Regulation of *microRNA-375* by cAMP in Pancreatic β-Cells

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MicroRNA-375 (*miR-375*) is necessary for proper formation of pancreatic islets in vertebrates and is necessary for the development of β-cells in mice, but regulation of *miR-375* in these cells is poorly understood. Here, we show that *miR-375* is transcriptionally repressed by the cAMP-protein kinase A (PKA) pathway and that this repression is mediated through a block in RNA polymerase II binding to the *miR-375* promoter. cAMP analogs that are PKA selective repress *miR-375,* as do cAMP agonists and the glucagon-like peptide-1 receptor agonist, exendin-4. Repression of the *miR-375* precursor occurs rapidly in rat insulinoma INS-1 832/13 cells, within 15 min after cAMP stimulation, although the mature microRNA declines more slowly due to the kinetics of RNA processing. Repression of *miR-375* in isolated rat islets by exendin-4 also occurs slowly, after several hours of stimulation. Glucose is another reported antagonist of *miR-375* expression, although we demonstrate here that glucose does not target the microRNA through the PKA pathway. As reported previously, *miR-375* negatively regulates insulin secretion, and attenuation of *miR-375* through the cAMP-PKA pathway may boost the insulin response in pancreatic β-cells. **(*Molecular Endocrinology* 26: 989–999, 2012)**

Over the past decade, the realization that microRNA (miRNA) control gene expression has been a paradigm shift in molecular genetics. Many studies have now investigated the expression profiles of miRNA in a variety of cells and cellular systems (1–5), but relatively few studies have investigated the mechanism of transcriptional regulation for the miRNA themselves (6–10). This is an especially important consideration because several miRNA-based clinical trials are being conducted that target miRNA expression (11), and understanding the endogenous regulatory mechanisms will be key to predicting effects of these interventions *in vivo*. Most miRNA are processed through a series of endoribonuclease cleavage steps from larger RNA molecules, which are themselves transcribed via RNA polymerase II (RNAP II) promoters (12). These parental RNA molecules are called primary miRNAs (pri-miRNA) and can be either protein-coding or noncoding transcripts (13). Thus, the rate of miRNA transcription will depend upon the rate of transcription of the parental pri-miRNA gene. In several cases, signaling pathways that regulate miRNA expression have been found to target transcription factors that bind to the promoter of the pri-miRNA (7, 9, 10). However, for the majority of miRNA the regulatory mechanisms are poorly understood.

Few miRNA have been investigated in pancreatic β-cells (5, 14–17), and little is known concerning their transcriptional regulation (18, 19). miRNA are necessary for β-cell development, as deletion of the enzyme Dicer results in a severe loss of these cells (20). *MiR-375* is one of the most abundant miRNA in β-cells (5) and is necessary for their proper development and maintenance (21, 22). In zebrafish, a model for vertebrate development, targeted disruption of *miR-375* by morpholinos severely

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Abbreviations: 6-Bnz-cAMP, N^6-Benzoyladenosine 3′,5′-monophosphate; ChIP, chromatin immunoprecipitation; 8-CPT-cAMP, 8-(4-chlorophenylthio)-adenosine 3′,5′-monophosphate; CREB, cAMP response element binding protein; CREM, cAMP response element modulator; db-cAMP, dibutyryladenosine 3′,5′-monophosphate; DMSO, dimethylsulfoxide; EPAC2, exchange protein directly activated by cAMP 2; FBS, fetal bovine serum; GFP, green fluorescent protein; GLP-1, glucagon-like peptide-1; *Glut2*, glucose transporter 2; GSIS, glucose-stimulated insulin secretion; GST, glutathione-*S*-transferase; HBSS, Hank's balanced salt solution; IBMX 3-isobutyl-1-methylxanthine; PKA, protein kinase A; PKI, PKA inhibitor; pre-*miR-375, miR-375* stem-loop precursor; pri-miRNA, primary miRNA; qRT-PCR, quantitative real-time RT-PCR; RNAP II, RNA polymerase II; SDS, sodium dodecyl sulfate; snRNA, small nuclear RNA; SSC, saline sodium citrate.

disrupts normal islet architecture (21). *MiR-375* knockout mice have a 30–40% reduction in β-cell mass compared with wild-type controls, whereas they exhibit an increase in α-cell mass (22). Increased levels of circulating glucagon in these mice lead to chronic hyperglycemia and the development of diabetes. In β-cell line cultures, *miR-375* inhibits insulin secretion, in part by inhibiting the translation of the mRNA for myotrophin (*5*) and phosphoinositide 3-kinase-dependent-kinase (*Pdk1*) (23). It is not clear, however, how blood sugar levels in diabetic patients would respond to *miR-375*-directed treatment. Based upon the current literature, one would predict that decreasing the amount of *miR-375* in diabetic patients would decrease glucose levels by stimulating insulin release after a meal. Thus, discovering cellular mechanisms that repress *miR-375* may lead to new avenues of type 2 diabetes therapy research.

cAMP is a key second messenger molecule in β-cells, because it potentiates glucose- and Ca^{2+}-dependent insulin secretion (24). cAMP is elevated in β-cells by various hormones, neurotransmitters, and other nutrient secretagogues, including the intestinal peptide hormones, glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide which enhance glucose-stimulated insulin secretion (GSIS) (25–27). A high-affinity agonist of the GLP-1 receptor, exendin-4, has received particular attention because it is now used clinically to ameliorate episodes of hyperglycemia in diabetic patients and to delay gastric emptying leading to reduced appetite (25, 28). Rises in cAMP concentration can also be triggered by rises in intracellular Ca^{2+} levels that occur after exposure to glucose (29, 30), as there are Ca^{2+}/calmodulin-stimulated adenylyl cyclases (31).

In β-cells, there are two types of cAMP-binding proteins that stimulate insulin secretion, protein kinase A (PKA) and exchange protein directly activated by cAMP 2/cAMP-guanine nucleotide exchange factor II (EPAC2/cAMP-GEFII) (24). Both these effector proteins directly target components of the exocytotic machinery and membrane channels responsible for β-cell depolarization. In addition to its role in exocytosis, cAMP also influences gene expression in β-cells (32) although, to date, only PKA (and not EPAC2) has been implicated in this process. The most ubiquitous cAMP-responsive transcription factor is cAMP-response-element binding protein (CREB) (32). CREB is phosphorylated by PKA, creating a binding site for the transcriptional coactivators CREB-binding protein or p300 (33, 34). Genes under control of CREB in β-cells include insulin (35) and *Irs2* (36). The reality is no doubt more complex, as studies in other cell types have shown that thousands of genes are concurrently regulated by CREB (37, 38).

In addition to gene activation, several studies in β-cells have shown that genes are down-regulated in response to cAMP as well (39–41). In one study, several genes necessary for insulin secretion were repressed by hyperglycemic conditions in a PKA-dependent manner (39). It was hypothesized that this mechanism could contribute to β-cell failure in type 2 diabetes. cAMP-mediated transcriptional repression occurs via the protein cAMP response element modulator (CREM) (32). In this study, we show that transcription of *miR-375* is strongly repressed by the cAMP-PKA pathway, and we propose that exendin-4 may boost insulin release in part through effects on *miR-375*.

Materials and Methods

Cell culture

Rat insulinoma INS-1 832/13 cells were a gift from Dr. Chris Newgard (Duke University Medical Center, Durham, NC). Cells were grown in RPMI-1640 media (Hyclone Laboratories, Logan, UT) containing 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, 100 U/ml streptomycin, 1 mM sodium pyruvate, and 50 μM β-mercaptoethanol, and were grown at 37 C in a humidified chamber containing 5% CO_2. Cells were transfected using the Amaxa Nucleofector electroporator (Lonza Group, Basel, Switzerland). The PKA-inhibitor (PKI) cDNA was a gift from Dr. Richard Maurer (Oregon Health & Science University).

Rat pancreatic islet preparation

Male Sprague Dawley rats aged 6 months were used for pancreatic islet preparations. Cold Hank's Balanced Salt Solution (HBSS, 5 ml) containing 1 g/liter glucose and 100 U/ml Liberase TL (Roche Applied Sciences, Indianapolis, IN) were injected into the pancreas via the common bile duct. The pancreas was dissected and placed into 10 ml cold HBSS, minced, and incubated at 37 C for 20 min. The suspension was vigorously shaken, and the reaction was stopped by adding 30 ml cold quenching buffer (HBSS + 10% FBS). The suspension was pelleted via centrifugation at 500 × *g* and washed an additional time with quenching buffer. Islets were visualized with 0.5 mg/ml diphenylthiocarbazone (Sigma-Aldrich, St. Louis, MO) and successively hand picked into quenching buffer and then into RPMI 1640 media containing 1 g/liter glucose, 2% FBS, 100 U/ml penicillin, and 100 U/ml streptomycin.

Reverse transcription (RT)

INS-1 832/13 cells were plated onto 12-well plates and grown overnight (5×10^5 per well). The cells were then incubated overnight in 2% FBS and 2 mM glucose unless otherwise noted before treatment with cAMP or cAMP agonists at the concentrations and times listed in the figure legends. Freshly isolated islets (25–30 per treatment) were incubated immediately with cAMP agonists. Forskolin, 3-isobutyl-1-methylxanthine (IBMX), exendin-4, H89 dihydrochloride hydrate, and dibutyryladenosine 3′,5′-monophosphate (db-cAMP) were

from Sigma-Aldrich. N^6-Benzoyladenosine 3′,5′-monophosphate (6-Bnz-cAMP), N^6-monobutyryladenosine 3′,5′-monophosphate, 8-(4-chlorophenylthio)-adenosine 3′,5′-monophosphate (8-CPT-cAMP) were from Biolog (Bremen, Germany). Total RNA was isolated by extraction with TRI reagent (Ambion, Austin, TX), and 500 ng were used for first-strand cDNA synthesis in a reaction containing 50 ng of random primers, 500 μM deoxynucleotide triphosphate, 1× first-strand buffer, 200 U of Moloney murine leukemia virus reverse transcriptase (Promega Corp., Madison, WI), and 20 U of RNasin ribonuclease inhibitor (Promega) in a 25-μl reaction volume. Samples were diluted 1:4 before using in quantitative PCR. Total RNA (100 ng) was used in the *microRNA-375* TaqMan assay (Life Technologies, Carlsbad, CA) using the manufacturer's reaction conditions. Samples were diluted 1:9 before using in quantitative PCR.

Chromatin immunoprecipitation (ChIP)

INS-1 832/13 cells (5×10^6) were plated on 10-cm dishes and the next day were treated with 0.1% dimethylsulfoxide (DMSO) control or a combination of 1 μM forskolin and 100 μM IBMX for 1 h. The ChIP assay was performed as described elsewhere (19) using anti-RNA polymerase II phosphoserine 2 and phosphoserine 5 polyclonal antibodies (Abcam, Cambridge, MA) and rabbit IgG (Sigma) as a control.

Quantitative PCR

ChIP DNA or cDNA (3 μl) was analyzed in a 15 μl real-time PCR (qPCR) containing 1× SYBR green mix (Thermo Scientific, Houston, TX) and 0.5 μM primer pairs. Reactions were run in a Realplex 2 (Eppendorf, Hamburg, Germany) for 15 min at 95 C, followed by 40 cycles of 15 sec at 95 C and 45 sec at 68 C. Primers against pre-*miR-375* (5′-CCTCGCACAAACCGGACCT-3′ and 5′-GCCTCACGCGAGCCGAAC-3′) were used for both RT and ChIP assays. Additional ChIP primers included *miR-375* upstream (5′-TCCTATCCCTGCCCTCCAGCTTT-3′ and 5′-CTTCACCATCCTCTTGCCCTGCT-3′), *miR-375* downstream (5′-TCTAACTCCCATCTCCTGTTCTTT-3′ and 5′-AGAGAGAAACCAGACACATGCAC-3′), and *MyoD* promoter (5′-GACGCGACTGCTTTCTTCACCAC-3′ and 5′-TTCCCTCCCTGTCCTGTCCTGTC-3′). Additional RT primers included 18S rRNA (5′-CGGACACGGACAGGATTGACAGA-3′ and 5′-ACCACCCACGGAATCGAGAAAGA-3′), U6 small nuclear RNA (snRNA) (5′-TTCGGCAGCACATATACTAAAATTGGA-3′ and 5′-AGGGGCCATGCTAATCTTCTCTGT-3′),*Nr4a2*(5′-GGACAGGTAGTTGGGTCGGTTCAA-3′ and 5′-AGAGACACGGGCTCAAGGAACC-3′), and glucose transporter (*Glut2*) (5′-TCACACCAGCACATACGACACCA-3′ and 5′-GACCATTCCGCCTACTGCAAAGC-3′). All primers and probes were from Integrated DNA Technologies (Coralville, IA). Data were from three independent experiments each repeated in triplicate unless otherwise noted. Statistics were performed using pair-wise, one-tailed Student's *t* tests.

Northern blots

Total RNA (10 μg) (from INS-1 832/13 cells was loaded onto a 15% polyacrylamide-urea denaturing gel and then transferred onto GeneScreen Plus hybridization transfer membrane (PerkinElmer, Waltham, MA) using a Trans-Blot SD Semi-Dry Transfer apparatus (Bio-Rad Laboratories, Inc., Hercules, CA). The RNA was cross-linked to the membrane using a Stratalinker UV cross-linker (Agilent Technologies, Santa Clara, CA) using the manufacturer's optimal setting (~45 sec) and then baked at 80 C in a vacuum oven for 30 min. Antisense locked-nucleic acid oligonucleotide probe (*miR-375*) or deoxyribonucleotide probe (U6 snRNA) was labeled using γ-^{32}P-ATP and T4 polynucleotide kinase (Promega). Membranes were incubated with probes overnight at 42 C in hybridization solution [5× saline sodium citrate (SSC), 20 mM Na_2HPO_4, 7% sodium dodecyl sulfate (SDS), 2× Denhardt's, and 0.04 mg/ml sheared salmon sperm DNA], and then washed in low-stringency wash buffer (2× SSC, 0.5% SDS) and high-stringency wash buffer (0.05× SSC, 1% SDS) for 30 min each. Washed membranes were then exposed to autoradiography film for several hours at −80 C.

cAMP RIA

INS-1 832/13 cells (5×10^5) or islets (25–30) were plated onto 12-well plates and grown overnight. Cells were treated with the indicated cAMP agonists, and RIA was performed according to the manufacturer's instructions (Linco Research, St. Charles, MO).

Rap1 activation assay

Active GTP-bound Rap1 was assayed with the glutathione-*S*-transferase (GST)-tagged Ras-binding domain of RalGDS as previously described (42). Monoclonal Rap1 antibody was from BD Transduction Laboratories (Franklin Lakes, NJ). GST-tagged RalGDS was a gift from Dr. Philip Stork (Oregon Health & Science University).

Results

The *miR-375* stem-loop precursor is repressed by cAMP-elevating agents

Previous work (19) suggested that *miR-375* might be regulated by cAMP. The *miR-375* proximal promoter contains a similar collection of transcription factor-binding sites as that of insulin, which is regulated by cAMP through CREB. To test whether *miR-375* was in fact regulated by cAMP, we treated rat insulinoma INS-1 832/13 (hereafter called INS-1) cells with forskolin, an adenylyl cyclase agonist (43), for 1 h and measured changes in the *miR-375* stem-loop precursor (pre-*miR-375*) expression levels by quantitative real-time RT-PCR (qRT-PCR). Surprisingly, pre-*miR-375* levels were reduced by forskolin treatment (Fig. 1A) in contrast to most known CREB target genes (32). Another cAMP-elevating drug, IBMX, which broadly inhibits phosphodiesterases and therefore inhibits cAMP degradation (44), also strongly repressed levels of pre-*miR-375* (Fig. 1A). El Ouaamari *et al.* (23) demonstrated that pre-*miR-375* was depleted by elevated glucose levels in INS-1 cells and islets. To isolate the effects of cAMP from glucose, cAMP agonists were added

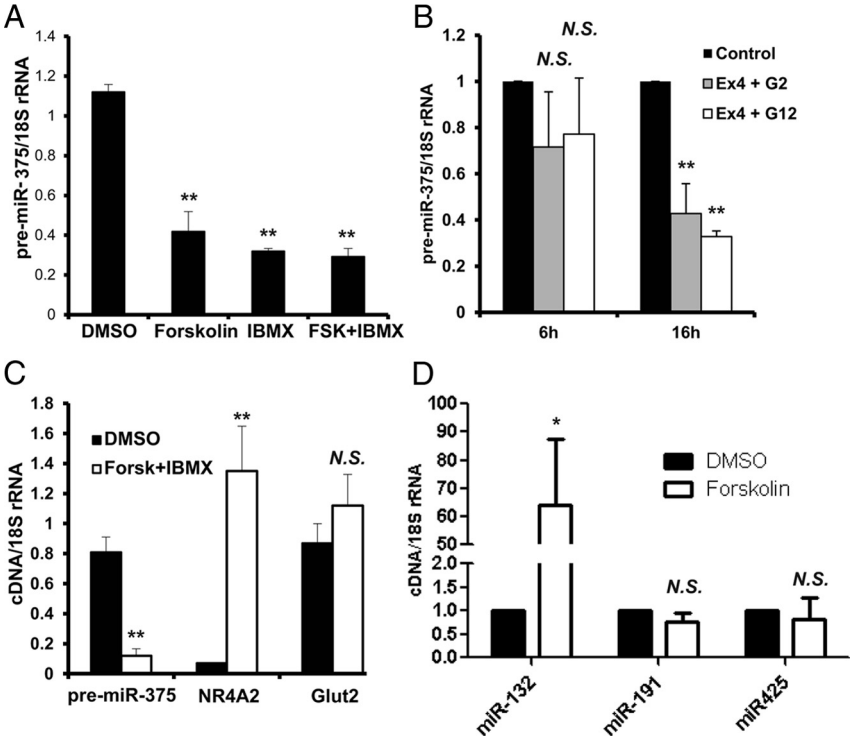

FIG. 1. The *miR-375* stem-loop precursor is repressed by cAMP-elevating agents. A, Rat insulinoma INS-1 832/13 cells were treated for 1 h with either vehicle control (DMSO), 10 μM forskolin (FSK), 100 μM IBMX, or a combination. qRT-PCR was performed using primers amplifying pre-*miR-375*. B, Pancreatic islets were isolated from 6-month-old Sprague Dawley rats and treated *in vitro* with or without 10 nM exendin-4 (Ex4) and 2 mM (G2) or 12 mM glucose (G12) as indicated for 6 and 16 h. The islets were then harvested for qRT-PCR. C, Pancreatic islets were treated with DMSO vehicle (*black bars*) or 1 μM FSK and 100 μM IBMX (*white bars*) for 1 h. qRT-PCR was performed using primers amplifying pre-*miR-375*, cAMP-responsive immediate early gene *Nr4a2*, or *Glut2*. D, INS-1 832/13 cells were treated for 1 h with either vehicle control DMSO or with 1 μM FSK and 100 μM IBMX combination. qRT-PCR was performed using primers amplifying pre-*miR-132*, pre-*miR-191*, and pre-*miR-425*. Samples were normalized to 18S rRNA or U6 snRNA, and data represent three independent experiments performed in duplicate. *Error bars* represent SD. Student's *t* tests were performed for each sample in comparison to control. N.S., $P > 0.05$; *, $P < 0.05$; **, $P < 0.01$.

in the presence of low glucose (2 mM) unless otherwise noted.

To test whether the entire *miR-375* locus is repressed by cAMP, we designed additional PCR primers against the predicted primary *miR-375* sequence (pri-*miR-375*) and used them in qRT-PCR. The results showed that pri-*miR-375* is depleted to the same extent as is pre-*miR-375* (Supplemental Fig. 1 published on The Endocrine Society's Journals Online web site at http://mend.endojournals.org), suggesting that the promoter of the pri-*miRNA* gene might be targeted by cAMP. Although forskolin is commonly used to stimulate cAMP levels in cells, it has off-target effects at a dosage close to that being used in this study (43, 45). 1,9-Dideoxyforskolin does not activate adenylyl cyclase but retains a similar spectrum of off-target effects as forskolin (43). Treatment of INS-1 cells with dideoxyforskolin had no effect on pre-*miR-375* (Supplemental Fig. 2), suggesting that the production of cAMP was responsible for the decrease in the miRNA.

Rat INS-1 cells are transformed and thus might not reflect the regulation in native islet cells. Additionally, their insulin release response to glucose and cAMP is blunted when compared with that of isolated pancreatic islets. Using a cAMP RIA, we compared levels of cAMP in INS-1 cells and pancreatic islets. Combined treatment with 1 μM forskolin and 100 μM IBMX raised cAMP levels 2-fold in INS-1 cells but more than 4-fold in pancreatic islets (Supplemental Fig. 3). To more accurately mimic conditions *in vivo*, we tested pre-*miR-375* depletion in isolated pancreatic islets with a physiological cAMP agonist, exendin-4. Exendin-4 stimulates cAMP production through binding and activating the glucagon-like peptide 1 receptor. Islets were isolated from adult rats and were treated with 10 nM exendin-4 for 6 or 16 h (Fig. 1B). Repression of pre-*miR-375* was first seen at 6 h and was significantly down-regulated by 16 h. In addition, no significant synergistic repression was seen when the exendin-4 treatment was combined with elevated glucose (12 mM, Fig. 1B), suggesting that the two pathways were converging on a single downstream target.

The reduction of pre-*miR-375* in response to stimuli is in marked contrast to CREB-regulated immediate-early genes such as *Nr4a2* (Fig. 1C), *c-Fos*, and *Mkp-1* (data not shown), which are up-regulated in islets by cAMP. The CREB-regulated miRNA *miR-132* (46) is also dramatically up-regulated by forskolin (Fig. 1D), which provides support that CREB is not targeting the *miR-375* promoter. *Glut2* serves as a control gene that is not significantly changed by forskolin after 1 h (Fig. 1C). miRNA *miR-191* and *-425* are bi-cistronic miRNA that are targeted by some of the same transcription factors as *miR-375* (19) but are not regulated by cAMP (Fig. 1D). Thus it appears that the cAMP-mediated repression of *miR-375* is specific for this miRNA.

Time course of *miR-375* repression by cAMP

Mature miRNA and the precursors from which they are derived can have dramatically different turnover rates

and thus *miR-375* and pre-*miR-375* may exhibit different turnover kinetics in response to cAMP. To determine the kinetics of pre-*miR-375* repression, we added forskolin and IBMX to INS-1 cells, extracted RNA at various times, and measured pre-*miR-375* levels by qRT-PCR. Figure 2A shows that the half-life is less than 15 min. 18S rRNA levels did not vary during the time course of the experiment (data not shown) and so were used to normalize data. Pre-*miR-375* never fell below the level of detection during the time course but instead reached a baseline below which it did not fall. Because mature miRNA are 19–25 nucleotides in length, conventional PCR cannot be used to detect them. Instead, we used a Northern blot in Fig. 2B to detect mature *miR-375*. In contrast to the precursor, mature *miR-375* gradually decreased over the course of the experiment, with a half-life between 8 and 16 h (Fig. 2B).

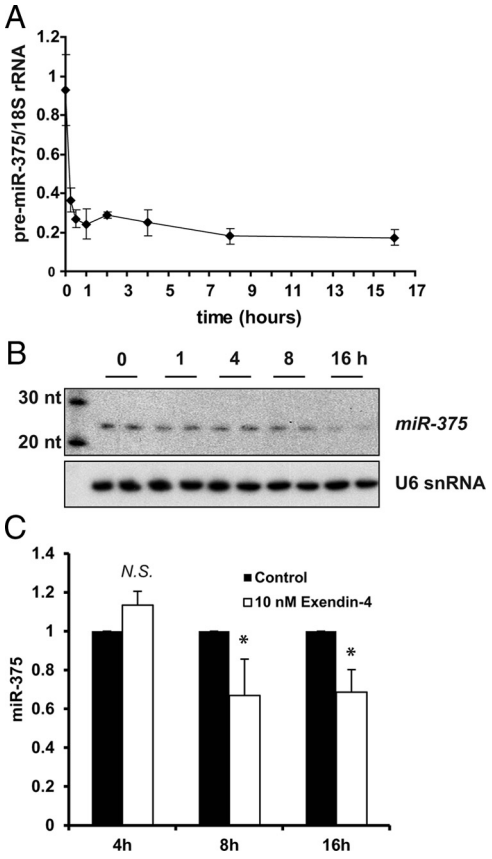

FIG. 2. The *miR-375* precursor is rapidly repressed by cAMP whereas processed mature *miR-375* shows delayed repression kinetics. A, INS-1 832/13 cells were treated for the indicated times with 1 μM forskolin and 100 μM IBMX. Primers amplifying pre-*miR-375* were used in qRT-PCR, and the data were normalized to 18S rRNA. *Error bars* represent SD (n = 3). B, INS-1 832/13 cells were treated as above, total RNA was extracted, and 10 μg was analyzed by PAGE. Northern blots were performed using radioactive probes against mature *miR-375* or U6 snRNA as a control. An RNA sizing ladder was radioactively labeled and run in the first lane. The samples are run in duplicate, and the data are representative of two independent experiments. C, The final processed *miR-375* was measured by TaqMan assay from rat pancreatic islets treated with 10 nM exendin-4 for the indicated times. Data are graphed as fold change with samples matched to controls at each time point. *Error bars* represent SD and the data represent the average of three independent experiments performed in triplicate. Student's *t* tests were performed for each sample in comparison with control. N.S., $P > 0.05$; *, $P < 0.05$; nt, nucleotide.

These data were supported by quantitatively measuring mature *miR-375* by a TaqMan real-time PCR assay. Rat pancreatic islets were isolated and treated with 10 nM exendin-4 for the indicated times (Fig. 2C), revealing a half-life of approximately 8 h.

Repression of pre-*miR-375* by cAMP occurs at the transcriptional level

If the *miR-375* precursor is short-lived, the rapid repression kinetics seen above suggested that it might be regulated at the transcriptional level. To test this possibility, INS-1 cells were treated with the transcriptional blocker, actinomycin D, either with or without forskolin and IBMX (Fig. 3A). As shown in the figure, actinomycin D depleted pre-*miR-375* with similar kinetics as forskolin and IBMX, suggesting that cAMP inhibited *miR-375* transcription.

Further evidence of a transcriptional block was obtained by ChIP using phospho-specific antibodies against the C-terminal domain of RNAP II. Serines 2 and 5 within the heptad amino acid repeat are phosphorylated selectively during transcription elongation and initiation, respectively. Using antibodies against these phosphorylation sites allowed us to determine whether transcription initiation or elongation or both were inhibited by cAMP agonists. At the precursor site/proximal promoter site 333 bp downstream from the transcription start site, initiation-selective phospho-Ser 5 and elongation-selective phospho-Ser 2 signals are reduced after treatment with 1 μM forskolin and 100 μM IBMX for 1 h (Fig. 3B, *upper left graph*). This suggests that promoter occupancy by RNAP II and transcription initiation are reduced after treatment with cAMP agonists. At the distal site 2609 bp downstream from the transcription start site, no detectable phospho-Ser 5 or -Ser 2 RNAP II signal was observed after cAMP agonist treatment (Fig. 3B, *upper right graph*), again supporting the idea that transcription initiation is inhibited. Control primer pairs against the inactive *MyoD* promoter and upstream of *miR-375* show no significant binding of RNAP II (Fig. 3B, *lower graphs*).

Repression of pre-*miR-375* is mediated by PKA

There are two major proteins that directly bind cAMP in pancreatic β-cells and mediate its effects. One of these is EPAC2, which works by stimulating the exocytotic machinery (47). Another is PKA, which phosphorylates and stimulates many proteins that regulate secretion (24) as

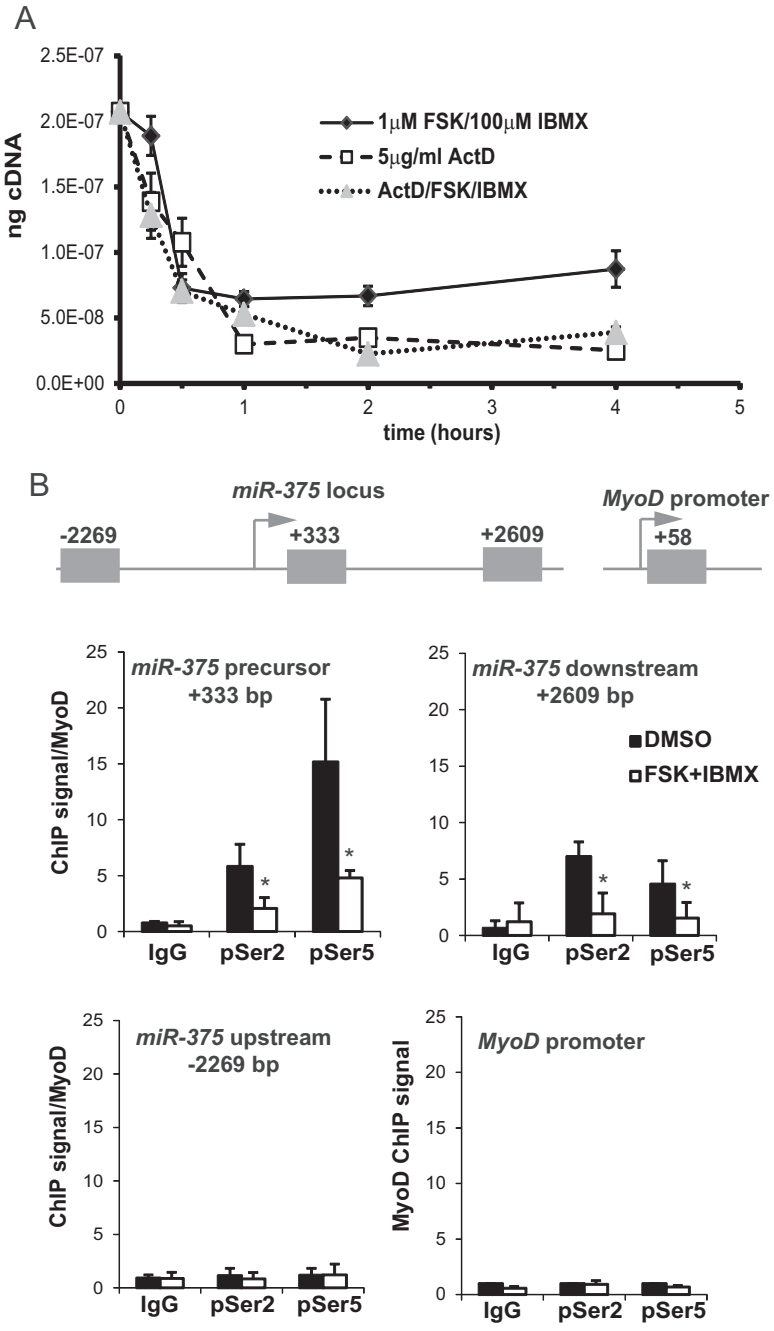

FIG. 3. Repression of pre-*miR-375* by cAMP occurs at the transcriptional level. A, INS-1 832/13 cells were treated for the indicated times with either forskolin (FSK, 1 μM) and IBMX (100 μM), actinomycin D (ActD, 5 μg/ml), or all three. Primers amplifying pre-*miR-375* were used in qRT-PCR. B, Cells were treated with vehicle control (DMSO) or FSK and IBMX as above for 1 h and then harvested for ChIP assay. Antibodies (0.5 μg) against control rabbit IgG, elongation-enriched RNAP II phosphoserine 2, or initiation-enriched phosphoserine 5 were used. Primers were used to amplify segments of the *miR-375* locus including an upstream region (−2269 bp), the precursor/promoter region (+333 bp), and a downstream region (+2609). Primers against the *MyoD* promoter were used as a negative control, and data were normalized to this signal. *Error bars* represent SD, and the data represent the average of three independent experiments performed in triplicate.*, $P < 0.05$.

well as proteins that regulate gene expression, *e.g.* CREB (32). One or both of these pathways may target *miR-375*. One way to distinguish between these two pathways is to use cAMP analogs that selectively bind to one or the other (48). 6-Bnz-cAMP and 6-MB-cAMP are cell-permeable cAMP analogs that bind selectively to PKA and were found to deplete pre-*miR-375* in INS-1 cells (Fig. 4A, lanes 5–7; and Supplemental Fig. 4A). A nonselective cAMP analog, db-cAMP, also depleted pre-*miR-375* in a dose-responsive fashion (Fig. 4, lanes 2–4). In contrast, two EPAC-selective cAMP analogs, 8-pCPT-cAMP and 8-pMeOPT-cAMP, did not affect pre-*miR-375* (Supplemental Fig. 4A). The effectiveness of the EPAC-selective cAMP analogs was tested by an *in vitro* protein-binding assay (42). In this assay, we incubated INS-1 cells with EPAC-selective cAMP analogs to stimulate the guanine-exchange factor activity of EPAC for its substrate, the small G protein Rap1. We prepared cell extracts and added recombinantly produced GST-RalGDS to the extracts as bait to pull down EPAC-activated Rap1-GTP. Addition of 8-pCPT-cAMP and 8-pMeOPT-cAMP stimulated binding of Rap1 to GST-RalGDS as detected by Western blot for Rap1 (Supplemental Fig. 4B), indicating that the EPAC-selective cAMP analogs were active, despite having no effect on *miR-375* levels. Increasing amounts of forskolin were also used to demonstrate the sensitivity of the assay (Supplemental Fig. 4B). Although the PKA-selective cAMP analogs were not as potent as nonselective, the differences can be attributed to their varying degrees of lipophilicity, because db-cAMP is approximately 3 times more lipophilic than 6-Bnz-cAMP, and 10 times more lipophilic than N^6-monobutyryladenosine 3′,5′-monophosphate (48). In contrast, EPAC-selective cAMP analogs are 2–3 times more lipophilic than db-cAMP. Thus through the use of these cAMP analogs, it appears that cAMP represses *miR-375* through the PKA pathway.

To obtain additional support for the cAMP-PKA model, we treated the cells with PKA inhibitors and mea-

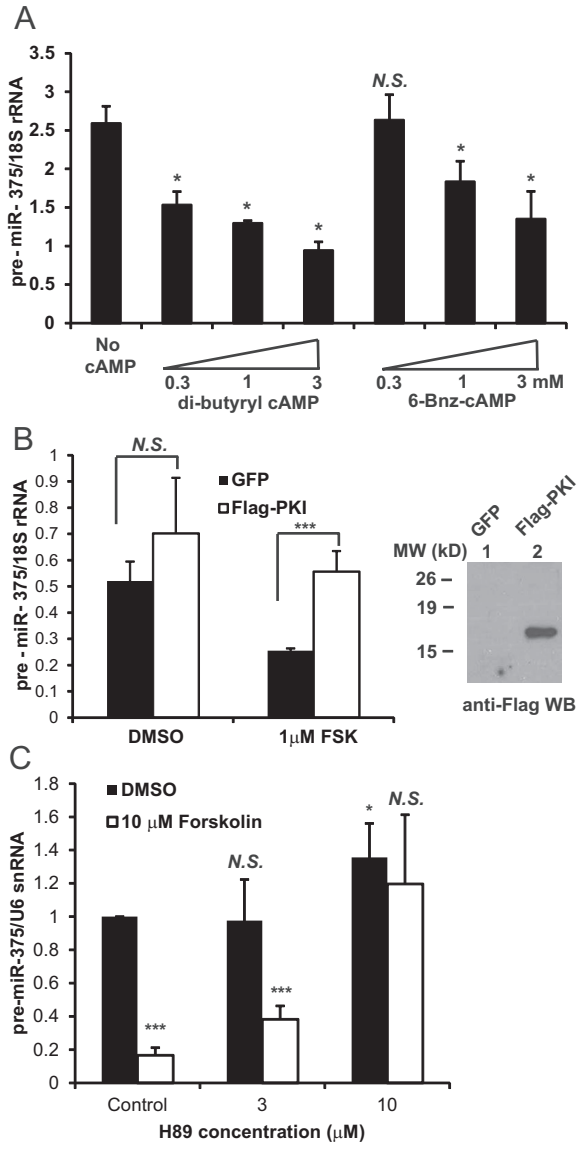

FIG. 4. Repression of pre-*miR-375* is mediated by PKA. A, INS-1 832/13 cells were treated for 1 h with membrane-permeable cAMP analogs as indicated. Cells were treated with either nonselective db-cAMP or PKA-selective 6-Bnz-cAMP at the concentrations indicated. B, INS-1 cells were transfected with DNA plasmids encoding either GFP or the PKA inhibitor PKI (Flag-PKI). After 48 h, the cells were treated for 1 h with either vehicle control (DMSO) or 1 μM forskolin (FSK). An anti-Flag Western blot (WB) shows Flag-PKI expression. C, INS-1 cells were treated for 20 h with increasing doses of H89 as indicated, followed by 1-h treatment with either vehicle control (DMSO) or 10 μM FSK. Primers amplifying pre-*miR-375* were used in qRT-PCR, and the data were normalized to 18S rRNA or U6 snRNA. *Error bars* represent SD (n = 3). In panels A and C, Student's *t* tests were performed for each sample in comparison with control. In panel B, a Student's *t* test was performed comparing samples as shown. N.S., $P > 0.05$. *, $P < 0.05$; ***, $P < 0.001$. MW, Molecular weight.

sured pre-*miR-375* in response to forskolin. First, we transfected INS-1 cells with a plasmid encoding the PKA-inhibitor (PKI) gene, the product of which was originally isolated from skeletal muscle (49) and which has no effect on EPAC (24). We transfected a mammalian expression vector containing PKI cDNA (Flag-PKI) into INS-1 cells and, 48 h later, treated the cells with or without forskolin for 1 h to stimulate cAMP production. We transfected control INS-1 cells with an expression vector containing green fluorescent protein (GFP) cDNA. As shown in Fig. 4B, addition of PKI to the cells blocked the depletion of pre-*miR-375* by cAMP, arguing that *miR-375* is under the regulatory control of PKA. Similar results were found when the cells were treated with a pharmacological inhibitor of PKA, H89. Cells were treated overnight with increased doses of H89 followed by a 1-h treatment with or without forskolin. The repression of pre-*miR-375* was relieved by H89 in a dose-dependent manner (Fig. 4C), again arguing that PKA is mediating repression of this miRNA. Interestingly, treatment with 10 μM H89 alone resulted in a significant increase in basal pre-*miR-375* levels, suggesting that there is a low level of PKA activity in these cells in the basal state. This is supported by our cAMP RIA (Supplemental Fig. 3B), which shows that INS-1 cells have detectable cAMP levels in the unstimulated state.

Glucose does not signal through PKA to repress *miR-375*

El Ouaamari *et al.* (23) demonstrated that pre-*miR-375* was depleted by elevated glucose levels in INS-1 cells and cultured islets, although the signaling pathway that mediates this effect is not known. cAMP potentiates GSIS, and the two have been reported to synergistically activate gene transcription (50). Additionally, CREM transcriptional repressors have been reported to be elevated by high glucose levels in cultured islets (39, 41). However, there is conflicting evidence as to whether glucose itself stimulates cAMP production in cells (31). To test whether glucose represses *miR-375* through the PKA pathway, we transfected INS-1 cells with the PKI plasmid and then incubated them in 20 mM glucose for 16 h, the time point that showed the maximal repression (data not shown). PKI expression had no effect on pre-*miR-375* repression due to glucose, suggesting that glucose is not regulating PKA directly (Fig. 5A). In a similar manner to H89 alone (Fig. 4C), PKI did cause a slight increase in basal expression of pre-*miR-375* after 16 h (Fig. 5A), again suggesting that there is a low level of PKA activity in these cells in the basal state. As a control, elevation of *Glut2* mRNA by glucose was attenuated by PKI (Fig. 5B), illustrating that the glucose and cAMP can work in concert in these cells, at least indirectly.

Discussion

Incretin hormones such as GLP-1 stimulate cAMP production in pancreatic β-cells, rapidly potentiating GSIS,

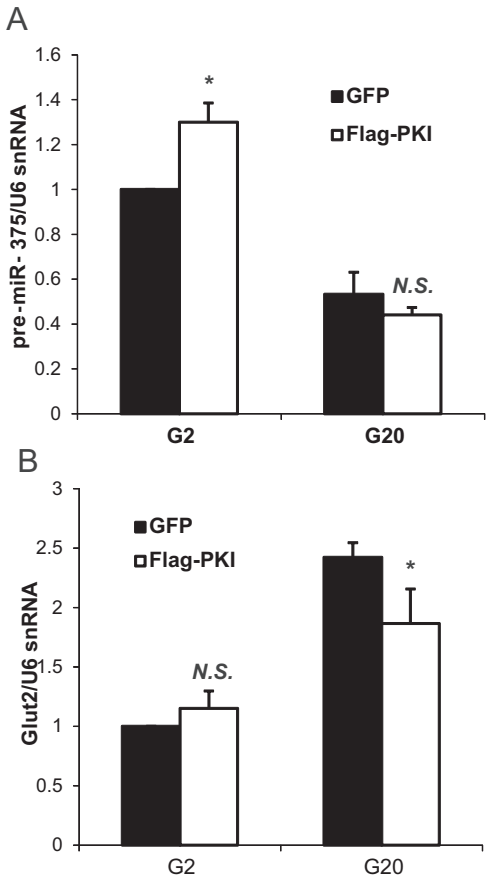

FIG. 5. Glucose does not repress pre-*miR-375* through the cAMP-PKA pathway. INS-1 832/13 cells were transfected with DNA plasmids encoding either GFP or Flag-PKI and 24 h later were pretreated with growth media containing 2 mM glucose and 2% FBS for 24 h. Half the cells were then treated for an additional 16 h with 20 mM glucose after which all cells were harvested. Quantitative RT-PCR was performed using primers for pre-*miR-375* (A) or *Glut2* mRNA (B), and the data were normalized to U6 snRNA. *Error bars* represent SD, and the data represent the average of three independent experiments performed in triplicate. N.S., $P > 0.05$. *, $P < 0.05$.

but also initiating changes in genetic programs that influence the future secretory response of the cells (12). We have identified *miR-375* as a target of cAMP signaling and propose that one way GLP-1 regulates the plasticity of β-cells is through the regulation of *miR-375*, the targets of which may number in the hundreds (51, 52). We hypothesized that the mechanism by which this occurs is through transcriptional repression in response to cAMP and that PKA is mediating this repression. In support of this idea, we found that PKA-selective cAMP analogs repress pre-*miR-375* in a dose-responsive fashion and that inhibitors of PKA attenuate this repression (Fig. 4).

Although the study of miRNA transcription regulation is still an emerging field, it appears that miRNA are transcribed by RNAP II and are regulated in a similar fashion as other class II genes (53, 54). In support of this model, a *miR-375* promoter has been identified that binds the transcription factors Pdx-1 and NeuroD1 (19) and that contains a TATA box and confines *miR-375* expression to the pancreatic islets in adult mice (18). Here we demonstrate that the PKA-signaling pathway targets the promoter of *miR-375*, as RNAP II is depleted from the promoter after forskolin treatment and transcription initiation is repressed (Fig. 3B). The transcription inhibitor actinomycin D represses the precursor with similar kinetics in INS-1 cells, lending support to the idea that transcription is repressed (Fig. 3A). In rat islets treated with exendin-4, pre-*miR-375* declined over a longer period of time (Fig. 1B) than it did in INS-1 cells treated with forskolin (Fig. 2A), and the reason for this is not known. Our hypothesis is that forskolin is a more potent agonist of adenylyl cyclase than is exendin-4, and thus a more robust response is seen with forskolin.

Interestingly, El Ouaamari *et al.* (23) reported that glucose could also repress the *miR-375* precursor, although the mechanism was not investigated. Our data support a model in which the two pathways, cAMP-PKA and glucose, are converging on a single factor to repress *miR-375* transcription (Fig. 6). Two lines of evidence support this model. First, we tested the possibility that cAMP and glucose were synergistically repressing pre-*miR-375* but found no evidence of enhanced repression when both treatments were combined (Fig. 1B), suggesting that a single factor is responsible for *miR-375* repression. Second, as shown in Fig. 5A, PKI did not relieve *miR-375* repression due to elevated glucose, again suggesting a single factor is responsible.

Although the transcription factor targeted by cAMP and glucose is unknown at this time, a leading candidate is the transcriptional repressor CREM. CREM is a member of the CREB family of b-ZIP transcription factors and is phosphorylated by PKA in response to cAMP (55). In β-cells, several repressing CREM splice variants have been identified (40, 56), and they appear to be responsible for the hyperglycemia and hyperlipidemia-induced gene

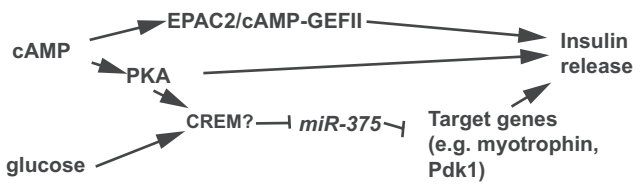

FIG. 6. Model for coordinated actions of cAMP and glucose on insulin release. Previous work has shown that cAMP potentiates insulin secretion in pancreatic β-cells after glucose stimulation through both the EPAC2/cAMP-GEF II and PKA pathways. Repression of *miR-375* via the cAMP-PKA and glucose pathways represents another way to enhance insulin release over the long term. The transcription factor CREM is a possible mediator in this pathway, because it is activated through phosphorylation by PKA and is transcriptionally up-regulated by glucose.

repression seen in diabetic rats and cell culture models of type 2 diabetes (39–41). Lending weight to the idea that CREM is repressing *miR-375* is the fact that CREM is overexpressed in rat islets treated with elevated glucose or free fatty acids (41). In this scenario, elevated glucose stimulates CREM expression and consequently leads to *miR-375* transcriptional repression.

Based upon the current literature, it is predicted that repression of *miR-375* will contribute to cAMP's ability to enhance insulin secretion and, in this regard, would provide a novel way for cAMP to potentiate GSIS, by repressing a miRNA. The evidence also expands on the model of how cAMP mediates GSIS potentiation (Fig. 6). The EPAC pathway is activated immediately upon binding of cAMP and promotes the rapid (within 80 msec) fusion of insulin granules with the plasma membrane (57). In contrast, PKA phosphorylates proteins such as the K_{ATP} channel (58) and L-type voltage-dependent calcium channel (59, 60), thereby stimulating insulin granule exocytosis from the reserve pool over the course of minutes to hours (24). PKA can also stimulate gene expression through phosphorylation of transcription factors such as CREM, which occurs over the course of minutes to hours (32).

The evidence presented here suggests a new, long-term (8–16 h) effect of the cAMP-PKA pathway on insulin release through repression of *miR-375*. Whether the cAMP agonist is forskolin or exendin-4, depletion of mature *miR-375* occurs over the course of hours ($t_{1/2}$ = 8–16 h). We hypothesize that this long-term effect will contribute to the plasticity of the β-cell-secretory response observed after exposure of the cells to glucose and incretin hormones such as GLP-1 (12). Administration of exendin-4 as a therapy for type 2 diabetes is currently before every meal, and this daily exposure would be expected to antagonize *miR-375* despite the mature miRNA's slow depletion kinetics. Furthermore, our data suggest that antagonizing *miR-375* could potentially enhance the effects of exendin-4 in patients.

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References

1. **Houbaviy HB, Murray MF, Sharp PA** 2003 Embryonic stem cell-specific microRNAs. Dev Cell 5:351–358
2. **Lagos-Quintana M, Rauhut R, Lendeckel W, Tuschl T** 2001 Identification of novel genes coding for small expressed RNAs. Science 294:853–858
3. **Lau NC, Lim LP, Weinstein EG, Bartel DP** 2001 An abundant class of tiny RNAs with probable regulatory roles in *Caenorhabditis elegans*. Science 294:858–862
4. **Lee RC, Ambros V** 2001 An extensive class of small RNAs in *Caenorhabditis elegans*. Science 294:862–864
5. **Poy MN, Eliasson L, Krutzfeldt J, Kuwajima S, Ma X, Macdonald PE, Pfeffer S, Tuschl T, Rajewsky N, Rorsman P, Stoffel M** 2004 A pancreatic islet-specific microRNA regulates insulin secretion. Nature 432:226–230
6. **Conaco C, Otto S, Han JJ, Mandel G** 2006 Reciprocal actions of REST and a microRNA promote neuronal identity. Proc Natl Acad Sci USA 103:2422–2427
7. **O'Donnell KA, Wentzel EA, Zeller KI, Dang CV, Mendell JT** 2005 c-Myc-regulated microRNAs modulate E2F1 expression. Nature 435:839–843
8. **Sachdeva M, Zhu S, Wu F, Wu H, Walia V, Kumar S, Elble R, Watabe K, Mo YY** 2009 p53 represses c-Myc through induction of the tumor suppressor miR-145. Proc Natl Acad Sci USA 106:3207–3212
9. **Farh KK, Grimson A, Jan C, Lewis BP, Johnston WK, Lim LP, Burge CB, Bartel DP** 2005 The widespread impact of mammalian microRNAs on mRNA repression and evolution. Science 310:1817–1821
10. **Zhao Y, Samal E, Srivastava D** 2005 Serum response factor regulates a muscle-specific microRNA that targets Hand2 during cardiogenesis. Nature 436:214–220
11. **Castanotto D, Rossi JJ** 2009 The promises and pitfalls of RNA-interference-based therapeutics. Nature 457:426–433
12. **Delmeire D, Flamez D, Moens K, Hinke SA, Van Schravendijk C, Pipeleers D, Schuit F** 2004 Prior in vitro exposure to GLP-1 with or without GIP can influence the subsequent β cell responsiveness. Biochem Pharmacol 68:33–39
13. **Lee Y, Jeon K, Lee JT, Kim S, Kim VN** 2002 MicroRNA maturation: stepwise processing and subcellular localization. EMBO J 21:4663–4670
14. **Baroukh N, Ravier MA, Loder MK, Hill EV, Bounacer A, Scharfmann R, Rutter GA, Van Obberghen E** 2007 MicroRNA-124a regulates Foxa2 expression and intracellular signaling in pancreatic β-cell lines. J Biol Chem 282:19575–19588
15. **Joglekar MV, Parekh VS, Mehta S, Bhonde RR, Hardikar AA** 2007 MicroRNA profiling of developing and regenerating pancreas reveal post-transcriptional regulation of neurogenin3. Dev Biol 311:603–612
16. **Plaisance V, Abderrahmani A, Perret-Menoud V, Jacquemin P, Lemaigre F, Regazzi R** 2006 MicroRNA-9 controls the expression of granuphilin/Slp4 and the secretory response of insulin-producing cells. J Biol Chem 281:26932–26942
17. **Tang X, Muniappan L, Tang G, Ozcan S** 2009 Identification of glucose-regulated miRNAs from pancreatic β cells reveals a role for miR-30d in insulin transcription. Rna 15:287–293
18. **Avnit-Sagi T, Kantorovich L, Kredo-Russo S, Hornstein E, Walker**

MD 2009 The promoter of the pri-miR-375 gene directs expression selectively to the endocrine pancreas. PLoS One 4:e5033
19. **Keller DM, McWeeney S, Arsenlis A, Drouin J, Wright CV, Wang H, Wollheim CB, White P, Kaestner KH, Goodman RH** 2007 Characterization of pancreatic transcription factor Pdx-1 binding sites using promoter microarray and serial analysis of chromatin occupancy. J Biol Chem 282:32084–32092
20. **Lynn FC, Skewes-Cox P, Kosaka Y, McManus MT, Harfe BD, German MS** 2007 MicroRNA expression is required for pancreatic islet cell genesis in the mouse. Diabetes 56:2938–2945
21. **Kloosterman WP, Lagendijk AK, Ketting RF, Moulton JD, Plasterk RH** 2007 Targeted inhibition of miRNA maturation with morpholinos reveals a role for miR-375 in pancreatic islet development. PLoS Biol 5:e203
22. **Poy MN, Hausser J, Trajkovski M, Braun M, Collins S, Rorsman P, Zavolan M, Stoffel M** 2009 miR-375 maintains normal pancreatic α- and β-cell mass. Proc Natl Acad Sci USA 106:5813–5818
23. **El Ouaamari A, Baroukh N, Martens GA, Lebrun P, Pipeleers D, van Obberghen E** 2008 miR-375 targets 3′-phosphoinositide-dependent protein kinase-1 and regulates glucose-induced biological responses in pancreatic β-cells. Diabetes 57:2708–2717
24. **Seino S, Shibasaki T** 2005 PKA-dependent and PKA-independent pathways for cAMP-regulated exocytosis. Physiol Rev 85:1303–1342
25. **Drucker DJ, Nauck MA** 2006 The incretin system: glucagon-like peptide-1 receptor agonists and dipeptidyl peptidase-4 inhibitors in type 2 diabetes. Lancet 368:1696–1705
26. **Fehmann HC, Göke R, Göke B** 1992 Glucagon-like peptide-1(7–37)/(7–36)amide is a new incretin. Mol Cell Endocrinol 85:C39–C44
27. **Holz IV GG, Kühtreiber WM, Habener JF** 1993 Pancreatic β-cells are rendered glucose-competent by the insulinotropic hormone glucagon-like peptide-1(7–37). Nature 361:362–365
28. **Göke R, Fehmann HC, Linn T, Schmidt H, Krause M, Eng J, Göke B** 1993 Exendin-4 is a high potency agonist and truncated exendin-(9–39)-amide an antagonist at the glucagon-like peptide 1-(7–36)-amide receptor of insulin-secreting β-cells. J Biol Chem 268:19650–19655
29. **Dyachok O, Isakov Y, Sågetorp J, Tengholm A** 2006 Oscillations of cyclic AMP in hormone-stimulated insulin-secreting β-cells. Nature 439:349–352
30. **Landa Jr LR, Harbeck M, Kaihara K, Chepurny O, Kitiphongspattana K, Graf O, Nikolaev VO, Lohse MJ, Holz GG, Roe MW** 2005 Interplay of Ca^{2+} and cAMP signaling in the insulin-secreting MIN6 β-cell line. J Biol Chem 280:31294–31302
31. **Willoughby D, Cooper DM** 2007 Organization and Ca^{2+} regulation of adenylyl cyclases in cAMP microdomains. Physiol Rev 87:965–1010
32. **Rosenberg D, Groussin L, Jullian E, Perlemoine K, Bertagna X, Bertherat J** 2002 Role of the PKA-regulated transcription factor CREB in development and tumorigenesis of endocrine tissues. Ann NY Acad Sci 968:65–74
33. **Chrivia JC, Kwok RP, Lamb N, Hagiwara M, Montminy MR, Goodman RH** 1993 Phosphorylated CREB binds specifically to the nuclear protein CBP. Nature 365:855–859
34. **Kwok RP, Lundblad JR, Chrivia JC, Richards JP, Bächinger HP, Brennan RG, Roberts SG, Green MR, Goodman RH** 1994 Nuclear protein CBP is a coactivator for the transcription factor CREB. Nature 370:223–226
35. **Bernardo AS, Hay CW, Docherty K** 2008 Pancreatic transcription factors and their role in the birth, life and survival of the pancreatic β cell. Mol Cell Endocrinol 294:1–9
36. **Jhala US, Canettieri G, Screaton RA, Kulkarni RN, Krajewski S, Reed J, Walker J, Lin X, White M, Montminy M** 2003 cAMP promotes pancreatic β-cell survival via CREB-mediated induction of IRS2. Genes Dev 17:1575–1580
37. **Impey S, McCorkle SR, Cha-Molstad H, Dwyer JM, Yochum GS, Boss JM, McWeeney S, Dunn JJ, Mandel G, Goodman RH** 2004 Defining the CREB regulon: a genome-wide analysis of transcription factor regulatory regions. Cell 119:1041–1054
38. **Zhang X, Odom DT, Koo SH, Conkright MD, Canettieri G, Best J, Chen H, Jenner R, Herbolsheimer E, Jacobsen E, Kadam S, Ecker JR, Emerson B, Hogenesch JB, Unterman T, Young RA, Montminy M** 2005 Genome-wide analysis of cAMP-response element binding protein occupancy, phosphorylation, and target gene activation in human tissues. Proc Natl Acad Sci USA 102:4459–4464
39. **Abderrahmani A, Cheviet S, Ferdaoussi M, Coppola T, Waeber G, Regazzi R** 2006 ICER induced by hyperglycemia represses the expression of genes essential for insulin exocytosis. EMBO J 25:977–986
40. **Inada A, Yamada Y, Someya Y, Kubota A, Yasuda K, Ihara Y, Kagimoto S, Kuroe A, Tsuda K, Seino Y** 1998 Transcriptional repressors are increased in pancreatic islets of type 2 diabetic rats. Biochem Biophys Res Commun 253:712–718
41. **Zhou YP, Marlen K, Palma JF, Schweitzer A, Reilly L, Gregoire FM, Xu GG, Blume JE, Johnson JD** 2003 Overexpression of repressive cAMP response element modulators in high glucose and fatty acid-treated rat islets. A common mechanism for glucose toxicity and lipotoxicity? J Biol Chem 278:51316–51323
42. **Franke B, Akkerman JW, Bos JL** 1997 Rapid Ca^{2+}-mediated activation of Rap1 in human platelets. EMBO J 16:252–259
43. **Laurenza A, Sutkowski EM, Seamon KB** 1989 Forskolin: a specific stimulator of adenylyl cyclase or a diterpene with multiple sites of action? Trends Pharmacol Sci 10:442–447
44. **Beavo JA, Rogers NL, Crofford OB, Hardman JG, Sutherland EW, Newman EV** 1970 Effects of xanthine derivatives on lipolysis and on adenosine 3′,5′-monophosphate phosphodiesterase activity. Mol Pharmacol 6:597–603
45. **Joost HG, Steinfelder HJ** 1987 Forskolin inhibits insulin-stimulated glucose transport in rat adipose cells by a direct interaction with the glucose transporter. Mol Pharmacol 31:279–283
46. **Vo N, Klein ME, Varlamova O, Keller DM, Yamamoto T, Goodman RH, Impey S** 2005 A cAMP-response element binding protein-induced microRNA regulates neuronal morphogenesis. Proc Natl Acad Sci USA 102:16426–16431
47. **Ozaki N, Shibasaki T, Kashima Y, Miki T, Takahashi K, Ueno H, Sunaga Y, Yano H, Matsuura Y, Iwanaga T, Takai Y, Seino S** 2000 cAMP-GEFII is a direct target of cAMP in regulated exocytosis. Nat Cell Biol 2:805–811
48. **Schwede F, Maronde E, Genieser H, Jastorff B** 2000 Cyclic nucleotide analogs as biochemical tools and prospective drugs. Pharmacol Ther 87:199–226
49. **Walsh DA, Ashby CD, Gonzalez C, Calkins D, Fischer EH** 1971 Krebs EG: Purification and characterization of a protein inhibitor of adenosine 3′,5′-monophosphate-dependent protein kinases. J Biol Chem 246:1977–1985
50. **Susini S, Roche E, Prentki M, Schlegel W** 1998 Glucose and glucoincretin peptides synergize to induce c-fos, c-jun, junB, zif-268, and nur-77 gene expression in pancreatic β(INS-1) cells. FASEB J 12:1173–1182
51. **Krek A, Grün D, Poy MN, Wolf R, Rosenberg L, Epstein EJ, MacMenamin P, da Piedade I, Gunsalus KC, Stoffel M, Rajewsky N** 2005 Combinatorial microRNA target predictions. Nat Genet 37:495–500
52. **Miranda KC, Huynh T, Tay Y, Ang YS, Tam WL, Thomson AM, Lim B, Rigoutsos I** 2006 A pattern-based method for the identification of microRNA binding sites and their corresponding heteroduplexes. Cell 126:1203–1217
53. **Cai X, Hagedorn CH, Cullen BR** 2004 Human microRNAs are processed from capped, polyadenylated transcripts that can also function as mRNAs. Rna 10:1957–1966
54. **Lee Y, Kim M, Han J, Yeom KH, Lee S, Baek SH, Kim VN** 2004 MicroRNA genes are transcribed by RNA polymerase II. EMBO J 23:4051–4060

55. **Sassone-Corsi P** 1994 Goals for signal transduction pathways: linking up with transcriptional regulation. EMBO J 13:4717–4728
56. **Inada A, Someya Y, Yamada Y, Ihara Y, Kubota A, Ban N, Watanabe R, Tsuda K, Seino Y** 1999 The cyclic AMP response element modulator family regulates the insulin gene transcription by interacting with transcription factor IID. J Biol Chem 274:21095–21103
57. **Renström E, Eliasson L, Rorsman P** 1997 Protein kinase A-dependent and -independent stimulation of exocytosis by cAMP in mouse pancreatic B-cells. J Physiol 502:105–118
58. **Ribalet B, Ciani S, Eddlestone GT** 1989 ATP mediates both activation and inhibition of K(ATP) channel activity via cAMP-dependent protein kinase in insulin-secreting cell lines. J Gen Physiol 94:693–717
59. **Britsch S, Krippeit-Drews P, Lang F, Gregor M, Drews G** 1995 Glucagon-like peptide-1 modulates Ca^{2+} current but not K+ATP current in intact mouse pancreatic B-cells. Biochem Biophys Res Commun 207:33–39
60. **Kanno T, Suga S, Wu J, Kimura M, Wakui M** 1998 Intracellular cAMP potentiates voltage-dependent activation of L-type Ca^{2+} channels in rat islet β-cells. Pflugers Arch 435:578–580