NICD/Hes1 pathway, and induced abnormal differentiation of IECs in normal mice. Conversely, treatment with miRNA-30e antagonist upregulated activity of the Dll4/NICD/Hes1 pathway *in vivo*, and normalized IEC differentiation in DM mice.

Conclusions: Increased levels of miRNA-30e downregulated activity of the Dll4/NICD/Hes1 signalling pathway by targeting the 3′-UTR of Dll4, which contributed to abnormal differentiation in small intestinal epithelia of DM mice.

Abbreviations

miRNA: microRNA

mRNA: messenger RNA

DM: diabetes mellitus

IEC: intestinal epithelial cell

IESC: intestinal epithelial stem cell

NICD: notch intracellular domain

Hes1: hairy and enhancer of split 1

STZ: streptozocin

QPCR: quantitative polymerase chain reaction

ATCC: American type culture collection

DTT: DL-dithiothreitol

HBSS: Hanks' buffered saline solution

3′-UTR: 3′-untranslated region

Dll4: delta-like 4

SI: sucrase-isomaltase

ChgA: chromogranin A

Tff3: trefoil factor 3

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Lyz1: lysozyme 1

Dll1: delta-like 1

Jag1: jagged 1

Jag2: jagged 2

DAB: 3, 3′-diaminobenzidine tetrahydrochloride

Introduction

microRNAs (miRNAs) are short, non-coding RNAs of 19-23 nucleotides, that modulate post-transcriptional protein-decoding genes by repressing translation of mRNA, or by promoting their degradation. Each miRNA can affect multiple target genes. miRNAs have been extensively studied for their roles in intracellular mechanisms such as differentiation, proliferation and apoptosis, in a wide range of eukaryotic organisms (1,2). They are a potential key to understanding the pathogenesis of chronic multifactorial diseases, such as cardiovascular disease, diabetes mellitus, obesity and cancer (3–5).

Diabetes mellitus (DM) is a chronic metabolic syndrome caused by factors relating to insulin deficiency (6). DM patients have higher risk of developing cardiovascular disease, stroke and cancers (7–9). Syndromes of DM, such as hyperinsulinaemia, activation of insulin-like growth factor receptors and hyperglycaemia, can activate mitogenic pathways (10). A common complication of DM is diabetic enteropathy and previous studies on this have focused on changes of gastrointestinal motility caused by hyperglycaemia (11). The mechanisms behind these changes involve pathological alterations to intestinal smooth muscle cells, interstitial cells of Cajal and the enteric nervous system (11). Previous studies have revealed significant changes to intestinal epithelial cells (IECs) in DM rats (12,13). Recently, researchers have found that changes leading to colorectal cancer may be closely related to DM (14,15), supporting the hypothesis that there is a relationship between DM and abnormal differentiation of IECs.

The small intestinal epithelium is composed very largely of absorptive cells, endocrine cells, goblet cells and Paneth cells (16). All IECs are derived from intestinal epithelial stem cells (IESCs) located at the bases of the crypts. Under physiological conditions, differentiation of IESCs is maintained in a dynamic balance in order to maintain integrity and physiological function of the intestinal epithelium (16–19). Notch signalling plays distinct roles in controlling cell lineage specification within the crypt compartment (20). Early Notch loss-of-function studies have shown that Notch inhibition results in goblet cell hyperplasia, while more recent research has revealed that Notch activation during intestinal development leads to amplification of IESCs (21–24). We have previously reported that abnormal differentiation of intestinal epithelium and intestinal barrier dysfunction in DM mice was associated with reduced Notch/Notch intracellular domain (NICD) transduction in Notch/hairy and enhancer of split 1 (Hes1) signalling (25); the reason for depression of the Notch/NICD/Hes1 signalling pathway in DM mice has remained unclear.

In this study, we investigated miRNA expression by microarray and bioinformatic analysis of IECs in DM mice, to identify candidate miRNAs associated with abnormal IEC differentiation, to characterize a mechanism for the depression of Notch/NICD/Hes1 signalling pathway activity.

Materials and methods

Streptozocin (STZ)-induced DM mice model

All experimental procedures were performed in accordance with animal protocols approved by the Animal Care Committee of Sun Yat-Sen University. Eight-week-old C57BL/6J mice HOW MANY? (Laboratory of Animal Center in Sun Yat-Sen University, Guangzhou, China) were housed individually in sterile microisolators for the duration of the experiment. Diabetes was induced by daily IP injection of STZ (Sigma, Saint Louis, MO, USA; 70 mg/kg) for 5 days (25,26); mice in the control group received IP injections of citrate buffer alone. Only mice with blood glucose levels above 16.7 mM were considered to have developed DM (26). Ten weeks after STZ administration, all mice were sacrificed. Small intestines were carefully removed, and proximal jejuna were flushed with 0.1 M PBS (pH 7.4) and used for isolation of primary IECs.

miRNA microarray and data analysis

Total miRNA was extracted from cultured cells and intestinal tissues using an miRNA isolation kit (TAKARA, Otsu, Shiga, Japan), and labelled with a miRCURY™ Hy3™/Hy5™ Power labelling kit (Exiqon, Vedbaek, Denmark) according to the manufacturers' guidelines. After labelling, samples were hybridized on a miRCURYTM LNA Array (v.18.0; Exiqon) according to the manufacturer's instructions, and bioinformatics analysis and visualization of microarray data were performed using MEV software (v4.6; TIGR, La Jolla, CA, USA)

miRNA extraction and real-time quantitative polymerase chain reaction (QPCR) detection for miRNA-30e

After isolating miRNA as above, cDNA was synthesized using an miRNA reverse transcription kit (TAKARA). Expression of miRNA-30e was quantified with a specific MiRNA assay kit (TAKARA) according to the manufacturer's protocol. Real-time QPCR was performed on a CFX-96 system (Bio-Rad, Hercules, CA, USA). Primer sequences are shown in Table S1. Data were analysed using the ΔΔCt method with U6 snRNA as the constitutive marker (27).

Cell culture

IEC-6 and 293T cell lines were purchased from ATCC (Manassas, VA, USA). IEC-6 cells were cultured in DMEM (Gibco, Grand Island, NY, USA), supplemented with 10% FBS (Gibco), 1% L-glutamine (Gibco), 0.01 mg/mL insulin (Sigma-Aldrich, St. Louis, MO, USA), 100 U/ml penicillin (Gibco) and 100 μg/ml streptomycin (Life Technologies, Grand Island, NY, USA). 293T cells were cultured in DMEM containing 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. All cells were incubated at 37 °C in 5% CO_2.

Primary IECs were isolated from the small intestines and cultured in Matrigel as described previously (28). Briefly, intestines were cut into 1 cm pieces and immersed in PBS with 30 mM EDTA (Beyotime, Shanghai, China) and 1.5 mM DTT (Beyotime) on ice for 20 min. Dissociated crypts and villi were pelleted by centrifugation at 2500 rpm for 5 min, and were resuspended in HBSS (Sigma-Aldrich) with 0.3 U/ml dispase. After treatment with 10% FBS and 100 μg DNaseI (Sigma-Aldrich), crypts were released by vigorous shaking.

Resuspended crypts were cultured in 50 μl Matrigel (BD Biosciences, San Jose, CA, USA) dissolved in advanced DMEM/F12 (Life Technologies) supplemented with 2 mM GlutaMax (Life Technologies), 50 ng/ml EGF (R&D Systems, Minneapolis, MN, USA), 5 ng/ml Wnt3a (R&D Systems), 1 μg/ml R-spondin 1 (R&D Systems) and 100 ng/ml noggin (PeproTech, Rocky Hill, USA). After polymerizing the solution at 37 °C for 10 min, 0.5 ml culture medium was added into each well and cultures were maintained at 37 °C in 5% CO_2.

Dual-luciferase reporter plasmid transfection and target analysis for 3′-untranslated region (3′-UTR) of mouse delta-like 4 (Dll4)

The 3′-UTR of mouse Dll4 was amplified from genomic DNA of NIH 3T3 cells and cloned into the pmiRRB-REPORT™ plasmid (Dll4-3′-UTR-wnt; RiboBio, Guangzhou, China). A plasmid containing the 3′-UTR sequence with a mutation in the miRNA-30e binding site was used as a negative control (Dll4-3′-UTR-mut; RiboBio). Mutations were introduced using the KOD-plus mutagenesis kit (Toyobo, Osaka, Japan). Firefly and Renilla luciferase activities were measured using the Dual-Luciferase Reporter Assay system (Promega, Madison, WI, USA). Sequences of primers used are given in Table S1. All primers were synthesized by RiboBio.

To investigate effects of miRNA-30e on Dll4 expression, miRNA-30e mimics (200 ng/μl, GenePharma, Shanghai, China) were cotransfected with Dll4-3′-UTR-wnt plasmid or Dll4-3′-UTR-mut plasmid into IEC-6, 293T and IECs using Lipofectamine 2000 (Life Technologies). Dll4-3′-UTR-wnt plasmid (200 ng/μl) was transfected separately into IEC-6, 293T and primary IECs as the control group. The luciferase reporter assay was performed as above. Data were then analysed with SpectraMax M5 (Molecular Devise, Sunnyvale, CA, USA).

Up- and downregulating expression of miRNA-30e

To investigate the relationship between miRNA-30e and Dll4, expression of miRNA-30 was up- and downregulated *in vitro* using oligonucleotides (miRNA mimic and inhibitor) designed and synthesized by GenePharma. The miRNA mimic (agomiRNA-30e) and inhibitor (antagomiRNA-30e) were transfected in IEC-6 and primary IECs using Lipofectamine 2000 (Life Technologies) according to the manufacturer's instructions; cells were harvested 48 hr after transfection for further analysis.

To assess effects of up- and downregulation of miRNA-30e *in vivo*, 96 C57BL/6J mice were randomly divided into four groups of 24, each of which was given one tail vein injection a day for 3 days. The Con-NS group consisted of control mice who received normal saline, the DM-NS group consisted of DM mice who received normal saline; the Con-agomiRNA-30e mice received injections of agomiRNA-30e (10 mg/kg body weight); and the DM-antagomiRNA-30e mice received injections of antagomiRNA-30e (80 mg/kg body weight) (29–31). In each group, six mice were sacrificed on day 0 (before injection), day 2, day 4 and day 6 for further investigation.

Total RNA extraction and real-time QPCR

Total RNA was extracted using TRIzol (Life Technologies). To generate cDNA, 1 μg of total RNA was reverse transcribed using a ReverTra Ace-α- kit (Toyobo, Japan).

Real-time PCR was performed using a Real-time™ PCR Master Mix kit (Toyobo, Japan) and a Rotor-Gene 6000 detector (Corbett Research, Sidney, Australia). Primers (forward and reverse) are shown in Table S1. Data were analysed using the ΔΔCt method with 18S ribosomal RNA as the constitutive marker (27).

Western blots analysis

All cells and tissues were crushed and incubated in RIPA lysis buffer (Thermo Fisher Scientific, Waltham, MA, USA). Protein (40 μg) was separated by SDS-PAGE on a 12% polyacrylamide gel. Proteins were transferred electrophoretically on to PVDF membrane and incubated with primary antibodies as shown in Table S2. Proteins were detected using ECL. Protein band integrated intensity was determined by scanning densitometry and analysed by Glyko BandScan 5.0. Data were analysed using relative intensity with β-actin as the constitutive marker.

Immunohistochemistry

Intestinal segments were fixed with 4% paraformaldehyde overnight at 4 °C, followed by embedding in paraffin wax, and sectioning at 4 μm. Sections were incubated with primary antibodies as shown in Table S3. After washing in PBS, tissue sections were incubated with EnVision+/HRP/Rb (DAKO, Glostrup, Denmark) for 30 min at room temperature. They were then incubated in 3, 3′-DAB (Maxim, Fuzhou, China) for 5 min and counterstained with haematoxylin for 30 s. All sections were photographed using a Nikon TE2000-U camera (Nikon, Tokyo, Japan) equipped with Nikon optics.

Statistical analysis

All analyses were performed using SAS 8 (SAS Institute; Cary, NC, USA) and data are presented as mean ± SEM. Data were evaluated by one-way ANOVA in which multiple comparisons were performed using the method of least significant difference. Differences were considered significant if $P < 0.05$.

Results

Abnormal differentiation of IECs in DM mice was associated with reduced Dll4/NICD/Hes1 pathway activity

Sucrase-isomaltase (SI), chromogranin A (ChgA), trefoil factor 3 (Tff3) and lysozyme 1 (Lyz1) were used as markers for absorptive cells, endocrine cells, goblet cells and Paneth cells respectively. The mRNA expressions of SI, Tff3 and Lyz1 in DM mice were significantly higher than those in control mice, while ChgA mRNA expression was significantly lower ($n = 6$, $P < 0.05$; Fig. 1a). Expression profiles of SI, ChgA, Tff3 and Lyz1 protein in the small intestines of DM mice were consistent with their mRNA expressions ($n = 6$, $P < 0.05$; Fig. 1b,c).

mRNA expression of delta-like 1 (Dll1), jagged 1 (Jag1), jagged 2 (Jag2) and Notch1 in DM mice was significantly higher than in control mice, and Dll4 and Hes1 mRNA expression was significantly lower ($n = 6$, $P < 0.05$; Fig. 1d). Protein expression of Dll1, Jag1, Jag2 and Notch1 in the small intestines of DM mice were higher than those of control mice ($n = 6$, $P < 0.05$; Fig. 1e,f). However, levels of Dll4, NICD and Hes1 protein in the small intestines of DM mice were significantly lower than those of controls ($n = 6$, $P < 0.05$; Fig. 1e,f).

Enhanced miRNA-30e expression in IECs of DM mice

Primary IECs were isolated from small intestines of DM and control mice. miRNA expression profiles were evaluated by microarray hybridization, and hierarchical clustering identified 107 miRNAs which were significantly altered in IECs of DM mice compared to the control group (Fig. 2a). Of the 107 miRNAs, 93 were significantly upregulated and 14 were significantly downregulated (Table S4). miRNA-30, which may target the Notch signal pathway, was identified by bioinformatic analysis, and considered to be a candidate for further investigation. QPCR results revealed that miRNA-30e expression in IECs of small intestine from DM mice was significantly upregulated compared to the control group ($n = 6$, $P < 0.05$; Fig. 2b). However, other miR-30 family members showed no difference in expression between the two groups ($n = 6$, $P > 0.05$; Fig. S1).

miRNA-30e targeted the 3′-UTR of Dll4 in primary IECs, IEC-6 and 293T cells

Analysis of the publicly available algorithms (TargetScan, www.targetscan.org; PicTar, pictar.mdc-berlin.de; miRanda, www.microrna.org) suggested that miRNA-30e has a highly conserved binding site in the 3′-UTR of Dll4 in several species (human, rat and mouse), including seed region of miRNA-30e (Fig. 3a). Sequence of the putative miRNA-30e binding site, TGT TTA C, was mutated into ACA AAT G in the Dll4-3′-UTR-mut vector (Fig. 3b). Activity analysis revealed that luciferase expression in primary IECs, IEC-6 and

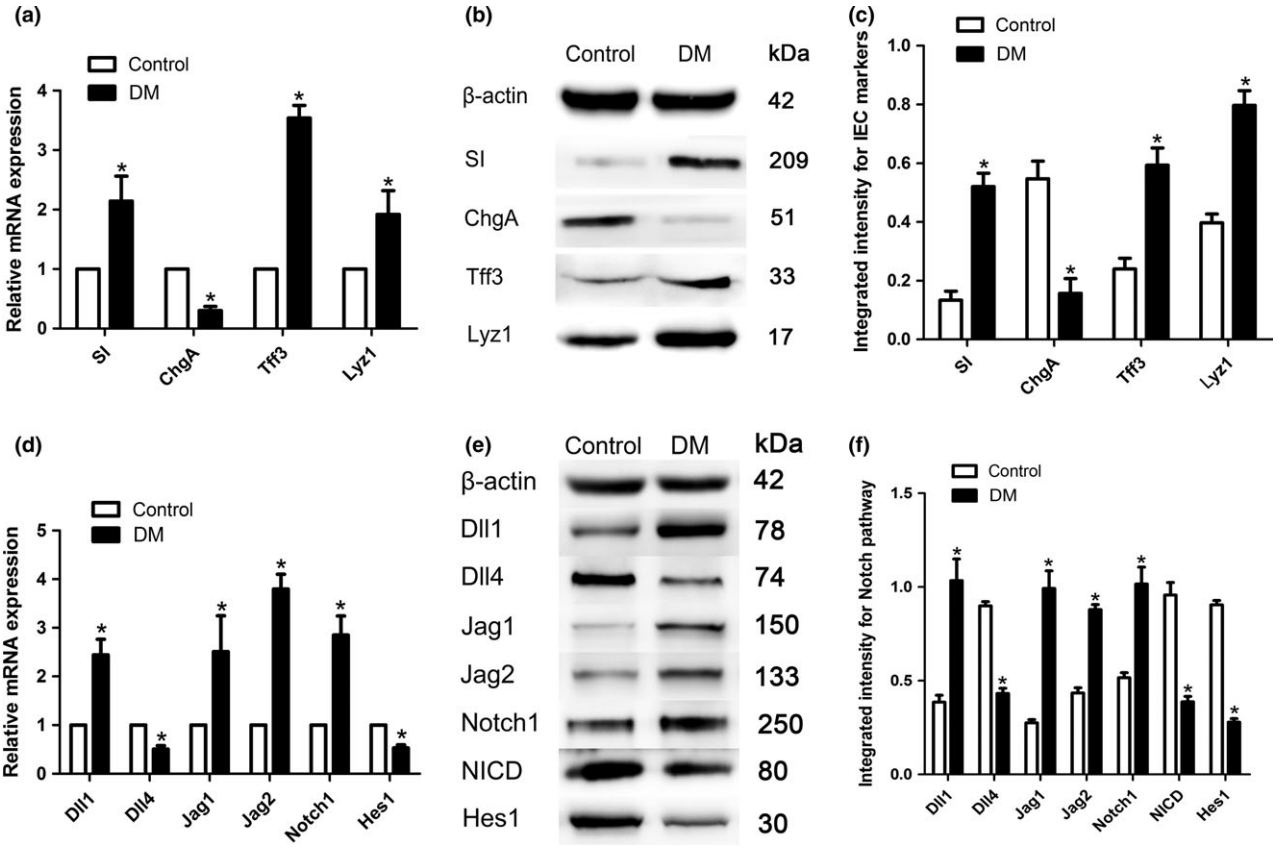

Figure 1. Abnormal differentiation of IECs in DM mice associated with reduced Dll4/NICD/Hes1 signalling. (a) QPCR analysis of mRNA expression of SI, ChgA, Tff3 and Lyz1 in DM and control mice. (b, c) Protein levels of IECs markers as assessed by western blot analysis in DM and control mice. (d) QPCR analysis of mRNA expression of Dll1, Dll4, Jag1, Jag2, Notch1 and Hes1 in DM and control mice. (e, f) Protein levels of Notch ligands and Notch pathway proteins as assessed by western blots analysis in DM and control mice. All results are presented as mean ± SEM, $n = 6$; *$P < 0.05$ versus the control group.

293T cells cotransfected with miRNA-30e mimic and the Dll4-3′-UTR-wnt plasmid was significantly lower than in cells cotransfected with miRNA-30e mimic and the Dll4-3′-UTR-mut plasmid, or transfected with Dll4-3′-UTR-wnt plasmid alone ($n = 6$, $P < 0.05$; Fig. 3c–e). These data indicate that miRNA-30e mimic specifically targetted the 3′-UTR of Dll4, and downregulate expression of the downstream reporter gene.

miRNA-30e regulated Dll4 expression in primary IECs and IEC-6 cells in vitro

Expression of Dll4 mRNA in primary IECs and IEC-6 cells after transfection of mimic was significantly lower than in the control group ($n = 6$, $P < 0.05$, Fig. 4a,b). Conversely, transfection of inhibitor into primary IECs and IEC-6 cells upregulated Dll4 expression at the mRNA level ($n = 6$, $P < 0.05$, Fig 4a,b).

Integrated intensities of bands for β-actin and Dll4 in primary IECs and IEC-6 cells after transfection of mimic and inhibitor are shown in Fig. 4c. Dll4 protein levels in primary IECs and IEC-6 cells after transfection of mimic were lower than those in the cells without transfection ($n = 6$, $P < 0.05$; Fig. 4c–e), while Dll4 protein expression was upregulated after transfection of inhibitor ($n = 6$, $P < 0.05$; Fig. 4c–e). These data further indicate that Dll4 can be regulated by miRNA-30e in primary IECs and IEC-6 cells *in vitro*.

miRNA-30e regulated the Dll4/NICD/Hes1 signalling pathway in small intestinal epithelium in vivo

Expression of miRNA-30e in IECs isolated from ConagomiRNA-30e treated mice was significantly higher 2, 4 and 6 days after administration compared to Con-NS mice ($n = 6$, $P < 0.05$; Fig. 5a); expression of miRNA-30e on the 4th day after administration was similar to that in DM-NS mice. Expression of miRNA-30e in IECs isolated from DM-antagomiRNA-30e mice was significantly lower 2, 4 and 6 days after administration

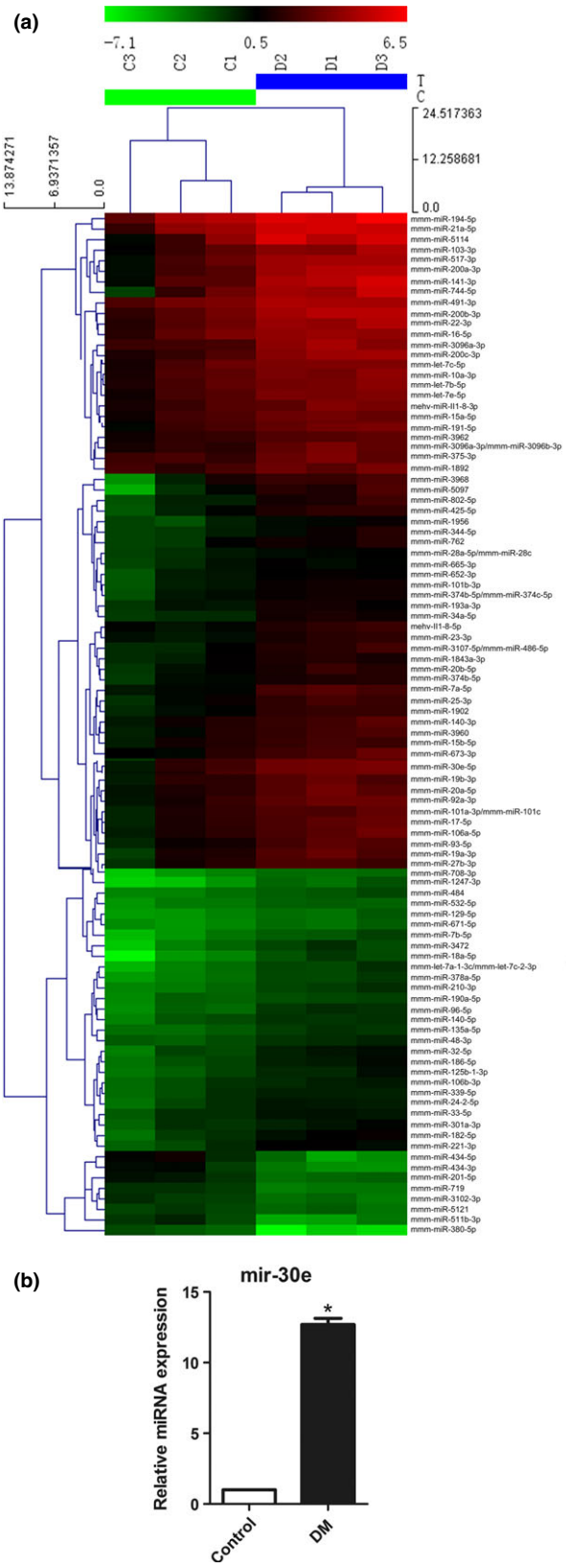

Figure 2. miRNA expression profile evaluated by microarray hybridization. (a) miRNA microarray analysis found 107 miRNAs significantly altered in IECs of DM mice (D1-3) compared to the control group (C1-3; $n = 3$). (b) QPCR analysis for miRNA-30e expression in IECs of small intestine from DM and control mice (mean ± SEM, $n = 6$; $^*P < 0.05$ versus control group).

compared to DM-NS mice ($n = 6$, $P < 0.05$; Fig. 5a); expression of miRNA-30e on the 4th day after administration was similar to that in Con-NS mice. These results indicate that exogenous agomiRNA-30e increased the level of miRNA-30e in IECs of normal mice to mimic levels seen in DM mice. Furthermore, expression of miRNA-30e in IECs isolated from DM mice was inhibited by treatment with antagomiRNA-30e, falling to control levels 4 days after administration.

QPCR results revealed that the Con-agomiRNA mice had significantly higher expression of Notch1 mRNA, and significantly lower expression of Dll4 and Hes1 mRNA 4 days after administration ($n = 6$, $P < 0.05$; Fig. 5b). In contrast, mRNA expressions of Dll4 and Hes1 in DM-antagomiRNA-30e mice were significantly higher than those in DM-NS mice, and were similar to the levels in Con-NS mice on the 4th day ($n = 6$, $P < 0.05$; Fig. 5b). Notch1 mRNA expression in DM-antagomiRNA-30e mice was significantly decreased compared to DM-NS mice, and was comparable to the Con-NS expression level ($n = 6$, $P < 0.05$; Fig. 5b).

Dll4, Notch1, NICD and Hes1 proteins were all detected in adult small intestinal crypts (Fig. 5c). NICD and Hes1 immunostaining was found largely in cell nuclei, while Notch1 appeared to be localized to cell membranes in DM-NS and Con-agomiRNA-30e mice. Expression of Dll4, NICD and Hes1 proteins in Con-agomiRNA-30e mice were lower than those of Con-NS mice 4 days after administration ($n = 6$, $P < 0.05$; Fig. 5d,e). However, expression of Notch1 protein in Con-agomiRNA-30e mice was significantly higher than Con-NS mice and was similar to DM-NS mice 4 days post-administration (Fig. 5d,e). Meanwhile, levels of Dll4, NICD and Hes1 protein in DM-antagomiRNA-30e mice were significantly higher than in DM-NS mice ($n = 6$, $P < 0.05$; Fig. 5d,e), but Notch1 expression was significantly lower than in DM-NS mice, and was similar to Con-NS mice (Fig. 5d,e).

Differentiation of small intestinal epithelium in vivo was altered by regulating miRNA-30e

On the 4th day after administration, mRNA expression of SI, Tff3 and Lyz1 in Con-agomiRNA-30e mice was significantly higher than those in Con-NS mice, while

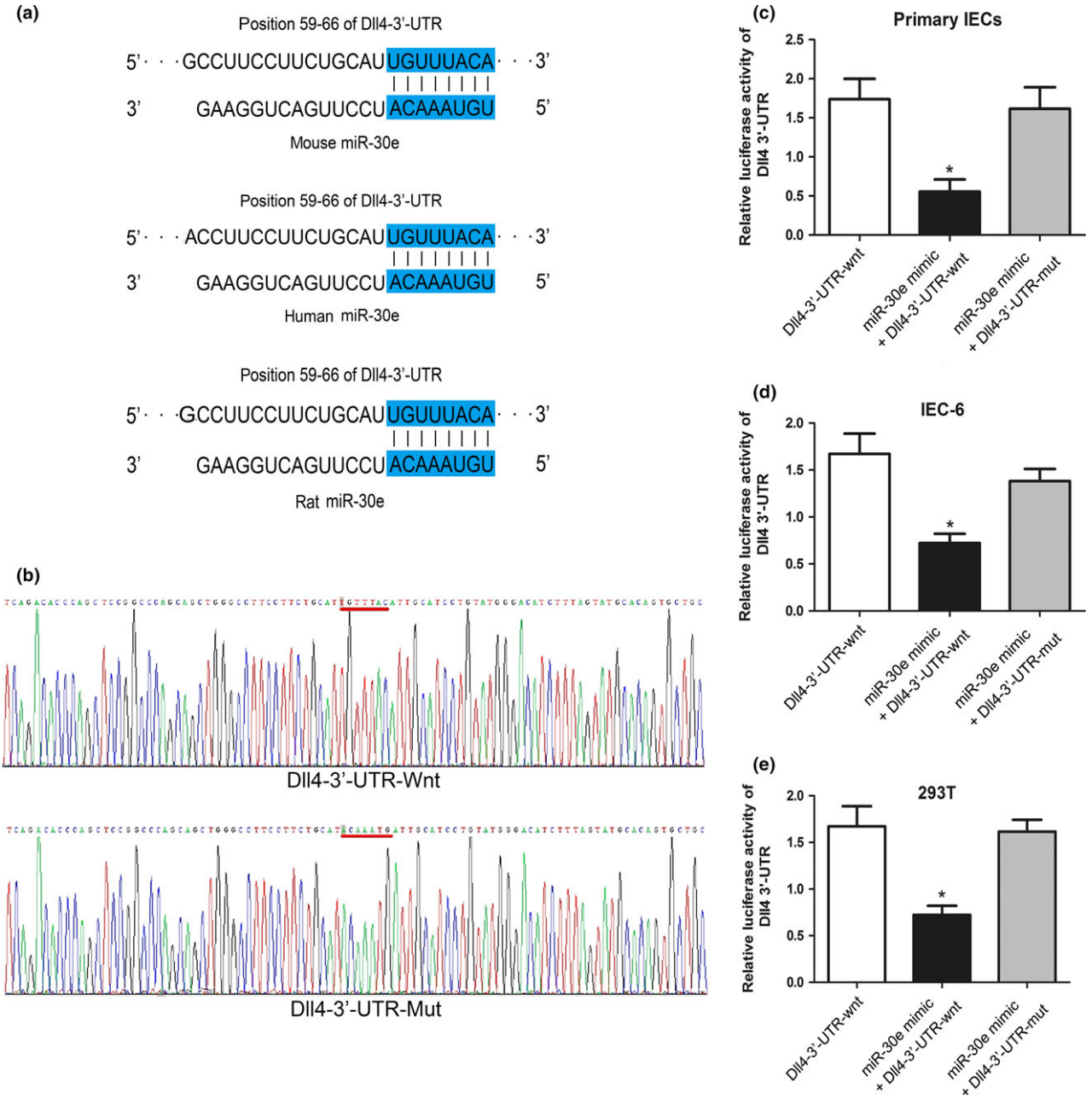

Figure 3. miRNA-30e targetted the 3′-UTR of Dll4 in primary IECs, IEC-6 and 293T cells. (a) The highly conserved binding site of miRNA-30e in the 3′-UTR of Dll4 in mouse, human and rat Dll4. (b) Sequence of the putative miRNA-30e binding site (TGT TTA C) was mutated into ACA AAT G. (c–e) Luciferase activity analysis in primary IECs (c), IEC-6 (d) and 293T cells (e). All luciferase assay results are presented as mean ± SEM, $n = 6$; *$P < 0.05$ versus control and mutant groups.

ChgA mRNA expression was significantly lower ($n = 6$, $P < 0.05$; Fig. 6a). Increased mRNA expression of SI, Tff3 and Lyz1 seen in DM was inhibited in DM-antagomiRNA-30e mice, and ChgA mRNA expression was increased ($n = 6$, $P < 0.05$).

Expression of IECs markers was detected by immunohistochemistry (Fig. 6b). Numbers of SI, Tff3 and Lyz1 positive cells in the small intestines of Con-agomiRNA-30e mice were significantly higher than in the Con-NS mice ($n = 6$, $P < 0.05$; Fig. 6c). The number of ChgA-positive cells in Con-agomiRNA-30e mice was significantly lower ($n = 6$, $P < 0.05$; Fig. 6c). Meanwhile, numbers of SI, Tff3 and Lyz1 positive cells in DM-antagomiRNA-30e mice were significantly lower,

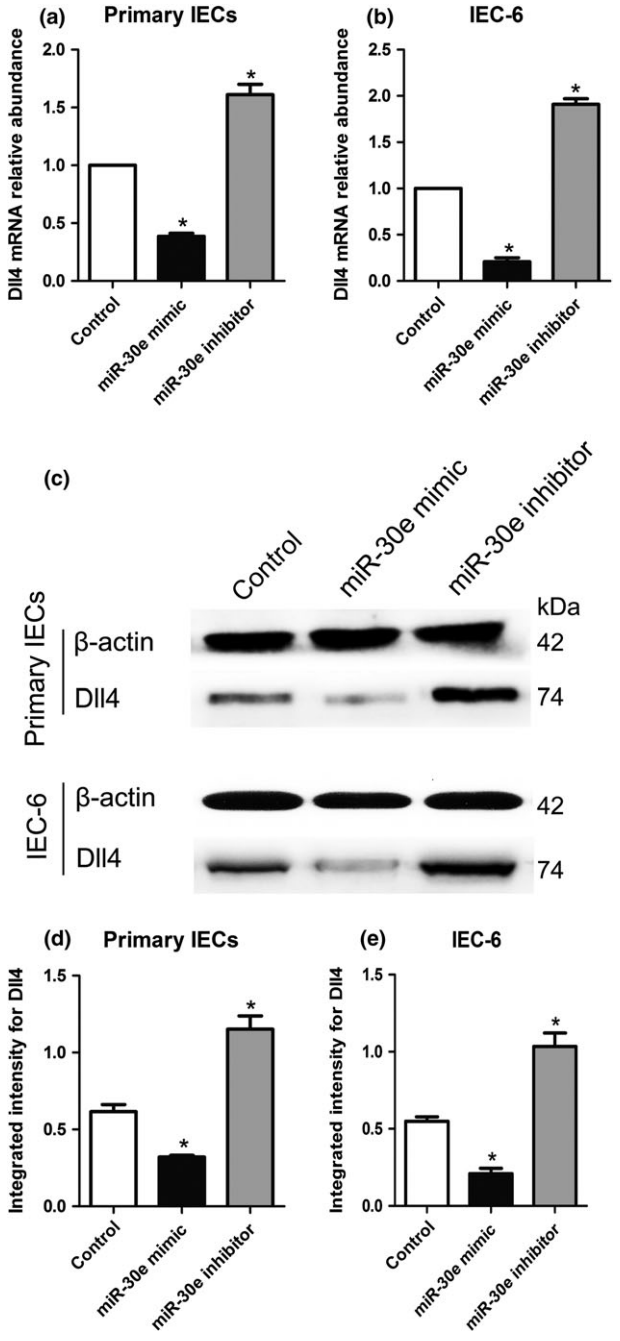

Figure 4. miRNA-30e regulates Dll4 expression in primary IECs and IEC-6 cells *in vitro*. (a, b) mRNA expressions of Dll4 in primary IECs (a) and IEC-6 cells (b) after transfection of mimic and inhibitor. (c) Western blots of β-actin and Dll4 in primary IECs and IEC-6 cells after transfection of mimic and inhibitor. (d, e) Dll4 protein levels in primary IECs (d) and IEC-6 cells (e). Results presented as mean ± SEM, $n = 6$; $^*P < 0.05$ versus control group.

and the reduced number of ChgA-positive cells seen in DM-NS mice was normalized after antagomiRNA-30e administration ($n = 6$, $P < 0.05$; Fig. 6c).

Western blotting was used to investigate protein expression levels of these differentiation-related markers (Fig. 6d). Expression of SI, Tff3 and Lyz1 protein in the small intestines of Con-agomiRNA-30e mice was significantly higher than in Con-NS mice ($n = 6$, $P < 0.05$; Fig. 6e). ChgA protein expression in Con-agomiRNA-30e mice was significantly lower compared to Con-NS mice ($n = 6$, $P < 0.05$; Fig. 6e). Meanwhile, protein expression of SI, Tff3 and Lyz1 protein in DM-antagomiRNA-30e mice was significantly reduced and close to the levels seen in Con-NS mice ($n = 6$, $P < 0.05$; Fig. 6e). Furthermore, downregulated ChgA protein expression in DM-antagomiRNA-30e mice was partially normalized after antagomiRNA-30e administration compared with DM-NS mice ($n = 6$, $P < 0.05$; Fig. 6e).

Discussion

Overproliferation and abnormal differentiation in diabetic intestinal epithelia have been observed in previous investigations (12,32,33), but little is known about the mechanisms involved. In these experiments, we investigated the role of the Dll4/NICD/Hes1 signalling pathway in IEC differentiation in DM mice. Our initial observations indicated that expression of SI, Tff3 and Lyz1 was significantly higher in DM mice, while ChgA expression was decreased (Fig. 1), suggesting that IECs in DM mice had an abnormal differentiation profile compared to control mice.

As the Notch signalling pathway has been implicated in small intestine differentiation (20), we then investigated expression of Notch1 and its ligands. Dll1, Jag1, Jag2 and Notch1 levels were higher in DM mice compared to controls (Fig. 1). However, levels of Dll4, NICD and Hes1, an important downstream target of Notch signalling, were significantly lower in DM mice. Hes1 is known to be downregulated in small IECs in DM (25). These results suggest that abnormal Notch signalling contributes to altered differentiation of small intestinal epithelia in DM. However, increase Dll1, Jag1 and Jag2 expression in DM mice does not correlate with suppressed Notch signalling seen in our previous study (25). This suggests that the key regulator of Notch signalling in IECs is Dll4, which was downregulated (Fig. 1).

Our data are in contrast to a study by Pellegrinet *et al.* (35). However, that study investigated the function of Notch ligands under physiological conditions, rather than in DM. While the exact function of Dll4 during DM is not well understood, we hypothesize that activity of Dll4 might be enhanced, or the ability of Dll1 to compensate for DM-induced loss of Dll4 might be reduced. This point requires further study.

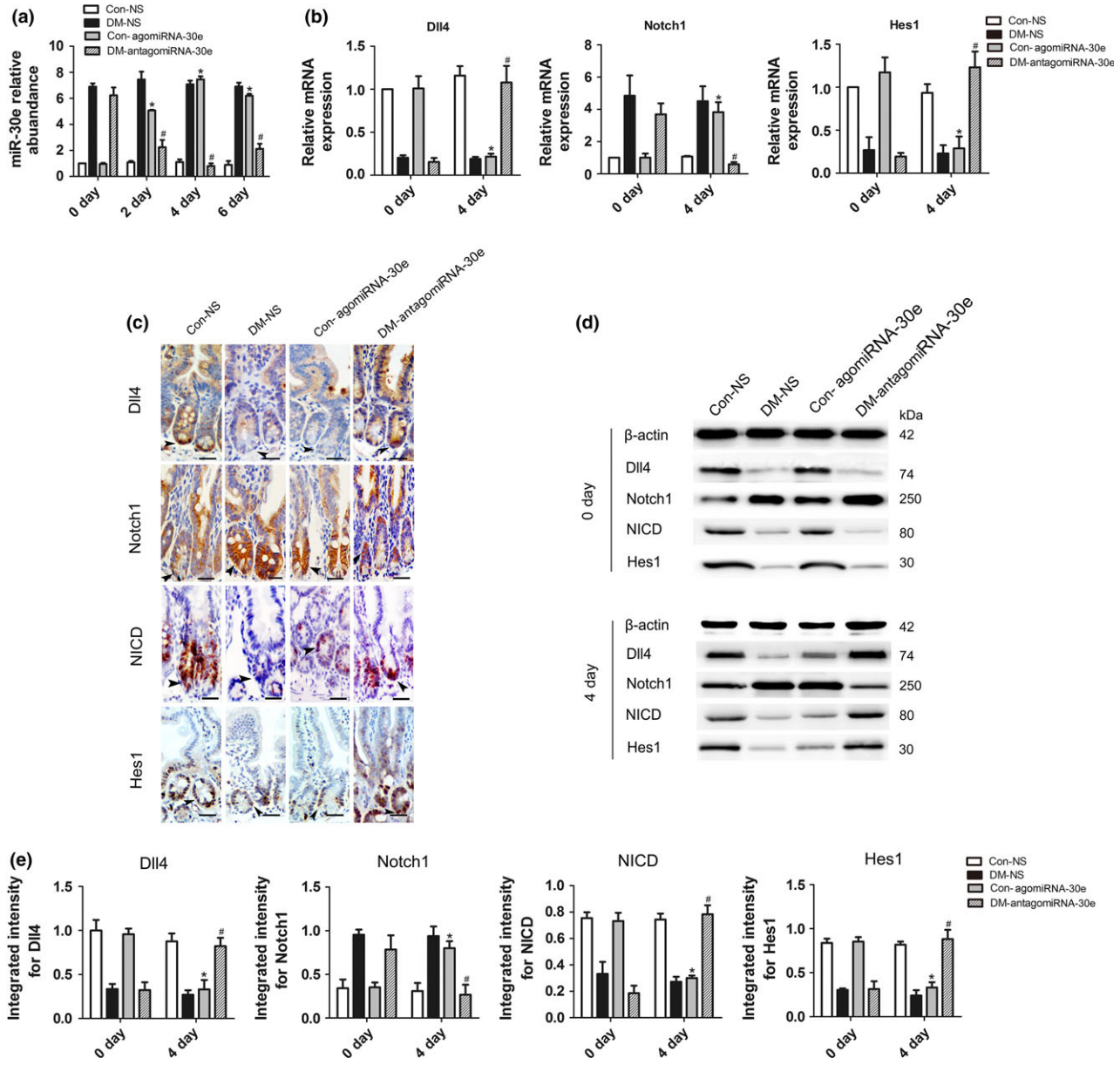

Figure 5. miRNA-30e regulating Dll4/Notch/Hes1 signalling pathway in small intestinal epithelium *in vivo*. (a) Levels of miRNA-30e detected by QPCR in the four groups of mice. (b) Levels of Dll4, Notch1 and Hes1 detected by QPCR before (0 day) and after tail vein injection (4 days). (c) Dll4, Notch1, NICD and Hes1 proteins located by immunohistochemistry in small intestines 4 days after the start of treatment (scale bars indicate 50 μm). (d, e) Western blot analysis for protein levels of Dll4, Notch1, NICD and Hes1 in the small intestines. Results are presented as mean ± SEM, $n = 6$; $^{*}P < 0.05$ compared to Con-NS group at same time point; $^{\#}P < 0.05$ compared with DM-NS group at same time point.

Dll4 plays a key role in development of some cancers, and in cardiovascular and metabolic diseases, by altering Notch activity (34,36–38). As previous reports have indicated that miRNA can regulate the Notch signalling pathway (39), we assessed miRNA expression profiles to find miRNAs that were differentially expressed in small intestinal epithelium in DM mice; we identified 107 miRNAs that were significantly altered in them (Fig. 2). Based on bioinformatics prediction and QPCR analysis, miRNA-30e was identified as a candidate miRNA for regulating Dll4 and the Notch1/Hes1 pathway in IECs.

Recent studies have confirmed the important role of miRNAs in differentiation of intestinal epithelium in the mouse (40,41); we therefore hypothesized that Dll4 was a target of miRNA-30e, resulting in downregulation of

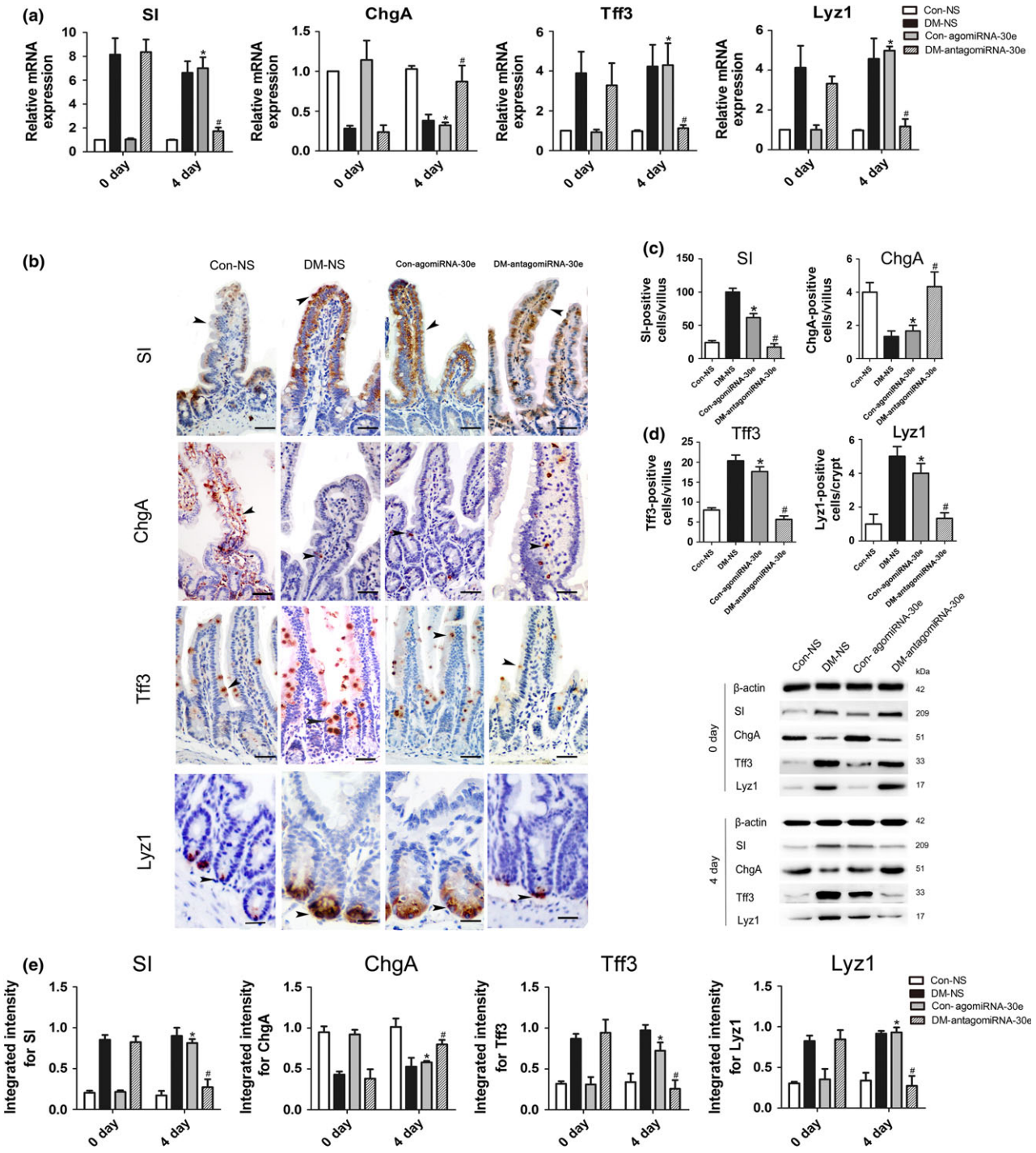

Figure 6. Regulating miRNA-30e controls differentiation of small intestinal epithelium *in vivo* during DM. (a) Levels of SI, ChgA, Tff3 and Lyz1 detected by QPCR before (0 day) and after tail vein injection (4 days). (b) SI, ChgA, Tff3 and Lyz1 protein were located by immunohistochemistry in the small intestines 4 days after the start of treatment (scale bars indicate 50 μm in SI, ChgA and Tff3, and 25 μm in Lyz1). (c) Numbers of SI-, ChgA-, Tff3- or Lyz1-positive cells per villus-crypt unit in these four groups were determined. Levels of SI-, Tff3- and Lyz1-positive cells were increased in Con-agomiRNA mice compared to Con-NS mice, and were reduced in DM-antagomiRNA mice compared to DM-NS mice. Conversely, ChgA-positive cells were low in Con-agomiRNA mice compared to Con-NS mice, and high in DM-antagomiRNA mice compared to DM-NS mice. (d, e) Western blot analysis for SI, ChgA, Tff3 and Lyz1 in small intestines. Results presented as mean ± SEM, $n = 6$; $^{*}P < 0.05$ compared to Con-NS group at same time point; $^{\#}P < 0.05$ compared to DM-NS group at same time point.

Notch1/Hes1 signalling. To confirm this hypothesis, we assessed luciferase activity after cotransfection with miRNA-30e mimic and Dll4-3′-UTR-wnt plasmid. The miRNA-30e mimic targeted the 3′-UTR of Dll4 and downregulated its expression (Fig. 3c–e). To further confirm Dll4 as a target of miRNA-30e, we examined Dll4 expression in primary IECs and IEC-6 cells after transfection of miRNA-30e mimic or inhibitor (Fig. 4). Expression of Dll4 in primary IECs and IEC-6 cells after transfection was significantly lower, while cells transfected with miRNA-30e inhibitor had significantly higher expression of Dll4. These data confirm that miRNA-30e can alter expression of Dll4, and suggest a potential mechanism for regulating Dll4 expression by promoting its mRNA degradation in primary IECs (42).

Notch signalling is critical in the maintenance of crypt epithelial cells (43), and numbers of studies have shown that miR-449a and miR-1 regulate Notch signals in the small intestine (44,45). The effect of miRNA-30e on the Dll4/NICD/Hes1 signalling pathway and IEC differentiation during DM had not been reported. Exogenous agomiRNA-30e was injected into normal mice with the goal of upregulating miRNA-30e *in vivo*. The results indicated that exogenous overexpression of miRNA-30e led to increase in inactive Notch1, and decreased signalling of the NICD/Hes1 pathway by downregulating Dll4 expression (Fig. 5). Our data further showed that overexpression of miRNA-30e induced abnormal differentiation *in vivo*, similar to pathological changes in IECs in DM mice (Fig. 6). However, there may be other cell signalling pathways associated with the miRNA-30e that contribute to pathologic differentiation of IECs in DM.

To further clarify the molecular mechanisms of abnormal differentiation of IECs in DM, the increased levels of miRNA-30e in DM were inhibited by injecting antagomiRNA-30e *in vivo*. After antagomiRNA-30e treatment in DM mice, Dll4 expression was normalized (Fig. 5). Furthermore, antagomiRNA-30e treatment normalized the expression of IEC markers (Fig. 6), indicating normal differentiation. These data show that inhibiting miRNA-30e could be an effective approach to preventing the progression of abnormal differentiation of IECs in DM mice.

In conclusion, abnormal differentiation in small intestinal epithelia of DM mice was found to be associated with reduced activity of the Dll4/NICD/Hes1 signalling pathway. Increased expression of miRNA-30e appears to be a key player in this pathological process, downregulating Dll4/NICD/Hes1 signalling by targeting the 3′-UTR of Dll4. We cannot assess whether miRNA-30e induces abnormal differentiation of IECs in human DM patients, but experiments in mice favour this view. Furthermore, these findings provide a new insight into the role of miRNA-30e in diabetic enteropathy, and serve as a foundation for the development of novel therapeutic strategies.

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Conflict of interest

The authors declare that there is no duality of interest associated with this manuscript.

Author's contributions

Ti-Dong Shan and Tao Yu carried out the molecular genetic studies, participated in the sequence alignment and drafted the manuscript. Jie-Yao Li carried out the immunoassays. Hui Ouyang and Can-Ze Huang participated in the induction of diabetes model. Wa Zhong and Hong-Sheng Yang participated in the sequence alignment. Tao Yu and Zhong-Sheng Xia participated in the design of the study and performed the statistical analysis. Qi-Kui Chen conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. MiRNA-30 family expression in IECs of DM mice. QPCR analysis revealed that there was no difference in the expressions of miRNA-30a, -30b, -30c, and -30d in IECs between the DM and control mice ($n = 6$; $P > 0.05$ compared with control group).

Table S1. The primers for real-time QPCR.

Table S2. Antibodies used for Western blots.

Table S3. Antibodies used for immunohistochemical staining.

Table S4. List of differentially expressed miRNAs in IECs of DM mice compared with normal control mice.