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ABSTRACT

Chronically elevated levels of glucose impair pancreatic beta-cell function while inducing beta-cell proliferation. MicroRNA-29a (miR-29a) levels are increased in several tissues in diabetic animals and mediate decreased insulin-stimulated glucose-transport of adipocytes. The aim was to investigate the impact of glucose on miR-29a levels in INS-1E beta-cells and in human islets of Langerhans and furthermore to evaluate the impact of miR-29a on beta-cell function and proliferation. Increased glucose levels up-regulated miR-29a in beta-cells and human and rat islets of Langerhans. Glucose-stimulated insulin-secretion (GSIS) of INS-1E beta-cells was decreased by forced expression of miR-29a, while depletion of endogenous miR-29a improved GSIS. Over-expression of miR-29a increased INS-1E proliferation. Thus, miR-29a up-regulation is involved in glucose-induced proliferation of beta-cells. Furthermore, as depletion of miR-29a improves beta-cell function, miR-29a is a mediator of glucose-induced beta-cell dysfunction. Glucose-induced up-regulation of miR-29a in beta-cells could be implicated in progression from impaired glucose tolerance to type 2 diabetes.

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1. Introduction

Type 2 diabetes mellitus is a complex metabolic disorder involving two core defects: insulin resistance and beta-cell dysfunction, both of which are present in pre-diabetic states and act in concert to progressively exacerbate glucose intolerance. Elevated plasma glucose levels (post-prandially or chronically) lead to decreased glucose-stimulated insulin secretion (GSIS) and beta-cell dysfunction [1]. The mechanisms underlying glucose-induced beta-cell dysfunction, or glucose toxicity, are incompletely understood, but involve beta-cell exhaustion from continued insulin release as well as mitochondrial dysfunction [2–4], however, an increased glucose level is also a powerful beta-cell mitogen [5].

MicroRNAs (miRNAs) are regulators of gene expression at the post-transcriptional level and function by partially binding to the 3′untranslated region (UTR) of their target gene transcripts either mediating transcript degradation or translational inhibition [6–8]. Several miRNAs have been implicated in beta-cell function: miR-9, miR-30d, miR-124, miR-133a and miR-375 are involved in glucose-dependent regulation of insulin transcription and/or insulin release in beta-cells by targeting beta-cell transcription factors and/or transcripts involved in insulin exocytosis [9–15]. Furthermore, fatty acid induced increase in miR-34a levels may cause beta-cell dysfunction [16].

miR-29a is up-regulated by glucose in skeletal muscle, liver and white adipose tissue, where it leads to insulin resistance [17–19]. We hypothesized that miR-29a could be similarly regulated by glucose in pancreatic beta-cells and mediate glucose-induced dysfunction. Thus, the aim of this study was to determine the glucose-dependent regulation of miR-29a in beta-cells, as well as effects on GSIS.

Our results indicate that glucose-mediated up-regulation of miR-29a in beta-cells mediates beta-cell dysfunction and increased beta-cell proliferation, while inhibition of miR-29a improves GSIS. Thus, the up-regulation of miR-29a by glucose could be a link between glucose-induced proliferation and beta-cell dysfunction.

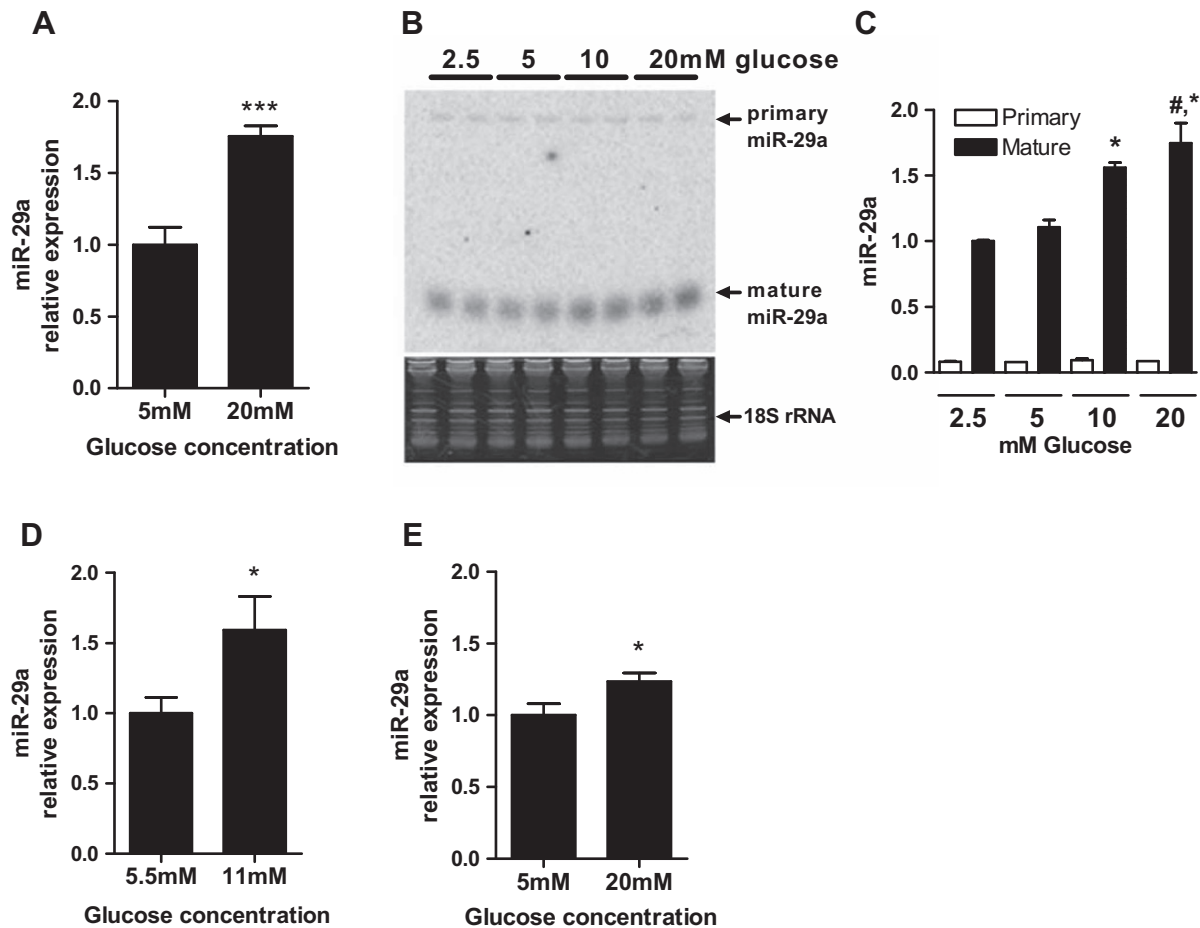2　　　　　　　　　　　　　　　A. Bagge et al./Biochemical and Biophysical Research Communications xxx (2012) xxx–xxx

Fig. 1. Regulation of miR-29a by glucose in INS-1E cells and human and rat pancreatic islets. (A) INS-1E cells were cultured for 48 h in RPMI with 5 mM or 20 mM glucose before RNA extraction. The expression of miR-29a was assessed as described for human islets (***$P < 0.001$, $N = 5$ experiments). (B) INS-1E cells were cultured in 2.5, 5, 10 or 20 mM glucose before RNA extraction. The levels of mature miR-29a in INS-1E cells in response to increasing levels of glucose were assessed by northern blotting. The [18]S rRNA band on ethidium bromide stained gels was used for loading control. (C) Quantification of the northern blot was done using ImageQuant ($N = 2$ experiments). Data are presented as mean ± SEM. (The P-value for one-way ANOVA for glucose-treatment was 0.009, (*$P < 0.05$ vs. 2.5 mM glucose, #$P < 0.05$ vs. 5 mM glucose by Tukeys post-test), (D) Human islets of Langerhans were cultured for 48 h in CMRL with 5 mM or 11 mM glucose before RNA extraction. The expression levels of miR-29a were assessed by RT-Q-PCR with primers specific for miR-29a. The level of *TFIIB* was used to normalize the data (*$P < 0.05$, $N = 5$ experiments). (E) Adult rat islets of Langerhans were cultured for 48 h in RPMI1640 with 5 mM or 20 mM glucose before RNA extraction. The levels of miR-29a were assessed by RT-Q-PCR with primers specific for miR-29a. The level of *TFIIB* was used to normalize the data (*$P < 0.05$, $N = 3$ experiments).

2. Materials and methods

2.1. Cell culture and nucleofection

INS-1E cells (gift from Claes Wollheim, Geneva, Switzerland) were cultured in RPMI as described previously [20]. Cells (4×10^6) were nucleofected using a Nucleofector (Amaxa, Lonza, Copenhagen, Denmark) with miR-29a LNA knock-down or scrambled LNA oligonucleotide (Exiqon, Vedbaek, Denmark), or with miRIDIAN miR-29a mimic or the miRIDIAN negative control #2 (Dharmacon, ThermoFisher Scientific, Slangerup, Denmark). Nucleofected cells were seeded in poly-lysine treated culture plates, and 24 h following nucleofection medium was changed according to experimental setup.

2.2. Islets of Langerhans

Human islets (average age of cadaveric donors 49.5 ± 8.9 years; range 40–58 years; $n = 4$) were provided through the Juvenile Diabetes Research Foundation (JDRF) Islet Distribution Program by Islet Cell Resource Centers in Geneva (Switzerland), Milan (Italy) and Lille (France). The use of human tissue for research was approved by the local ethical committee. Islets were cultured in 5 mM or 11 mM glucose with 5% human serum in CMRL 1066 medium for 48 h before RNA extraction. Rodent islets were isolated as described previously [21,22]. Db/db islets were harvested for RNA extraction immediately following isolation, and adult rat islets were cultured for 48 h in RPMI1640 with 5 mM or 20 mM glucose and 10% fetal bovine serum.

2.3. RNA extraction and northern blotting

RNA was extracted using TRI-Reagent (Sigma–Aldrich, Broendby, Denmark) according to the manufacturer's instructions, quantified using NanoDrop and stored at −80 °C until cDNA synthesis or northern blotting. Total RNA (5 μg) was resolved in 15% TBE-Urea gels (Invitrogen, Novato, CA, USA), photographed and blotted to Hybond-XL membrane (GE Healthcare, Broendby, Denmark) by capillary transfer. RNA was cross-linked to membranes in UV Stratalinker (Stratagene/Agilent Technologies, Hoersholm, Denmark) at 120,000 μJ/cm² and baked for 60 min at 80 °C. Complementary LNA probe (Exiqon) for mature miR-29a was end-labeled with [γ-³²P] dATP (Perkin Elmer, Waltham, MA, USA). Hybridization was performed using the ULTRAhyb-Oligo protocol (Ambion, Foster City, CA, USA).
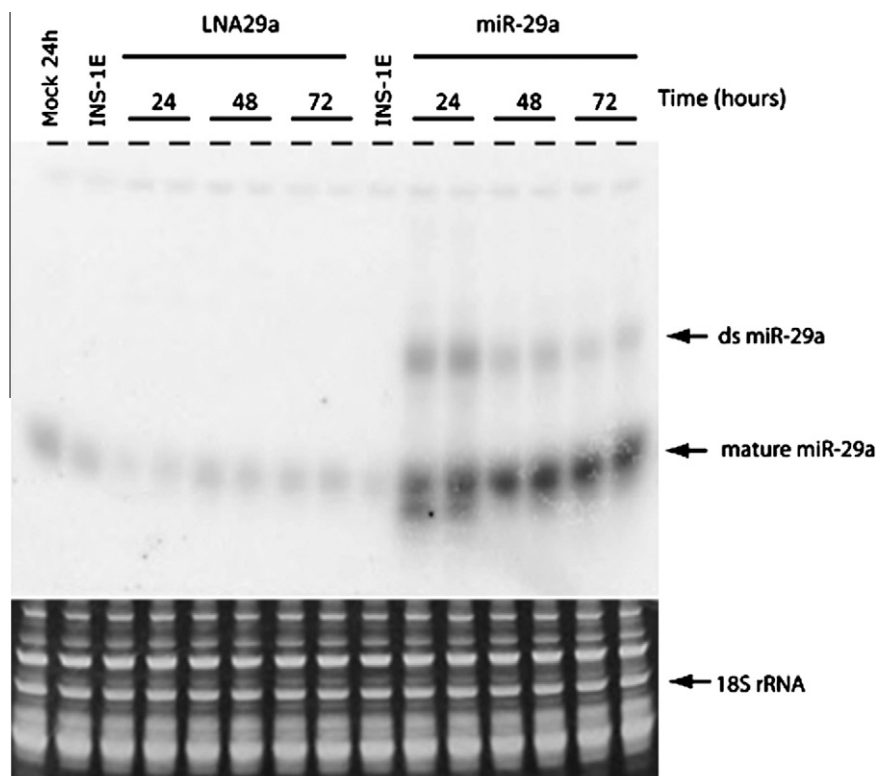
Fig. 2. Efficiency of miR-29a over-expression and inhibition in INS-1E cells over time. INS-1E cells were nucleofected with either miR-29a mimic (miR-29a) or miR-29a LNA inhibitor (LNA29a). After 24, 48 and 72 h duplicate wells were harvested and RNA was isolated. The levels of functional miR-29a were assessed by northern blotting (5 μg total RNA/lane). Mock 24hrs is RNA from mock control nucleofected INS-1E cells, lanes with INS-1E are RNA from INS-1E cells that were not nucleofected. Equal loading was assessed by the intensity of the ^{18}S rRNA band on the ethidium bromide dyed gel before blotting.

2.4. Real-time RT-Q-PCR

Gene-specific stem-looped RT-primers were used to prime cDNA synthesis of miR-29a as described previously [23]. Gene-specific cDNA syntheses were multiplexed with primer for the normalizing gene *TFIIB*. Q-PCR was performed using Quantitect SYBR reagent (Qiagen, Ballerup, Denmark) with melting curve detection using a MX3000P instrument (Agilent Technologies). *TFIIB* was used for normalization as its levels do not change when glucose levels are changed [24], whereas the levels of ^{18}S and ^{5}S rRNA levels were dependent on glucose treatments. Oligonucleotide sequences are available on request.

2.5. Proliferation rate

The xCELLigence System (Roche Diagnostics, Copenhagen, Denmark) was used to estimate INS-1E growth curves following nucleofection with miR-29a mimetic and negative control oligos. Measurements were performed according to the manufacturer's protocol. Briefly: poly-lysine treated E-plates were calibrated with 50 μl complete growth medium before nucleofected cells were seeded in triplicate wells and growth followed for 48 h. Experiments were performed in multiples of 4–8 of each treatment condition. Proliferation rates (Cell index) for wells were calculated from the slope of the linear part of the curve and the average level of 20 mM glucose with Neg. ctrl. was set to 100% and data normalized to this. Data were normalized, because the Cell index changes between experiments. For statistical analysis data from all experiments were compared using one-way ANOVA followed by Tukey's multiple comparison tests.

2.6. Determination of average cell size

Nucleofected cells were seeded in 24-well plates and phase contrast images were obtained after 24 and 48 h. Single cells were outlined in ImageJ [25] and area determined. At least 30 cells per nucleofection were measured.

2.7. GSIS assay

Nucleofected cells were seeded in 24-wells in normal medium. The following day medium was changed to medium containing low (5 mM) or high (20 mM) glucose followed by culture for 48 h. Three hours before the GSIS assay, medium was changed to RPMI 1640 containing 3 mM glucose. Cells were subsequently stimulated for 2 h with 3 mM or 20 mM glucose in Krebs–Ringer buffer with 10 mM HEPES, 5 mM NaHCO$_3$, 2.54 mM CaCl$_2$ and 0.2% BSA. Insulin content in the buffer was determined with a rat insulin ELISA kit (Mercodia, Uppsala, Sweden). All glucose-treatments and stimulations were prepared in quadruplicate wells. Due to variance in cell numbers following nucleofection between individual experiments both relative (left side) and raw values (right side) are presented in Fig. 4. For relative values changes in GSIS are expressed as fold the value observed in control wells in order to facilitate comparison of data from several experiments.

2.8. Statistics

Students *t*-test was used to compare pairs of data. Multiple groups were analyzed using ANOVA with TUKEYs correction for multiple comparison. The significance level was 0.05. Data are

168 presented as a pool of replicate measurements from independent
169 experiments.

3. Results

3.1. Regulation of miR-29a levels by glucose in human and rat islets of Langerhans and INS-1E cells

173 The expression levels of miR-29a in human islets of Langerhans
174 and INS-1E cells treated with low (5 mM) or high glucose (11 mM
175 and 20 mM respectively) for 48 h were assessed by real-time RT-Q-
176 PCR or northern blotting. The expression levels of mature miR-29a
177 in INS-1E cells (Fig. 1A–C), rat and human pancreatic islets
178 (Fig. 1D–E) increased 1.7 ± 0.1 ($P < 0.001$), 1.5 ± 0.3 ($P < 0.05$) and
179 1.23 ± 0.06-fold ($P < 0.05$), respectively, in response to increased
180 glucose levels, and in INS-1E cells miR-29a up-regulation was
181 dose-dependent (Fig. 1B–C). There was no effect of glucose on
182 miR-29a primary transcript levels (Fig. 1B–C). Thus, both in the
183 rat beta-cell line INS-1E and in rat and human islets of Langerhans
184 mature miR-29a expression levels were increased by elevated glu-
185 cose levels.

3.2. Over-expression and inhibition of miR-29a in INS-1E cells

187 Because the biological effects of high levels of glucose on INS-1E
188 cells are normally observed after at least 48 h of glucose treatment,
189 the efficiency of over-expressing and inhibiting miR-29a over time
190 in INS-1E cells was assessed. Synthetic mature miR-29a (miR-29a)
191 or miR-29a LNA inhibitor (LNA-29a, which binds and sequesters
192 the endogenous miR-29a) was delivered to INS-1E cells by nucleo-
193 fection and the levels of functional miR-29a were followed for up
194 to 72 h after nucleofection and examined by northern blotting.
195 As seen in Fig. 2 inhibition and over-expression of miR-29a was
196 detectable for the 72 h of treatment, while inhibition decreased
197 48 h after nucleofection. Because the reduction by LNA-29a treat-
198 ment appeared modest by northern blotting, the levels of
199 miR-29a was also quantified by RT-Q-PCR. Mature miR-29a levels
200 in LNA-29a treated cells was on average $42 \pm 17\%$ of the level in
201 Scr ctrl. treated cells ($n = 4$), which is similar to the level of inhibi-
202 tion observed previously with miR-21 in INS-1E cells [26]. The syn-
203 thetic miR-29a is delivered as a modified dsRNA species, which is
204 visible on the blot as a band above the mature miR-29a band
205 (Fig. 2, 'ds miR-29a'). Thus, 48 h was chosen as the most appropri-
206 ate time-point for assessment of the effects of miR-29a.

3.3. Proliferation and size of clonal INS-1E beta-cells over-expressing miR-29a

209 MiR-29a has been reported to be associated with cancer and/or
210 increased proliferation in various cell types. MiR-29a over-expres-
211 sion increased proliferation in INS-1E beta-cells by $41.9 \pm 8.0\%$
212 ($P < 0.01$) in 3 mM glucose medium and by $55.9 \pm 13.9\%$
213 ($P < 0.001$) in 20 mM glucose medium compared with cells trans-
214 fected with the negative control miR (Fig. 3A). The area of cells
215 was unchanged by over-expression or depletion of miR-29a
216 (Fig. 3B).
217 The level of miR-29a was assessed in islets from db/db mice and
218 controls at the age of 8–15 weeks, at which time the mice have
219 developed diabetes (Fig. 3C). The relative level of miR-29a was ap-
220 peared lower in db/db vs. db/+ islets at 15 weeks of age, however,
221 this did not reach statistical significance (db/db: 1.1 ± 0.5 vs. db/+:
222 4.1 ± 1.9, $P = 0.13$, $N = 5$–6 islet preparations). Thus, although
223 speculative diabetic db/db mouse islets may have decreased
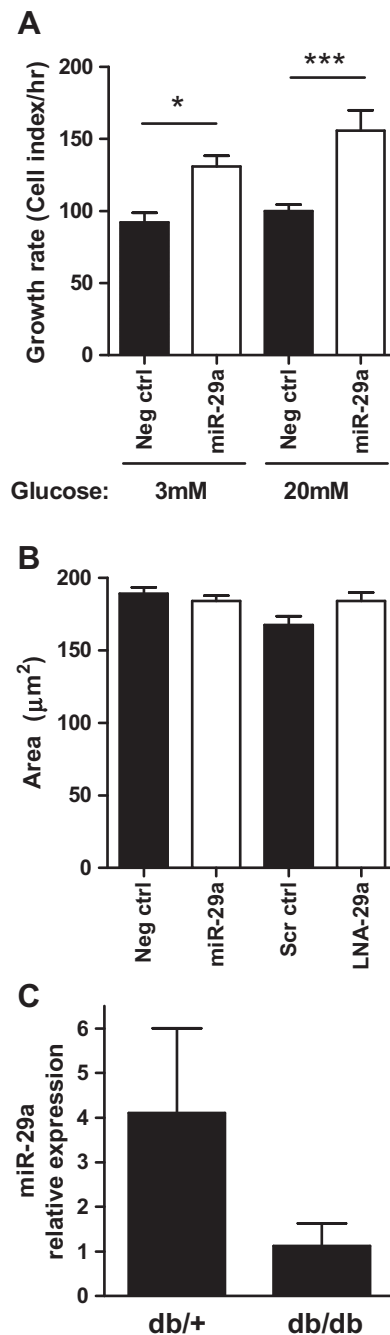224 miR-29a levels, which corresponds well with the very decreased

Fig. 3. Effects of miR-29a on proliferation and cell size of INS-1E beta-cells. (A) Proliferation rate (cell index) of nucleofected INS-1E cells assessed at 3 mM glucose medium and 20 mM glucose medium. (B) Cell area of nucleofected INS-1E cells was determined as described in research design and methods. Mean ± SEM from 3 independent experiments, $^*P < 0.05$, $^{***}P < 0.001$ as indicated by bars. (C) MiR-29a levels were assessed by RT-Q-PCR in isolated islets from db/db or db/+ mice aged 15 weeks.

225 proliferation of β-cells in this model, when diabetes has developed
226 [27]. However, further studies are needed to confirm this.

3.4. Effects of miR-29a over-expression and depletion on glucose-stimulated insulin secretion

229 To test if the glucose-induced up-regulation of miR-29a affected
230 insulin secretion, INS-1E beta-cells were transfected with miR-29a
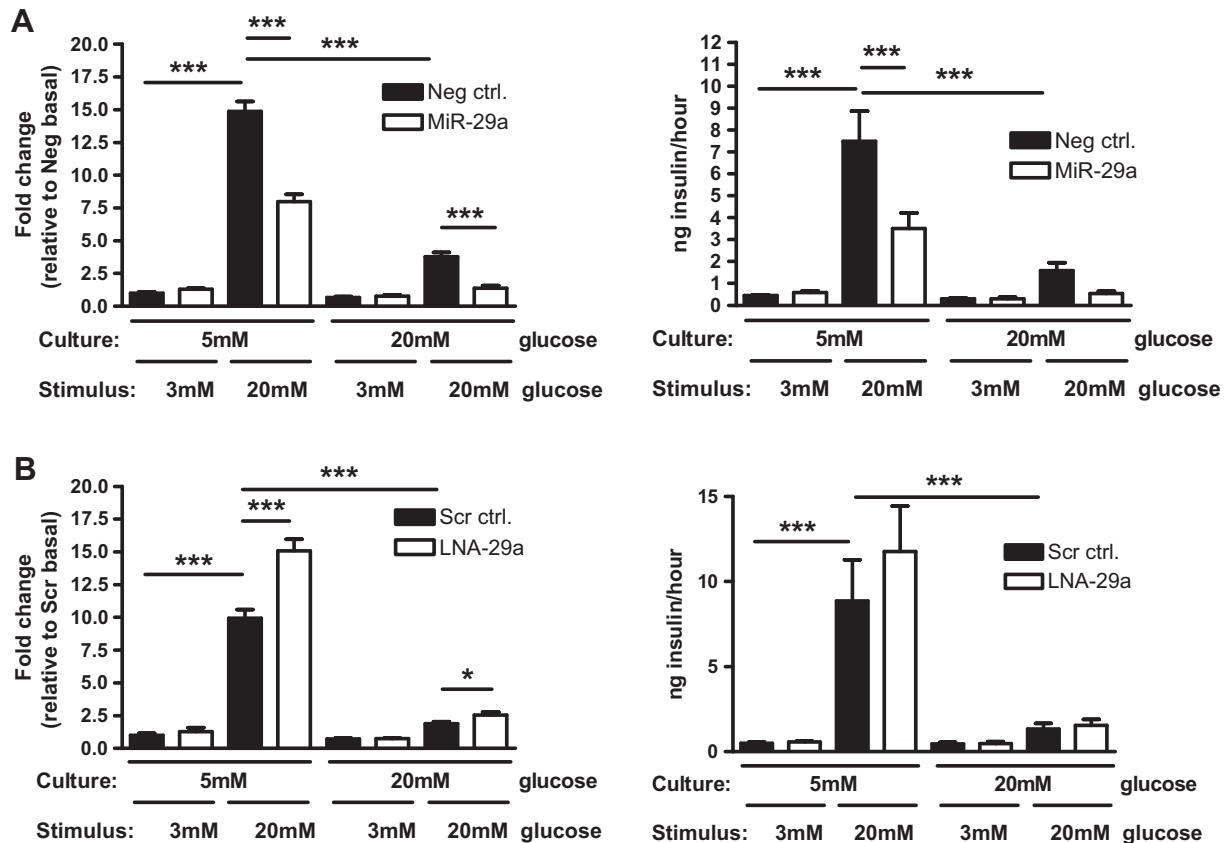231 mimic for over-expression or with LNA-modified antisense

Fig. 4. Glucose-stimulated insulin secretion in INS-1E cells. (A) Over-expression of miR-29a compared with negative ctrl. (Neg. ctrl.). INS-1E cells were nucleofected followed by culture in low (5 mM) or high (20 mM) glucose for 48 h ("Culture") before basal (3 mM glucose) or glucose stimulated (20 mM glucose) insulin secretion was determined ("stimulus") (ANOVA P-value <0.0001, N = 3 experiments). In order to facilitate comparison between individual experiments, each experiment was normalized to basal secretion in low glucose in Neg./Scr. ctrl. cells and relative values are shown on the left side of the figure while absolute values are shown on the right side of the figure. (B) MiR-29a LNA inhibitor compared with scrambled ctrl. (Scr. ctrl.). Cells were treated as described in A. Data is presented as mean ± SEM. (ANOVA P-value <0.0001, N = 4 experiments) (*P < 0.05, ***P < 0.001).

miR-29a for depletion of endogenous miR-29a. Cells were cultured in either 5 mM (low) or in 20 mM glucose (high) medium to induce glucose toxicity. INS-1E beta-cells cultured in 5 mM glucose elicit a robust insulin secretion response from 1.0 ± 0.2 to 14.9 ± 0.8-fold (P < 0.001) (Fig. 4A left side, Culture: 5 mM, miRNA mimic Neg. ctrl. transfection) or from 1.0 ± 0.2 to 9.9 ± 0.7-fold (P < 0.001) (Fig. 4B left side, Culture: 5 mM, LNA Scr. ctrl. transfection) when stimulated with 20 mM glucose compared with basal insulin secretion at 3 mM glucose. Absolute values for GSIS are displayed in the right side panels of Fig. 4.

Over-expression of miR-29a significantly decreased insulin secretion at 20 mM glucose (Fig. 4A left side, Culture: 5 mM, miR-29a: 8.0 ± 0.6-fold vs. Neg. ctrl.: 14.9 ± 0.8-fold of basal insulin secretion (P < 0.001)). The decrease in GSIS by miR-29a over-expression is similar to but not quite as large as the effect of chronically increased glucose concentration on insulin secretion in INS-1E beta-cells ((Fig. 4A left side, Culture: 5 mM Neg. ctrl.: 14.9 ± 0.8 vs. Culture: 20 mM Neg. ctrl.: 3.8 ± 0.3 (P < 0.001)) [28]. Moreover, over-expression of miR-29a further decreased GSIS (Fig. 4A left side, Culture: 20 mM, miR-29a: 1.4 ± 0.2-fold vs. Neg. ctrl.: 3.4 ± 0.3-fold of basal secretion (P < 0.001)).

Depletion of endogenous miR-29a in INS-1E cells cultured in 5 mM glucose markedly increased GSIS compared with Scr. ctrl. (Fig. 4B left side, Culture 5 mM, Scr. ctrl.: 9.9 ± 0.7-fold vs. LNA-29a: 15.1 ± 0.9-fold of basal insulin secretion, (P < 0.001)). There was a tendency that transfections of INS-1E cells with LNA-oligos decreased maximal GSIS, but compared with earlier observations

from our laboratory, this change was not significant [20]. Furthermore, inhibition of miR-29a in cells cultured with 20 mM glucose also increased GSIS slightly (Fig. 4B left side, Culture: 20 mM, LNA-Scr. ctrl.: 1.9 ± 0.1-fold vs. LNA-29a: 2.5 ± 0.2-fold of basal Scr. ctrl. upon 20 mM glucose stimulation (P < 0.05)), but due to variations between experiments, this was not significant when data are presented with absolute values (Fig. 4B, right side). Thus, miR-29a up-regulation impairs GSIS while its inhibition improves insulin secretion. The insulin content of cells was not determined, and it cannot be excluded that miR-29a also has an impact on insulin content. However, in the mouse beta-cell line MIN6, insulin content is not affected by miR-29a over-expression [29]. It is possible that mir-29a affects glucose uptake into beta-cells and in this way influences insulin secretion, but there are no predicted miR-29a targets which should influence glucose uptake or glycolysis, and the mRNA for the rate-limiting enzyme for glycolytic flux, glucokinase, does not change in response to miR-29a over-expression (Fig. 1S). However, in order to investigate this issue further, glucose transport and rate of glycolysis should be determined.

4. Discussion

Prolonged exposure of beta-cells to high levels of glucose decreases GSIS [2,30]. The current results show that over-expression of miR-29a, up-regulated by glucose in human islets of Langerhans and in INS-1E beta-cells, decreased GSIS markedly in INS-1E cells that normally exhibit a robust insulin secretion

response. Inhibition of miR-29a increases GSIS, suggesting that endogenous miR-29a exert a tonic inhibition on GSIS. Even though inhibition of miR-29a increased GSIS in cells cultured in 20 mM glucose, inhibition of endogenous miR-29a function did not compensate for the negative effects of high glucose on insulin secretion, which may be due to incomplete knock-down at increased glucose concentrations, where endogenous miR-29a levels are increased. However, it is likely that activation of other glucotoxic pathways such as UCP2 induction also takes place [31].

Increased glucose levels is a powerful mitogen for beta-cells [5]. Concordant with this miR-29a increased INS-1E cell growth. Therefore, one effect of glucose-induced up-regulation of miR-29a may be to promote proliferation as the demand for more beta-cells increases upon prolonged exposure to elevated levels of glucose. MiR-29a is preferentially expressed in β-cells in islets [26,32], and the possible lower level of miR-29a in db/db islets may also in part be due to a decreased number of beta-cells per islet in db/db diabetic islets.

It was recently shown that a certain normal level of miR-29a expression is critical for maintaining a low expression of monocarboxylate transporter 1 (Mct1) in beta-cells denying entrance of lactate into beta-cells [32]. Our data suggest that an increased amount of miR-29a is unfavorable for insulin secretion while it promotes beta-cell proliferation.

MiR-29 family members also promote apoptosis of mouse beta-cells [29]. Although there may be a species or cell-line difference, it is highly likely that the increased proliferation happens in context with increased cell death, which is a phenomenon of cancer cells [33].

Our results demonstrate that glucose-induced miR-29a impairs beta-cell function by decreasing GSIS. He et al. [17] showed that miR-29a up-regulation in adipocytes caused insulin resistance. As miR-29a has been shown to be up-regulated by glucose in several tissues [18] and increased in serum of type 2 diabetic patients [34] it is possible that miR-29a up-regulation promotes development of type 2 diabetes by at least two mechanisms: via decreased insulin secretion in beta-cells and via peripheral insulin resistance. This suggests that therapy with miR-29a LNA inhibitor could be beneficial for type 2 diabetic patients in order to improve both beta-cell function as well as glucose uptake in peripheral tissues. As shown recently, miR-29 family members may also be involved in cytokine-mediated beta-cell dysfunction [29].

The molecular mechanisms causing the alterations in normal function of beta-cells upon prolonged exposure to increased levels of glucose are still incompletely understood. Here, we show that forced expression of the glucose-up-regulated miR-29a mimics the effect of high glucose levels on beta-cells. Further, inhibition of miR-29a improves beta-cell function, even when beta-cell function is impaired by prolonged culture at increased glucose levels. At the same time, miR-29a over-expression increases beta-cell proliferation. Therefore, the up-regulation of miR-29a by glucose could be a link between glucose-induced proliferation and beta-cell dysfunction. These findings suggest that glucose-induced up-regulation of miR-29a may constitute a mechanism for beta-cell dysfunction and may contribute to the progression from impaired glucose tolerance to overt type 2 diabetes.

Acknowledgments

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.08.082.

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A. Bagge et al./Biochemical and Biophysical Research Communications xxx (2012) xxx–xxx

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