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MicroRNA-124a regulates Foxa2 expression and intracellular signaling in pancreatic β-cell lines

Nadine Baroukh [1], Magalie A. Ravier [2], Merewyn K. Loder [2], Elaine V. Hill [2], Ali Bounacer [3], Raphaël Scharfmann [3], Guy A. Rutter [2], and Emmanuel Van Obberghen [1]*.

[1] INSERM U145, IFR 50, Faculté de Médecine, Université de Nice-Sophia Antipolis, 28 Avenue de Valombrose, 06107 Nice Cedex 2 France.
[2] Department of Cell Biology, Division of Medicine, Faculty of Medicine, Imperial College London, Exhibition Road, London SW7 2AZ, U.K.
[3] INSERM EMI 0363, Faculté Necker, 156 rue Vaugirard, 75015, Paris, France.

*Corresponding author. INSERM U145, IFR 50, Faculté de Médecine, Université de Nice-Sophia Antipolis, 28, Avenue de Valombrose, 06107 Nice Cedex 2 France.
Tel: 33-(0)4-93-81-54-47; Fax: 33-(0)4-93-81-54-32
E-mail: vanobbeg@unice.fr

MicroRNAs (miRNAs) are short non-coding RNAs that have been implicated in fine-tuning gene regulations, although the precise roles of many are still unknown. Pancreas development is characterized by the complex sequential expression of a gamut of transcription factors. We have performed miRNA expression profiling at two key stages of mouse embryonic pancreas development, e14.5 and e18.5. miR-124a2 expression was strikingly increased at e18.5 compared to e14.5, suggesting a possible role in differentiated β-cells. Amongst the potential miR-124a gene targets identified by bio-computation, Foxa2 is known to play a role in β-cell differentiation. To evaluate the impact of miR-124a2 on gene expression, we over-expressed or down-regulated miR-124a2 in MIN6 β-cells. As predicted, miR-124a2 regulated Foxa2 gene expression, and that of its downstream target, pancreatic duodenum homeobox-1 (PDX-1). It has been described that Foxa2 is a master regulator of pancreatic development and also of genes involved in glucose metabolism and insulin secretion, including the ATP-sensitive K^+ (K_{ATP}) channel subunits, Kir6.2 and SUR1. Correspondingly, miR-124a2 over-expression decreased, and anti-miR-124a2 increased Kir6.2 and SUR1 mRNA levels. Moreover, miR-124a2 modified basal and glucose- or KCl-stimulated intracellular free Ca^{2+} concentrations in single MIN6 and INS-1 (832/13) β-cells, without affecting the secretion of insulin or co-transfected human growth hormone, consistent with an altered sensitivity of the β-cell exocytotic machinery to Ca^{2+}. In conclusion, while the precise role of microRNA-124a2 in pancreatic development remains to be deciphered, we identify it as a regulator of a key

transcriptional protein network in β-cells responsible for modulating intracellular signaling.

Studies implicating small regulatory RNAs in the control of gene expression have demonstrated that transcriptional regulation is controlled not only by protein factors, but also by small endogenous RNA molecules of ~ 19-23 nucleotides in length called microRNAs (miRNAs or miRs) (1,2). The first miRNAs discovered were *lin*-4 and *let*-7, which are crucial for regulating the developmental timing in the nematode, *Caenorhabditis elegans* (1,3). Since this initial report, several hundreds of miRNAs have been identified in plants and animals that regulate diverse biological processes ranging from cell metabolism to cell differentiation and growth, apoptosis and immune responses (4-8). Moreover, it has been shown that miRNAs are characterized by differential spatial and temporal expression patterns supporting their role in such processes (3,9). miRNAs serve as regulators of gene expression by binding to complementary sites on their target transcripts and, by an ill-defined mechanism, significantly induce the cleavage of mRNA or the repression of translation, depending on the partial or complete sequence homology, respectively (2,10-12). It has been estimated that miRNA genes represent approximately 1% of the genome of complex organisms. It appears that they share a certain degree of structural similarity and are highly conserved between species (13).

Recently, the development of different computational approaches provides reliable prediction for discovering novel miRNAs and their target genes, the identification of which is crucial to understand miRNA regulatory function (for review see Brown et *al*. (14)). Several miRNA do not have an obvious role in vertebrate development, but rather act in diverse physiological and cellular processes. This is the case for the miR-375, which specifically expressed in murine pancreatic cells, regulates the Myotrophin (Mtpn) gene and insulin exocytosis (15). More recently, it has been described that an over-expression of miR-9 in insulin-secreting cells causes a reduction in insulin exocytosis through an indirect effect on Granuphilin/Slp4 levels (16). In the present work, we have used computational analysis and the available databases to uncover microRNAs regulating the expression of genes important for pancreatic β-cell function. A review indicates that miR-375 may act synergistically with miR-124a, both of which are abundantly expressed in islet cells and may coordinately repress myotrophin and regulate insulin exocytosis (17,18). Initially miR-124 was described as a brain-specific miRNA in mammals, the function of which is presumably to help define and maintain cell-specific characteristics (19). It is also well represented in the mouse pancreatic MIN6 β-cell line, in which a large number of miR molecules have been found by sequencing of the library (15). In this study, we have focused on miR-124a in an effort to uncover regulated genes important for pancreatic β-cell function. Several transcription factors have been demonstrated to be critical regulators of pancreatic development through disruption of the corresponding gene in mice (20). One of these factors is the winged-helix transcription factor Foxa2 (Forkhead boxa2 or formerly named HNF3-β for Hepatocyte Nuclear Factor 3β), which is required for β-cell specific functions. Thus, conditional deletion of *Foxa2* in murine pancreatic β-cells results in hyperinsulinemic hypoglycemia, mimicking the pathophysiological features of familial hyperinsulinism (21). Foxa2 is

therefore a part of the transcriptional network downstream of the insulin receptor, and improves insulin resistance in peripheral tissues. Foxa2 is known to control the expression of many key genes involved in β-cell glucose sensing such as *PDX-1* gene. Moreover, Foxa2 plays a central role in insulin release via the regulation of genes coding for both *Kir6.2* (inward rectifier potassium channel member 6.2) and *SUR1* (sulfonylurea receptor 1) genes, the two subunits of the ATP-dependent K^+ (K_{ATP}) channel. In brief, it appears that Foxa2 plays a pivotal role in key β-cell functions and physiology (22-25).

In the present work, we have analyzed the expression of the miR-124a in adult mouse organs and in the developing mouse pancreas. We found that miR-124a2 is increased at embryonic (e) stage e18.5 compared to stage e14.5. We computationally identify the *Foxa2* gene product as a potential miR-124a2 target, and reveal that increasing the level of miR-124a2 negatively regulates the level of the Foxa2 protein. This, in turn, decreases the level of its downstream targets of Foxa2 including PDX-1, Kir6.2 and SUR1, resulting in an impairment of insulin biosynthesis and changes in glucose signaling in pancreatic β-cell lines.

Experimental Procedures

Total RNA isolation

Adult mouse organs, dissected mouse pancreas from e14.5 and e18.5 embryos (about 8-10 of each), or β-cells were homogenized in TRIzol reagent (Invitrogen), followed by RNA isolation using chloroform extraction and isopropanol precipitation. After being washed with 70% (v/v) ethanol, RNA pellets were dissolved in DEPC-treated H_2O. The RNA quality was analyzed by 1% (v/v) agarose gel and properly quantified with a bioanalyzer. For MicroRNA array, 5µg of total RNA (1µg/µl) of e14.5 and e18.5 stages was used. All RNA samples were conserved at -80ºC until used in assays.

MicroRNA array hybridization and data analysis

The microRNA array contains 226 mouse microRNA sequences. miRNA sequences were selected from a public database (26). The 35mer probes were designed by the Genosensor Company (Genosensor, Tempe, AZ), based on mature miRNA sequences and their flanking sequences. The synthesized probes were immobilized on the chips. RNA processing procedures were as described in the MicroARN-Chip Expression Analysis Technical Manual. Briefly, 5 µg total RNA was isolated at stage e14.5 and e18.5 from pancreata of mouse embryos and was directly labeled with a biotin molecule. Labeled RNA was then used as a target for on-chip hybridization assays under optimized conditions. A streptavidin-Alexa dye was used to stain the hybridized targets, and the fluorescent signals were then scanned and analyzed. tRNA and 5S rRNA functions were used as loading controls for normalization.

Identification of microRNA-124 targets

To identify genomic targets of miR-124a, we used an algorithm named miRanda (http://www.microrna.org/mammalian/index_new.html) as described in Enright et *al.* (27), and Pic Tar (http://pictar.bio.nyu.edu/cgi-bin/PicTar) (17).

Reporter vectors and DNA constructs

Putative miRNA124a-recognition element (MRE) (as single copy) from Foxa2 gene (miR-124a-RE Foxa2) was cloned in the 3'UTR of the Renilla luciferase reporter vector following the protocol already described in Cheng et *al.* (28). Briefly, the sense and anti-sense strands of the oligonucleotide sequences were annealed

by adding 1 mM of each oligonucleotide to 46 µl of annealing buffer (100 nM potassium acetate, 30 mM HEPES, pH7.4 and 2mM Magnesium Acetate) and incubated at 90ºC for 5 min and then 37C for 1h. The oligonucleotide sequences were designed to carry the *Hind*III and *Spe*I sites at their extremities facilitating the ligation into the *Hind*III and *Spe*I sites of pmir-Report luciferase (Ambion, Inc.). The oligonucleotides used in these studies were miR-124aRE Foxa2 (pLuc- miR-124a-RE): 5'-CTAGTATAAAGCACGAGAAACCAA-3' and 5'-AGCTTTGGTTTCTGCGTGCTTTATA-3'; scrambled miR-124a-RE Foxa2 (pLuc-scrambled miR-124a-RE): 5'-CTAGTGTCTTTGGCGTGTTTATA -3' and 5'-AGCTTATAAAGCACGACCAAAGAC-3'; mature mir-124a sequence (pLuc-miR-124-RE as a positive control of regulation): 5'-CTAGTTGGCATTCACCGCGTGCCTTAA -3' and 5'-AGCTTTTAAGGCACGCGGTGAATGCCAA -3'.

Expression vectors directing the synthesis of miR-124a2 were prepared either by introducing oligonucleotides corresponding to the murine precursor sequence of mir-124a2 into the pcDNA6.2 (Invitrogen), or into human growth hormone expressing vector (pXGH5) (29). In the former case, the oligonucleotide sequences were as follows: sense: 5'-TGCTGATCAAGATCAGAGACTCTGCTCTCCGTGTTCACAGCGGACCTTGATTTAATGTCATACAATTAAGGCACGCGGTGAATGCCAAGAGCGGAGCCTACGGCTGCACTTGAA-3' and antisense: 5'-CCTGTTCAAGTGCAGCCGTAGGCTCCGCTCTTGGCATTCACCGCGTGCCTTAATTGTATGACATTAAATCAAGGTCCGCTGTGAACACGGAGAGCGAGATCTCTGATCTTGATC- 3'. For miR-9, the sequences were as follows: sense: 5'-GATCCTATCTTTGGTTATCTAGCTGTATGAGTATATTGGTGTTCATAAAGCTAGATAACCGAAAGTTTTTA-3' and antisense: 5'-AGCTTAAAAACTTTCGGTTATCTAGCTTTATGAAGACCAATACACTCATACAGTAGATAACCAAAGATAG-3'. For cloning into pXGH5, the above sequences were inserted into vector pSUPER after the addition of *Bgl*II and *Hind*III sites, and the H1 promoter and miR sequences inserted into pXGH5 after excision with *Xba*1 and *Hind*III.

Cell culture and transfections

Mouse islets were isolated from pancreata of female CD1 mice by ductal injection of collagenase, as described (30).

The murine pancreatic MIN6 β-cell line was a kind gift of Dr Jun-ichi Miyazaki (Osaka University, Osaka, Japan). MIN6-cells were maintained in Dulbecco's modified Eagle's medium containing 25 mM glucose supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS), 100 units/ml penicillin, 100 µg/ml streptomycin, 100 µg/ml glutamine, and 50 µM β-mercaptoethanol in humidified 5% CO_2, 95% air at 37ºC (31) and used between passages 20 and 30.

The rat β-cell lines INS-1E (between passages 70 and 75) and INS-1 (832/13), passages 50-64 (32) were cultured as described in Merglen et *al*. (33), in a humidified atmosphere containing 5% (v/v) CO_2 in complete medium composed of RPMI 1640 supplemented with 5% (v/v) heat-inactivated fetal calf serum, 1mM sodium pyruvate, 50 µM 2-β mercaptoethanol, 2mM glutamine, 10mM HEPES, 100U/ml penicillin, and 100 µg/ml streptomycin.

LipofectAMINE 2000 transfection reagent (Invitrogen, Life Technologies) was used

to transfect the MIN6 and INS-1 (832/13) cells according to the manufacturer's instructions. A total of 5 pmol to 250 pmol of miR-124a2 precursor or miR-124a2 inhibitor (Ambion, Inc) (28) and 2 µl of lipofectAMINE 2000 were used per well, containing $2.5x10^5$ cells (6-well plate). The effects of miR transfection were tested in at least three separate experiments.

Transient transfection of plasmids was performed using the same methodology as described above, and luciferase activities were measured 2 days after transfection with the dual-luciferase reporter assay system (Promega, Madison WI).

RNA reverse-transcription, real time PCR and Northern blot experiments

RNA from transfected β-cells was isolated as previously described using TRIzol reagent. 1 µg of RNA from each transfected wells was reverse-transcribed at 42ºC for 15 min with 1 µg Oligo(dT) Primer, 5X first Strand Buffer, 100 mM DTT, 10 mM dNTP, RNAsin and SuperScript II Reverse Transcriptase (Promega Reverse Transcription System, Charbonnière, France). cDNA was used at a dilution of 1:50 in water in subsequent real-time PCR reactions.

For miR-124a precursor detection, RT was performed as described in Schmittgen et *al*. (34) and cDNA was used at a dilution of 1:100 in real-time PCR. PCR reaction mixtures included the Brilliant SYBR Green QPCR Master Mix (Stratagene), 10 µM primers, and the included reference dye at a dilution of 1:200 according to manufacturer's instructions in a total reaction volume of 20 µl. Reactions were performed with the SYRB Green (with dissociation Curve) program on the 7000 Sequence Detection System ABI Prim 7000 (Applied Biosystem, Courtaboeuf, France). Cycling parameters were 95ºC for 10 minutes and then 40 cycles of 95ºC (30s), 60ºC (1min), and 72ºC (30s), followed by a melting curve analysis.

All reactions were performed in triplicate with reference dye normalization (β-actin or U6 RNA), and the median Ct (Cycle threshold) value was used for analysis. Results were expressed relative to the control condition, which was arbitrarily assigned a value of 1. Each RT-PCR quantification experiment was performed in triplicate.

Primer sequences used to quantify mRNA by real-time RT-PCR were designed by using the Primer Express software from Applied Biosystems. The following forward and reverse primers were used for amplification:

Mouse Foxa2: Forward: 5' - CACCATCAGCCCCACAAAAT - 3'
Reverse: 5' - GGGTAGTGCATGACCTGTTCG - 3'

Mouse PDX-1: Forward: 5' - AAAACCGTCGCATGAAGTGG - 3'
Reverse: 5' – CCCGCTACTACGTTTCTTATCTTCC - 3'

Mouse Insulin-1: Forward: 5' - CAGAGACCATCAGCAAGCAGG - 3'
Reverse: 5' - GTGCACCAACAGGGCCAT - 3'

Mouse Kir6.2: Forward: 5' - CGCTTCGTGTCCAAGAAAGG - 3'
Reverse: 5' - TCGAATGTTCTTGTGGGCG - 3'

Rat SUR-1 (isoform D): Forward: 5' - TCCTGGTCACACCGCTGTT - 3'
Reverse: 5' - CCTTGACAGTGGACCGAACC - 3'

mmu-miR-124a: Forward: 5' - TCCGTGTTCACAGCGGAC - 3'
Reverse: 5' - CATTCACCGCGTGCCTTA - 3'

mmu-miR-124a2: Forward: 5' - ATCAAGATCAGAGACTCTGCTC - 3'
Reverse: 5' – GTGCAGCCGTAGGCTCCGCTC - 3'

Rat β-actin: Forward: 5' - CGAGCGTGGCTACAGCTTC - 3'
Reverse: 5' - GTCACGCACGATTTCCCTCT- 3'
U6: Forward: 5' - CTCGCTTCGGCAGCACA - 3'
Reverse: 5' - AACGCTTCACGAATTTGCGT - 3'
Cyclophilin: Forward: 5' - TATCTGCACTGCCAAGACT - 3'
Reverse: 5' - CCACAATGCTCATGCCTTC - 3'

For mature miR-124a detection, RT was performed as described in Applied Biosystems TaqMan MicroRNA Assays protocol using specific RT-primers and cDNA was used at a dilution of 1:50 in real-time PCR. PCR reaction mixtures included the TaqMan mouse microRNA-124a and TaqMan Universal PCR Master Mix in a total reaction volume of 20 µl. Reactions were performed with the 9600 emulation (Default) program on a Sequence Detection System ABI Prism 7000 (Applied Biosystem, Courtaboeuf, France) or 7500 (Applied Biosystem, UK). Cycling parameters were 95ºC for 10 minutes and then 40 cycles of 95ºC (15s), anneal/extend 60ºC (1min). All reactions were performed in triplicate. Results were expressed relative to the control empty vector for (mouse) MIN6 cells and for (rat) INS-1 (832/13) cells and arbitrarily assigned a value of 1.

Total RNA from the same transfected MIN6 cells was used for Northern blot analysis. Twelve µg total RNA was separated on a 12% denaturing polyacrylamide gel containing 7 M urea, at 25 Watts for 1h in 0.5X TBE. The RNA was then transferred to a Nytran N membrane (Schleider & Schuell, Germany) using a Trans-blot SD semi-dry transfer cell (Bio-Rad, Hercules, CA) at 20 Volts for 30 min using 0.5X TBE as a transfer buffer. Membrane was UV crosslinked and baked at 80ºC for 2 hours. Twenty pmol of miR-124a antisense oligonucleotide was end labelled by γ^{32}P dATP with T4 polynucleotide kinase for 1h at 37 ^{o}C (Amersham). The reactions were stopped with 2 µl 0.5 M EDTA. The probe was then purified with a G-25 MicroSpin column (Amersham). Pre-hybridization and hybridization were carried out at 30ºC using ULTRAhyb-Oligo hybridization buffer (Ambion), according to the manufacturer's manual. After hybridization the membrane was washed twice with 2X SSC 0.5% SDS (w/v) for 30 minutes at 30ºC. The membrane was then exposed for autoradiography. 5S rRNA was probed as a loading control and exposed for autoradiography for 20 min.

Whole cell extracts and Western blotting analysis

Forty eight hours after transfection, MIN6 cells were washed twice with ice-cold phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM NaH_2PO_4, 1.7 mM KH_2PO_4, pH 7.4), scraped from the dishes, and homogenized at 4°C in 100 µl (for one 6-well plate) of lysis buffer (50 mM NaF, 1 mM EDTA, 1 mM EGTA, 10 mM KH_2PO_4, 0.2% (v/v) Tween 20, 1 mM sodium orthovanadate, 1 mM 1.4-dithiothreitol, and 50 mM β-glycerophosphate, pH 7.4) containing the protease inhibitors: 5 µg/ml leupeptin, 150 µg/ml aprotinin, and 250 µg/ml 4-(2-aminoethyl)-benzene-sulfonylfluoride.

Protein extracts were then agitated at 4°C for 20 min and centrifuged at 14,000 x *g* for 15 min at 4 °C and the supernatants were used for the experiments described below.

For Western blotting, total proteins were separated by electrophoresis using 7.5% (v/v) polyacrylamide 0.1% (v/v) SDS gels and transferred to polyvinylidene difluoride membranes (Immobilon-P,

Millipore) followed by immunoblotting. Immunodetection was performed using an affinity purified goat polyclonal antibody raised against a peptide located at the carboxyl terminus of Foxa2 (HNF-3β) of mouse origin or an affinity purified rabbit polyclonal antibody against a peptide mapping near the C-terminus of CREB-1 of human origin (Santa Cruz Biotechnology, Inc.). After an overnight incubation at 4ºC, membranes were washed with PBS wash buffer (1X PBS, 0.1% (v/v) Tween 20, pH 7.4). Incubation with the secondary anti-goat or anti rabbit peroxidase-conjugated antibody (from Jackson Laboratories, Inc., Copenhagen, Denmark) was then performed for 1h at room temperature and membranes were washed again with wash buffer. Antibody binding was detected by enhanced chemiluminescence using an ECL kit (Amersham) following the manufacturer's protocol. To assess the total amount of protein, the membranes were stripped (for 30 min at 60ºC in buffer containing 100 mM 2-β mercaptoethanol, 2% (v/v) SDS and 62.5 mM Tris-HCl, pH 6.7) and reprobed with antibody to β-actin or β-tubulin (Sigma-Aldrich, St. Quentin-Fallavier, France).

Primary antibodies used in this study include anti-Foxa2, anti-CREB-1 (Santa Cruz Biotechnology, Inc.), and anti-PDX-1 (kind gift from M. Montminy of the Salk Institute, La Jolla, CA, USA).

Immunocytochemistry

MIN6 or INS-1(832/13) cells were transfected with 1 µg pcDNA6.2-miR-124a2 plasmids or the corresponding pcDNA6.2 empty vector (Invitrogen, Life Technology). The pcDNA6.2-miR-124a2 was obtained by cloning the miR-124a2 precursor sequence (see Sanger data base; http://www.sanger.ac.uk/Software/Pfam/index.shtml) into the empty vector following instructions of Invitrogen Technical Manual.

After transfection, MIN6 or INS-1(832/13) cells were fixed and incubated with primary and secondary antibodies as described before (35,36). Foxa2 was probed with the same antibody used for Western blotting (Santa Cruz Biotechnology, Inc.; 1:2500) and revealed with Alexa Fluor 568 donkey anti-goat IgG (Molecular Probes, Eugene, OR; 1:35000). Guinea pig polyclonal antibodies to anti-insulin were from Dako (Cambridge, UK, 1:1000) and revealed using an Alexa Fluor 568 donkey anti-guinea pig antibody (1:2000). Images were captured on an upright Leica SP2 laser-scanning confocal or a Zeiss 200M inverted optics spinning disc confocal microscope (63X oil immersion in both cases) and analyzed with appropriate software (Leica, Heldelberg, Germany or Volocity 4.0™, Improvision, Coventry, UK).

Intracellular free Ca^{2+} measurements and **assay of human growth hormone (hGH) release**

MIN6 or INS-1 (832/13) cells were transfected with 1 µg pcDNA6.2-miR-124a2 plasmids or the corresponding empty vector (pcDNA6.2) (Ca^{2+} experiments) or co-transfected together with 0.5 µg hGH-encoding plasmid pXGH5 (37) (assay of hGH release), using 2 µg/ml lipofectAMINE 2000 (Invitrogen, Life Technology) in Optimem I medium for 4 h in 12 well plates. Cells were cultured for 36 h in complete growth medium (containing 25 or 11 mM glucose for MIN6 and INS-1 (832/13) cells respectively) which was then replaced with a 3 mM glucose-containing DMEM or RPMI-based medium respectively 12 hours prior to experiments.

Changes in cytosolic concentration of Ca^{2+} ($[Ca^{2+}]_c$) were measured after loading cells with 5 µM Fura-red AM (Molecular

probes) for 30 min in 3mM glucose. Cells were perifused at 37°C in Krebs-Ringer buffer (KRB) containing 125 mmol/l NaCl, 3.5 mmol/l KCl, 1.5 mmol/l $CaCl_2$, 0.5 mmol/l NaH_2PO_4, 0.5 mmol/l $MgSO_4$, 3 mmol/l glucose, 10 mmol/l HEPES, 2 mmol/l $NaHCO_3$, pH 7.4 and equilibrated with O_2 / CO_2 (95:5) and supplemented with 0.1 % (w/v) bovine serum albumin. Stimulation with KCl was achieved using 50 mmol/l KCl-containing KRB (NaCl was reduced to maintain the osmolarity). Cells were imaged using an Olympus IX-70 microscope (x63 oil immersion objective) with an Imago charge-coupled device camera (Till Photonics, Grafelfing, Germany) controlled by TILLvisION software (Till Photonics). Cells were illuminated alternatively for 20 min at 430 and 480 nm, and the emitted light was filtered at 600 nm. The ratio images were used to calculate $[Ca^{2+}]_c$ off-line.

Assay of hGH release was essentially performed as previously described (35). Cells were pre-incubated for 30 min in KRB containing 3mM glucose and then incubated for 30 min subsequently at the glucose or KCl concentrations given. Released and total hGH content were assayed using a colorimetric sandwich ELISA method according to the manufacturer's instructions (Roche Diagnostics).

Insulin Secretion Assay

Cells were incubated in DMEM or RPMI as appropriate and supplemented as given above but containing 3 mM glucose for 16 h prior to experiments. The cells were preincubated for 30 min in KRB buffer as above and containing 3 mM glucose. The cells were then treated with 1.0 ml of KRB buffer containing the glucose or KCl concentrations given. Insulin secreted into the medium, and total cellular insulin, were measured by radioimmunoassay (Linco).

Statistical analysis

Results are expressed as arbitrary units and are presented as the means ± S.D or means ± S.E.M as stated. In each experiment, all determinations were performed at least in triplicate. Statistical significances were assessed using the Student's *t*-test or by ANOVA followed by Newman-Keul's test for experiments with multiple groups.

Results

miR-124a expression in mouse adult organs and developing pancreas.

Three genes encode the miR-124a mature sequence, miR-124a1, miR-124a2 and miR-124a3 respectively located on chromosomes 14, 3 and 2 in the mouse genome. We first analyzed pre-miR-124a (isoform 1, 2 and 3) expression by RT-PCR in different organs in mouse. Using specific primers (F' and R') designed for amplifying pre-miR-124a2 (Fig.1A), we revealed that pre-miR-124a2 is preferentially expressed in brain, lung, pancreas, kidney and muscle (Fig.1B, left panel). In this study, we were interested in identifying a role for miR-124a in pancreatic β-cell. To that aim, we first confirmed the expression of pre-miR-124a in two insulin-secreting cell lines, Min-6 and Ins-1E, and in primary pancreas islets (Fig.1B, right panel). To continue, we analyzed miR-124a expression during pancreas development by microRNA array GenoSensor Technology. We selected the embryonic stage e14.5 and e18.5 because of their importance in β-cell differentiation in terms of their difference in expression of transcription factors. miR-124a2, characterized by more than a six-fold change in expression level, was clearly differentially expressed during pancreas development (stage e14.5 compared to e18.5), whereas the two other isoforms, miR-124a1 and miR-124a3, were unchanged (Fig. 1C). The miR-124a2 up regulation in developing pancreas was

confirmed by RT-PCR analysis (data not shown). miR-124a2 expression thus appears to be turned-on at e18.5 indicating its potential role in the regulation of genes involved in pancreatic β-cell-specific functions. Moreover, the microRNA chip array revealed a specific pattern of expression between these 2 key stages in embryonic pancreas development. 45 of the 226 mouse microRNAs analyzed in this array exhibited a variation in expression, representing 20% of all miRNAs analyzed (Supplemental Table 1).

Prediction of microRNA-124a targets using bio-informatics.

The striking upregulation of miR-124a2 at e18.5 suggested that this microRNA might participate in or simply be associated with regulatory events involved in the modulation of gene products having a role at this stage of pancreas development. Using bio-informatic tools (miRanda and Pic Tar), we searched for potential mRNA targets of human miR-124a. This analysis identified several genes as being potentially targeted by miR-124a, from which we selected a series of genes anticipated to have a key role in pancreatic β-cells: Forkhead box protein A2 (Foxa2 or HNF3-β), cAMP responsive element binding protein 1 (CREB-1), ISL1 transcription factor (ISL1), myotrophin (Mtpn), neurogenic differentiation (NeuroD1) and vesicle associate membrane 3 (VAMP-3) genes based on their acknowledged role in pancreas (Supplemental Table 2). In the listed genes, one emerges as the gene encoding for a key protein in endodermal development in most species and corresponds to the forkhead transcription factor boxa2 gene (Foxa2) (38).

Foxa2 protein regulation identified by miR-124a2 functional analysis.

To investigate the influence of miRNA-124a on predicted mRNA targets, we transfected the miR-124a2 precursor (pre-miR-124a2) or inhibitor (anti-miR-124a2) into murine pancreatic MIN6 β-cells and we searched for changes in Foxa2 protein levels using Western blotting with an antibody to Foxa2. Introduction of miR-124a2 precursor decreased Foxa2 protein levels and, conversely, miR-124a2 inhibitor increased Foxa2 protein amounts (Fig. 2A). Quantification of the signals shown in Figure 2B revealed a direct dose-dependent relationship between the amount of anti-miR-124a2 and the Foxa2 protein level, and an inverse relationship between pre-mir-124a2 and Foxa2.

To investigate the influence of microRNAs on transcript levels, we transfected miR-124a2 precursor (pre-miR-124a2) or inhibitor (anti-miR-124a2) into MIN6-cells and examined changes on Foxa2 mRNA levels. We showed that delivering miR-124a2 causes no significant change in Foxa2 mRNA expression, whereas the anti-miR-124a2 induced more than a 2-fold increase in the Foxa2 transcript (Fig. 2C).

Most miRNAs are thought to control gene expression by base pairing with the microRNA-recognition element (miR-RE) found in their messenger RNA target (Fig. 3A). To verify this hypothesis, the miR-124a-RE (as a single copy) of Foxa2 has been cloned into the 3'untranslated region (3'UTR) of the luciferase gene of the pmiR-Report Luciferase vector. We tested the activity of the miR-124a by transfecting the luciferase reporter vector bearing the miR-124a-RE of Foxa2, or the scrambled miR-124a-RE of Foxa2 (as a negative control vector that does not target any known cellular miRNA), with the pcDNA6.2-miR-124a2 and the pmiR-Report β-gal vector as a control for transfection efficiency. Following transfection into HeLa cells, both luciferase and β-gal activity were

measured and the relative level of luciferase was normalized against the β-gal reading to control the transfection variation. To evaluate the activity of miR-124a, the mature miR-124a sequence was cloned in the anti sense orientation in the 3'UTR of the luciferase reporter gene. This construct (pLuc-miR-124a-RE) allows a perfect match between mir-124a and its target sequence located in the 3'UTR of the luciferase mRNA. Importantly, pcDNA6.2-miR-124a2 was able to inhibit, by approximately 20 % ($p<0.01$), the expression of the reporter gene containing the miR-124a-RE of Foxa2 (Fig. 3B). Further, pcDNA6.2-miR-124a affected the activity of a reporter gene containing the canonical response element for miR-124a.

Role of miR-124a2 in Foxa2 gene regulation

Foxa2 is known to regulate hepatic and/or pancreatic gene expression and to play a central role in maintaining glucose homeostasis (39-42). Foxa2, which is expressed in islets, is a major upstream trans-activator of pancreatic duodenal homeobox-1 (PDX-1) (22,43,44), a homeobox gene essential for pancreatic development and for the maintenance of β-cell functions (39,40).

To further explore whether miR-124a2 regulates the Foxa2 protein, we analyzed PDX-1 expression levels in MIN6-cells transfected with microRNA corresponding to miR-124a2. We found that PDX-1 mRNA levels were down- or up- regulated ($p<0.01$ and $p<0.05$, respectively) in the presence of miR-124a2 precursor or inhibitor, respectively (Fig. 4A). At the protein level, exposure of MIN6-cells to microRNA induced a pattern of regulation in which pre-miR-124a2 decreased PDX-1 protein level ($p<0.05$), whereas anti-miR-124a2 increased it ($p<0.05$). Collectively our data provide evidence that modulation of Foxa2 protein by miR-124a2 has an impact on its downstream gene *PDX-1* (Fig. 4B). Importantly, PDX-1 is known to play a key role in regulating insulin gene transcription. Therefore, we decided to analyze the expression of the insulin gene, which lies downstream of PDX-1. As expected, we found that insulin mRNA levels were down- or up-regulated ($p<0.001$ and $p<0.05$, respectively) in the presence of miR-124a2 precursor or inhibitor, respectively (Fig. 4C).

K_{ATP} channels are heteromultimers composed of four SUR1 (Sulfonylurea receptor 1) subunits surrounding an inner core of four Kir6.2 (Inward rectifier K^+ channel member 6.2) subunits (45). ATP-sensitive K^+ channels are found in β-cells and play an important role in the regulation of insulin secretion (46-48). Importantly, the expression of the β-cell K_{ATP} channel subunits SUR1 and Kir6.2 have been described as being Foxa2-dependent (23). To verify such a relationship, we examined *Kir6.2* and *SUR1* gene expression in MIN6-cells transfected with miR-124a2. We found that both genes are indeed regulated, as pre-miR-124a2 significantly decreases the mRNA level of both *Kir6.2* and *SUR1* ($p<0.05$) and, conversely, anti-miR-124a2 increases the corresponding mRNA levels ($p<0.05$) (Fig. 4C).

miR-124a2 regulates stimulus-dependent increases in intracellular free Ca^{2+} concentration but not hormone secretion.

To determine whether miR-124a2 over-expression may affect the response to single β-cells stimulation by glucose or other agents, we subcloned miR-124a2 into a plasmid vector (pcDNA6.2) from which the miRNA was expected to be produced by processing of a precursor mRNA also encoding the Green Fluorescent Protein (GFP). Green fluorescence could thereafter be used to identify individual transfected cells. First, we confirmed the correct

processing of the mature miR-124a from the precursor transcripts of pcDNA6.2-miR-124a2 expression by quantitative RT-PCR and/or Northern blot analysis (Supplemental Fig. 1). Correspondingly, transfection with pcDNA6.2-miR-124a2 caused a small but statistically significant decrease in Foxa2 immunoreactivity in transfected (GFP-positive) MIN6-cells (Fig. 5).

Consistent with the decrease in Foxa2 level and likely decrease in K_{ATP} channel subunit expression, we observed a significant increase in basal cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_c$) (fura-red 430/480 fluorescence ratio: 0.618 ± 0.024, n=35 in empty pcDNA6.2-transfected cells and 0.716 ± 0.014, n=54 cells in pcDNA6.2-miR-124a2 transfected cells; $p<0.001$; see also Fig. 6A). When assessed in single cells, over-expression of miR-124a2 led to an increase in the proportion of cells in which an increase in $[Ca^{2+}]_c$ was apparent in response to elevated (30 vs 3 mM) glucose (Fig. 6B). Thus, 29/54 (54%) of miR-124a2-expressing cells displayed a significant increase in $[Ca^{2+}]_c$ in response to high glucose, compared to only 8/35 (22.85%) of empty vector-transfected cells (6-9 independent experiments). However, analysis of the magnitude of the increase in those cells which responded to glucose in each case, by measurement of the "Area under the curve" (AUC, arbitrary units) for the fura red ratio revealed a smaller increase in miR-124a2-expressing cells (empty vector, 0.048 ± 0.024, n=8 versus 0.021 ± 0.003, n=25, microRNA-expressing cells; $p<0.05$, 3-8 independent experiments; Fig. 6A). Likewise, the increase in $[Ca^{2+}]_c$ in response to depolarisation with 50 mM KCl in cells pretreated at 30 mM glucose was reduced by about 50 % in miR-124a2-expressing versus empty vector expressing cells (AUC values: 0.035 ± 0.009, n=19 cells, and 0.070 ± 0.013, n =19 cells, respectively, three independent experiments, $p<0.05$; Fig. 6B).

Assessed in transfected cell populations, no change was apparent in basal, high glucose- or KCl-stimulated release of human growth hormone (hGH), used as a surrogate for insulin (35) (hGH secreted as % cell content / 30 min for miR-124a2-transfected versus empty vector- (GFP alone) transfected cells respectively at 3 mM glucose: 8.4 ± 1.9, and 6.7 ± 1.6; at 30 mM glucose: 13.7 ± 2, 12.6 ± 1.6; at 50 mM KCl: 31.7 ± 2.4 and 30.7 ± 2.8; three independent experiments performed in triplicate in each case) (Fig. 7A). Moreover, introduction of miR-124a2 oligonucleotides directly had no effect on regulated insulin secretion in MIN6 β-cells at either 3 mM or 30 mM glucose, or in the presence of 50 mM KCl (Fig.7B).

Similar experiments to the above were also performed in INS-1 (832/13) cells. In contrast to MIN6 cells, INS-1 (832/13) cells displayed oscillatory increases in $[Ca^{2+}]_c$, similar to those apparent in primary mouse β-cells (49) (Fig. 8A) in response to high glucose. Whereas the amplitude of these oscillations was not significantly affected in cells expressing miR-124a2 from plasmid pcDNA6.2 compared to empty vector, basal $[Ca^{2+}]_c$ was slightly but significantly increased in miR-124a2 expressing cells (Fig. 8B, C). No change was apparent in the extent of the $[Ca^{2+}]_c$ response to depolarisation with 50 mM KCl of control or miR-124a2 expressing cells (~14 % in each case) (Fig. 8C). As also observed in MIN6 cells, expression of miR-124a2 exerted no significant effect on the regulated release of hGH, when both were expressed from the same vector (Fig. 9 and see Methods). By contrast, miR-9 augmented basal hGH release without affecting release in response to 20 mM glucose (Fig. 9),

consistent with a recent study in which the effects of miR-9 were examined on the secretory response to a combination of secretagogues (16). Note that the release of hGH was quantitated above (Fig. 9) as percentage of total cellular hGH content in each case: total hGH content was identical in empty vector-transfected and miR124a2 vector expressing cells (data not shown). Correspondingly, immunocytochemical analyses revealed that insulin staining was not different between single vector-transfected (fluorescence intensity ratio = 0.87 ± 0.18, for 15 INS-1(832/13) transfected *versus* 16 non-transfected cells in a total of five separate fields) and miR124a2 plasmid transfected (1.05 ± 0.05; 19 transfected *vs* 18 control cells) cells. Thus, the absence of detectable changes in hormone release in miR124 expressing cells is unlikely to be due to the "masking" of any such changes through alterations in total hormone content.

Discussion

Regulation of β-cell gene expression by miR-124a2

MicroRNAs form a growing family of small non-protein-coding regulatory genes (~ 22 nucleotides) that regulate the expression of homologous target-gene transcripts. Over the last couple of years, the list of reported miRNA functions and targeted genes has extended at an impressive pace. miRNAs appear to decrease protein production by two different mechanisms: either by increasing the degradation of mRNA (RNA interference-like effect) or by blocking translation of mRNA into protein (genuine microRNA effect) (2,10-12).

Here, we report a biological function of miR-124a2 to regulate gene expression and Ca^{2+} signaling in pancreatic β-cells. As previously described, miR-124a is mainly expressed in the brain and pancreas (11,15). Using microRNA array technology, we found that miR-124a2 is up-regulated between stage e14.5 and e18.5 in the developing mouse pancreas. This microRNA exists in three isoforms, miR-124a 1, 2 and 3, respectively located on chromosome 14, 3 and 2 in the mouse. Each isoform possesses the same mature microRNA sequence, but each precursor has a distinct sequence. Interestingly, we observed a 6-fold increase exclusively for miR-124a2 expression at e18.5 compared to e14.5, whereas miR-124a1 and miR-124a3 are unchanged. These results suggest a specific pattern of microRNA regulation during pancreas development, which is likely to be controlled at the transcriptional level.

To identify genes that may be regulated by miR-124a, we applied algorithms designed to search for matching base pairs in miRNAs and in mRNA targets. We found that Foxa2 is a potential candidate for regulation by miR-124a. Foxa2 contributes to the complex transcriptional circuitry within the pancreas and is involved in β-cell development and function. Therefore, we studied the expression of the Foxa2 gene by immunoblotting cell extracts obtained from MIN6 cells transfected with either the miR-124a2 precursor or the miR-124a2 inhibitor, which induce an enhancement or an inhibition of miR-124a functions, respectively. Expression of pre-miR-124a2 led to a reduced level of Foxa2 protein and, conversely, transfection with miR-124a2 inhibitor increased the Foxa2 protein level.

The mechanism(s) of regulation by miR-124a of Foxa2 expression in β-cells remain(s) unclear. No change was detected in Foxa2 mRNA level in pre-miR-124a2-transfected cells compared to controls, and miR-124a2 inhibitor induced a two-fold increase in Foxa2 mRNA levels. One may speculate that the regulation of this target gene by miR-124a2 is controlled by a

cis/trans-regulation. Based on this hypothesis, and using genomic comparison, we investigated whether the transcription factor CREB-1 may be a regulated target of miR-124a, as predicted (Supplemental Table 2). CREB-1 is a basic leucine zipper transcription factor, expressed widely and regulating many rapid response genes (50). Using Western (immuno) blotting with a polyclonal antibody to CREB-1, we found that miR-124a2 precursor decreased CREB-1 protein levels, whereas miR-124a2 inhibitor increased the amount of CREB-1 protein (Supplemental Fig. 2A and B). Next, we were interested to determine whether a relationship existed between CREB-1 and Foxa2 gene regulation. To this aim, we used genomic comparison (http://ecrbrowser.dcode.org/) to identify non-coding sequences that are evolutionarily conserved in human, mouse and frog, and that in addition are located upstream of the Foxa2 gene (Supplemental Fig. 3A). The rationale for using inter-species sequence comparisons to identify non-coding regulatory elements is based upon the observation that sequences that exert important biological functions are frequently conserved between species. We localized an Evolutionary Conserved Region (ECR), with 208 bp in length and 83.2% identity between human and mouse, and 111 bp in length and 73.9% identity in frog. We run the rVISTA 2.0 program (http://rvista.dcode.org/), which helped us to localize a potential regulatory element for CREB on this ECR (Supplemental Fig. 3B). Together, our data suggest that CREB-1 may be a transcription factor regulating Foxa2 expression which, in part, might explain the observed up-regulation of the gene when cells are treated with anti-miR-124a2. Moreover, Foxa2 has been shown recently to be a direct target for CREB using genome wide analysis by Chip on Chip (51). However, much work needs to be done to precisely determine whether CREB-1 binds to Foxa2 promoter and trans-activates its gene expression. In any case, it remains conceivable that the regulation of Foxa2 expression by miR-124a2 is via both direct and indirect effects.

Foxa2 is known to activate gene transcription in pancreatic β-cells and to regulate the expression of several genes including those encoding for the transcription factor PDX-1, a key regulator of β-cell gene expression (24). We show here that Foxa2 down-regulation in microRNA-124a2 transfected MIN6 cells induces a reduction in PDX-1 mRNA and subsequent reduction in PDX-1 protein level. A similar situation is observed with preproinsulin gene expression, presumably reflecting the decrease in PDX-1 level (52). As microRNA-124a is not predicted to interact directly with PDX-1, Kir6.2, SUR1 or preproinsulin gene transcripts, we suspect that the decrease in the levels of each of these reflects a loss of the transcriptional stimulation of the respective genes by Foxa2. Consistent with this view, PDX-1, Kir6.2, SUR1 and preproinsulin mRNA levels were closely correlated to Foxa2 protein levels. Thus, an increase in Foxa2 protein level induced an up-regulation of PDX-1, Kir6.2, SUR1 and preproinsulin mRNA expression whereas the reverse was observed when Foxa2 protein levels were decreased. To summarize, our data are compatible with a scenario in which miR-124a2 acts on Foxa2 expression, which consequently regulates PDX-1, Kir6.2, SUR1 and preproinsulin gene transcription.

Impact of miR-124a2 on stimulus-dependent Ca^{2+} changes in β-cells.

ATP-sensitive K^+ channels are key regulators of β-cell membrane potential and insulin release (48). Inhibition of

Foxa2 function, as observed here after miR-124a2 over-expression, has previously been shown to decrease the expression of each K_{ATP} channel subunit (22,24,25,43) and to lead to persistent hyperinsulinemic hypoglycaemia in *Foxa2* knockout mice (23). These changes seem likely to reflect a more depolarised membrane potential, increased Ca^{2+} influx through voltage-gated Ca^{2+} channels, and hence elevated basal free $[Ca^{2+}]_c$. This, in turn, is expected to lead to constitutive activation of insulin release. Correspondingly, we observed here that basal $[Ca^{2+}]_c$ was significantly elevated in miR-124a2 expressing MIN6 and INS-1 (832/13) cells. In the case of MIN6 cells, the proportion of cells which subsequently responded to elevated glucose concentrations was substantially increased (Results and Fig. 6B), albeit with a decreased glucose-induced change in $[Ca^{2+}]_c$ in the responding cells (Fig. 6A). Moreover, depolarisation-dependent increases in $[Ca^{2+}]_c$ were markedly lowered in miR-124a2 expressing MIN6 (Fig. 6) or INS-1 (832/13) (Fig. 8), the latter of which displayed an oscillatory increase in $[Ca^{2+}]_c$ as observed in dissociated mouse β-cells (49). Since none of the subunits of L-type voltage-gated Ca^{2+} channels (the principle means of Ca^{2+} entry in stimulated β-cells) (53) are predicted to be direct targets of miR-124a2 (assessed using http://cbio.mskcc.org/cgi-bin/mirnaviewer/mirnaviewer.pl), these observations suggest that the apparent changes in the activity of this channel may reflect an indirect consequence of the decreases in the levels of Foxa2, or other transcription factors targeted by miR-124a.

Surprisingly, none of the above alterations in intracellular free Ca^{2+} concentration were translated in the present study into differences in basal or stimulated hGH release, in either MIN6 (Fig. 7) or INS-1 (832/13) cells (Fig. 9), when measured either as the release of hGH (Fig. 7A), used as a surrogate for insulin in co-transfected cells, or via direct measurements of insulin release itself after treatment with miR duplexes (Fig. 7B). By contrast, over-expression of miR-9 (16), used as a positive control, caused a significant increase in basal hGH release from INS-1 (832/13) cells, and a tendency towards decreased glucose-stimulated release (Fig. 9). Similarly, we observed no effects of miR-124a2 over- or under-expression on hGH or insulin release from a third, distinct β-cell line, INS-1E cells, whilst miR-375, again used as a positive control (15), caused a clear inhibition of glucose-stimulated secretion from these as well as MIN6 cells (data not shown).

There are several plausible, and mutually inclusive, explanations for the apparent discordance between the effects of miR-124a on glucose-stimulated changes in $[Ca^{2+}]_c$ and secretory activity. Firstly, decreases in Foxa2 level, likely reflecting the increased levels of miR-124a2 achieved after plasmid transfection, were relatively small in the single cell assay required to measure Ca^{2+} changes (Fig. 6A). Given that miR-124a is estimated to comprise as much as 11.5 % of the total microRNA profile in MIN6 cells (17) large changes in the level of this microRNA were difficult to achieve by transfection with an exogenous plasmid so that relatively small changes in the levels of target genes are anticipated (see Results). Importantly, direct measurements of mature miR-124 (Supplemental Fig. 1) revealed clear and substantial increases in the levels of this species in both MIN6 and INS-1 (832/13) cells after transfection with the corresponding plasmid. Secondly, in MIN6 cells, an increase in the proportion of cells responding to glucose with a detectable increase in $[Ca^{2+}]_c$ was

accompanied by a decrease in the overall $[Ca^{2+}]_c$ increase in these cells, two opposing effects which may have led to no net change in secretion. However, this was not the case in INS-1 (832/13) cells where there was no change in the proportion of cells responding to the sugar after the introduction of miR-124, and only a small though significant increase in basal $[Ca^{2+}]_c$. Finally, and perhaps most plausibly, miR-124a2 may have affected directly or indirectly the expression of a wide range of target genes, some of which may act to oppose the alterations in Ca^{2+} homeostasis. In particular, the smaller glucose- or depolarisation-induced $[Ca^{2+}]_c$ increase in miR-124a2 expressing cells may be compensated by relative increases in the expression of components of the vesicle trafficking and exocytotic machinery in β-cells, as in nerve cells (54). Whilst leaving exocytosis at basal $[Ca^{2+}]_c$ unaffected by the small increase in the latter parameter, this alteration may sensitise insulin release to the smaller overall excursion in $[Ca^{2+}]_c$ in response to cell stimulation. Intriguingly, the results of the present study suggest that such a sensitisation to smaller changes in $[Ca^{2+}]$, the principal intracellular trigger for insulin release (55,56), may be one of the properties acquired during the normal differentiation of pancreatic precursor cells into mature β-cells.

Conclusions. We have identified a regulated pancreatic microRNA, miR-124a2, that regulates preproinsulin message levels. This might reflect Foxa2-directed decreases in Pdx-1 activity, and down-regulation of the genes encoding the K_{ATP} channel components, Kir6.2 and SUR1, as reflected by alterations in basal and/or stimulated Ca^{2+} levels. It should be emphasised, however, that we were unable to detect changes in regulated insulin secretion, as observed in Foxa2 null mice (21). This observation presumably reflects additional actions of miR-124, including a "resetting" of the sensitivity of the exocytotic machinery to changes in Ca^{2+} (see above). Interestingly, miR-124a seems to share features with the miR-375, which has been shown to regulate insulin secretion, albeit at distal steps in the process, through the targeting of the Myotrophin gene (15). It is tempting to envision the possible occurrence of cooperation between miR-124a2 and miR-375, which leads to the concerted regulation of a series of target genes involved in insulin secretion, such as the Mtpn gene (also predicted to be regulated by miR-124a). Moreover, as described by Bartel *et al.*, the dosage of gene expression regulated by miRNA is adjusted in a particular manner during cellular differentiation and development and it is unique to a specific cell type (57). Such fine orchestration may thus add a new dimension to the complexity of the mechanisms controlling gene expression.

However, the real picture seems likely to be substantially more complex as an individual protein is likely to be regulated by several microRNAs and, conversely, a particular microRNA is known to affect several proteins. Therefore, because of the multiplicity of possible interactions between one microRNA and its gene targets predicted by bio-computation, an urgent challenge is to define the particular function of each microRNA within an integrated response in a given cell system. In any case, the fast-accumulating evidence in favor of a key role of microRNAs in the regulation of a series of developmental and physiological programs probably represents only a glimpse of the complex network of gene regulation by microRNAs, given that they represent a large part of the human genome.

Although microRNA-124a has so far been described to help maintain cell-specific characteristics of neurons (19, Conaco, 2006 #146), we now present direct evidence for the prediction (17) that this microRNA is also a key regulator of β-cell physiology. Since the inappropriate regulation of microRNAs is likely to be involved in processes leading to human disease (58,59), we speculate that miR-124a may provide a novel target for cell-based treatments of type 2 diabetes characterized by β-cell failure.

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Footnotes

Keywords: microRNAs, gene regulation, Foxa2, β-cells, insulin secretion, exocytosis, Ca^{2+}

Abbreviations: MicroRNA, miR or miRNA; miR-124a2 precursor, pre-miR-124a2; miR-124a2 inhibitor, anti-miR-124a2; pcDNA6.2, empty vector; pcDNA6.2-miR-124a2, miR-124a2; forkhead transcription factor boxa2 gene, Foxa2; inward rectifier potassium channel member 6.2, Kir6.2; sulfonylurea receptor 1, SUR1; ATP-dependent K^+ channel, K_{ATP}; intracellular free Ca^{2+} concentrations, $[Ca^{2+}]_c$; embryonic, e; pancreatic duodenal homeobox-1, PDX-1; cAMP responsive element binding protein, CREB; Myotrophin, Mtpn; Cycle threshold, Ct; Evolutionary Conserved Region, ECR; human, hm.

Conflict of interest: The authors have declared that no conflict of interest exists.

Legends to figures

Figure 1. Specific expression of microRNA-124a in mouse tissues. *A*. Primer sequences to miR-124a isoforms. The sequences of the precursor and mature members of the mouse miR-124a family of isoforms are shown. Underlined, sequences of the mature miRNA-124a. F and R, sequences of the forward/reverse PCR primers amplifying miR-124a (corresponding to a 60 bp); F' and R', sequences of the forward/reverse PCR primers amplifying miR-124a2 specific isoform (corresponding to a 105 bp); in bold, sequences that differ among isoforms. Sequences are presented in the 5' to 3' direction.
B. RT-PCR of mouse total tissues and β-pancreatic models. Representative abundance of miR-124a2 (upper panel) and miR-124a (lower panel) and corresponding U6 SnRNA in tissue. Br, brain; Ht, heart; Lg, lung; Lv, liver; Pn, pancreas; Kd, kidney; Sp, spleen; Ms, muscle; Min-6, mouse insulinoma β-cells; Ins-1E, rat β-pancreatic cells; Islet, mouse pancreatic islets. ***C.*** microRNA-124a expression levels at stage e14.5 and e18.5 in developing pancreas of mouse embryos. The results are expressed as relative units normalized with tRNA and are the means ± S.D. from 3 samples. **p<0.01.

Figure 2. Foxa2 protein and mRNA gene expression levels in MIN6 β-cells treated with pre-mir-124a2 or anti-miR-124a2. *A*. Analysis of Foxa2 protein levels. MIN6-cells were transfected with miR-124a2 precursor or inhibitor (5 to 250 pmol) and left as such for 48h. Proteins were extracted and subjected to SDS-PAGE. Representative immunoblots with anti-Foxa2 antibodies (*upper panel*) and anti-β-actin antibodies (*lower panel*) are shown. * over-exposure of the immunoblots done in presence of the anti miR-124a2. ***B.*** Relative quantification of Foxa2 protein levels. Five independent experiments were performed and three gels showing Foxa2 protein levels were scanned and quantified using Scion image program. The values were corrected for differences in the total amount of β-actin. ***C.*** Analysis of Foxa2 gene expression levels. MIN6-cells were transfected with miR-124a2 precursor or inhibitor (250 pmol) and left as such for 48h. RNA was extracted and subjected to reverse transcription followed by Real-time-PCR. Data represent three independent experiments ± S.D. with n=3. *p<0.05.

Figure 3. **Analysis of miRNA-124a activity**. ***A.*** Bioinformatic prediction of miRNA-124a and Foxa2 transcript interaction using human miRNA target predictions (version 2005 at http://cbio.mskcc.org/cgi-bin/mirnaviewer/mirnaviewer.pl). ***B***. Relative expression of miR-124a-RE- reporter by miRNA-124a2. miR-124a-RE of Foxa2 was cloned into the pmiR-Report luciferase vector in the 3'UTR region. Once cloned the miR-124a-RE vector is co-transfected with the pmiR-Report β-gal vector as a control for

transfection efficiency and the pcDNA6.2-miR124a2. HeLa cells were plated at 50 000 cells/well in 6-wells plates. Cells were transfected using lipofectamine 2000 in triplicate with pmiR-Report β-gal, a luciferase reporter construct that contained one target site for miR-124a and either plasmid expressing miR-124a2 (pcDNA-miR-124a2) or the empty vector (pcDNA6.2) as a negative control. Fourty eight hours post-transfection cells were assayed for luciferase and β-gal expression, and β-gal is used to normalize for differences in transfection efficiency. Data represent four independent transfection ± S.E.M. with n=3. **p<0.01, ***p<0.001.

Figure 4. miR-124a effect on cascade genes related to Foxa2. ***A.*** PDX-1 mRNA levels in MIN6 β-cells transfected with miR-124a2 precursor or with anti-miR-124a2 (250 pmol). *p<0.05, **p<0.01 ***B.*** PDX-1 protein levels in MIN6-cells transfected with miR-124a2 precursor or anti-miR-124a2 (250 pmol). Three independent experiments were performed and gels showing PDX-1 protein levels were scanned and quantified using Scion image program. The values were corrected for differences in the total amount of β-actin. *p<0.05. ***C***. Insulin, Kir6.2 and SUR-1 mRNA gene expression. MIN6-cells were transfected with miR-124a precursor or inhibitor (250 pmol) and left as such for 48h. RNA was extracted and subjected to reverse transcription followed by Real-time-PCR. Data represent three independent experiments ± S.D. with n=3. *p<0.05, ***p<0.001.

Figure 5. Impact of miR-124a2 expression on Foxa2 immunoreactivity. MIN6 cells were transfected with either empty pcDNA6.2 or pcDNA6.2-miR-124a2 (see Experimental Procedures) and, 48 hrs later, the immunoreactivity of Foxa2 determined by confocal microscopy using a goat anti-Foxa2 antibody (1:200 dilution) and Alexa Fluor 568-labeled anti-goat secondary antibody (1:500). GFP fluorescence was monitored by excitation at 488 nm and emission at 500-520 nm, and Alexa 568 at 568 (excitation) and 560-600 (emission). Foxa2 immunoreactivity was normalised in individual fields to the fluorescence in non-transfected cells (taken as 100%) to account for the non-specific effects of GFP expression alone. Cells 1 and 2 displayed ~20% and ~50% decrease in the expression of Foxa2, respectively. Scale bar = 10 μm. *p<0.05.

Figure 6. Impact of miR-124a2 on glucose- and KCl-induced $[Ca^{2+}]_c$ in single MIN6 β-cells. ***A.*** Single cells transfected as in Fig. 5 were perifused initially in KRB at the indicated glucose concentrations before changing to media with the concentrations of glucose shown or 50 mM KCl. Cells were selected as showing a significant increase in $[Ca^{2+}]_c$ in response to glucose. ***B.*** Distribution of $[Ca^{2+}]_c$ increases in response to a step increase in [glucose] from 3 to 30 mM in pcDNA6.2 alone or pcDNA6.2-miR-124a2-transfected cells, relative to the fold-increase indicated. Cells were stimulated during perifusion in KRB medium as described (Experimental Procedures). The black column illustrates the percentage of cells that did not display any statistically significant change in $[Ca^{2+}]_c$ under glucose stimulation. The total number of cells in each group was 35 and 54, respectively and data are from 6-9 independent experiments.

Figure 7. Effect of miR-124a2 expression on hGH or insulin secretion from MIN6 cells. ***A.*** MIN6 cells were co-transfected with pXGH5 and pcDNA6.2-miR124a2 or empty vector (pcDNA6.2). After 48hrs, cells were treated as described in the Experimental Procedures and stimulated for 30 min in KRB containing 3 mM glucose (clear bars) 30 mM glucose (filled bars) or 20 mM glucose + 50 mM KCl (hatched bars). Data are expressed as the average of three separate experiments ± SEM. *p<0.05, **p<0.005. ***B.*** MIN6 β-cells were treated with pre-miR-124a2 or with anti-miR-124a2

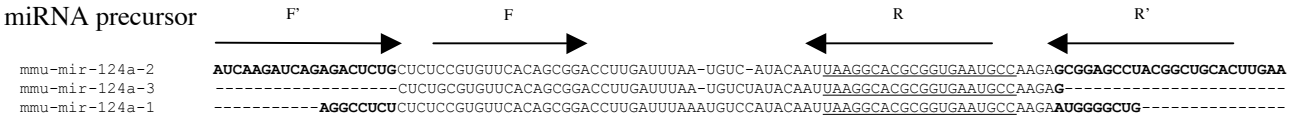
(250 pmol) and stimulated with glucose (30 mM) or 50 mM KCl. Data are expressed as average of three experiments ± SEM. *p<0.05.

Figure 8. Impact of miR-124a2 on glucose- or KCl-induced $[Ca^{2+}]_c$ increases in INS-1 (832/13) cells. Cells were transfected as described under Experimental Procedures. Single cells were perifused at the indicated glucose concentrations before changing to media containing 20 mM glucose (A) or 50 mM KCl (C). ***A***. Traces of $[Ca^{2+}]_c$ increase in response to a step increase in glucose in two representative single cells respectively transfected with the empty vector, pcDNA6.2 alone (left) or the vector expressing the miR-124a2, pcDNA6.2-miR124a2 (right). ***B***. Effect of miR-124a2 on $[Ca^{2+}]_c$ increases in response to glucose. Average of n=39 (pcDNA6.2) and 34 (pcDNA6.2-miR124a2) cells selected as showing a significant $[Ca^{2+}]_c$ response to glucose (fold response >1.10) and 5-6 independent experiments. Top left: Effect of miR-124a2 on basal $[Ca^{2+}]_c$; top right: effect of miR-124a2 on sensitivity to glucose; bottom left: effect of miR-124a2 on total $[Ca^{2+}]_c$ response; bottom right: effect of miR-124a2 on maximal $[Ca^{2+}]_c$ response. ***p<0.001. ***C.*** Effect of miR-124a2 on $[Ca^{2+}]_c$ increase in response to stimulation with KCl. Left: average response n=39 (pcDNA6.2) and 41 (pcDNA6.2-miR124a2) cells and 3 independent experiments. Right: effect of miR-124a2 on peak fold increase in $[Ca^{2+}]_c$. *p<0.05.

Figure 9. Effect of miR-124a2 on hGH secretion in INS-1 (832/13) cells. Effect of microRNA expression on hGH secretion as % total hGH transfected in INS-1 (832/13) cells with plasmid pXGH5 or pXGH-mir-124a2 or pXGH-mir-9. After pre-incubation as described, cells were incubated for 30 min in KRB containing 3 mM glucose (clear bars), 30 mM glucose (filled bars) or 3 mM glucose + 50 mM KCl (hatched bars). **p<0.01; ***p<0.001; ns= non significant.

Figure 1

A.



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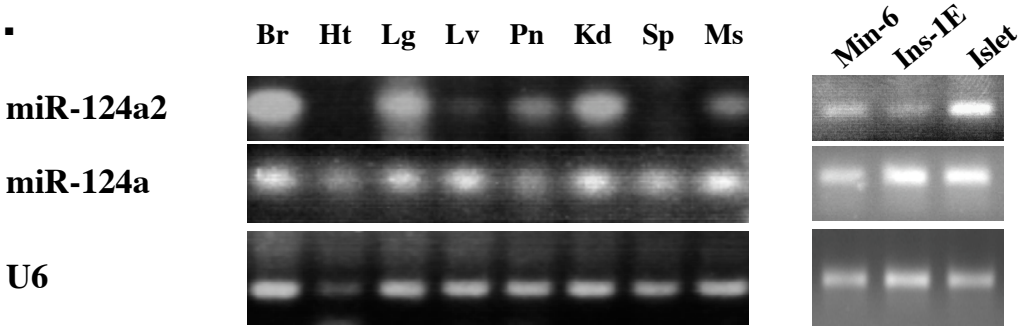

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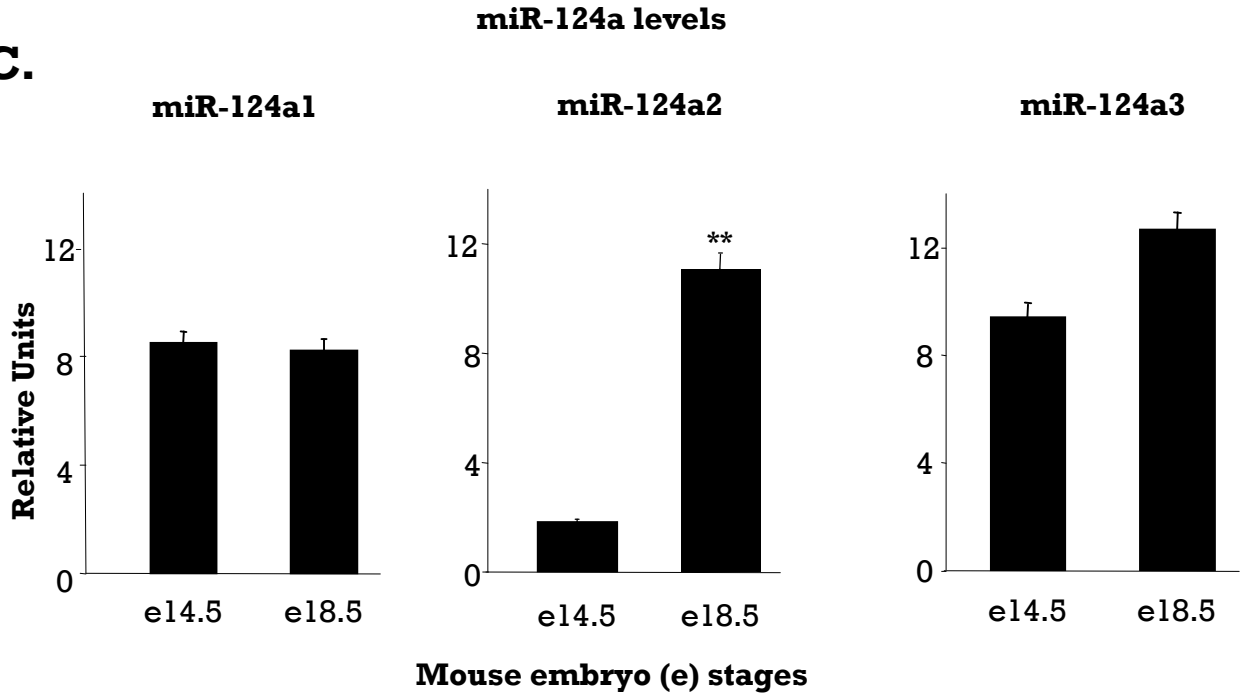
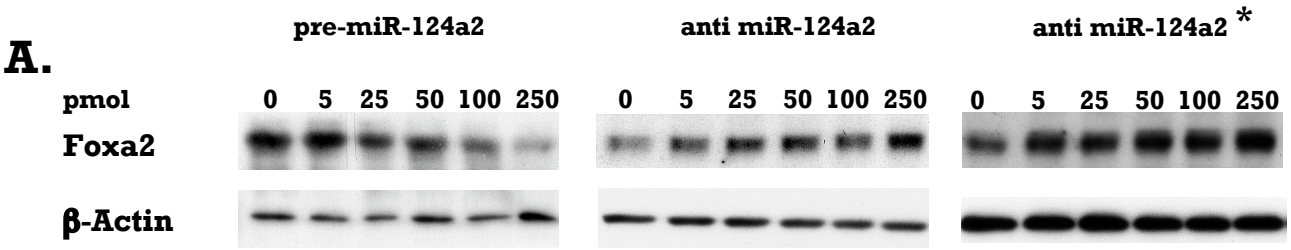
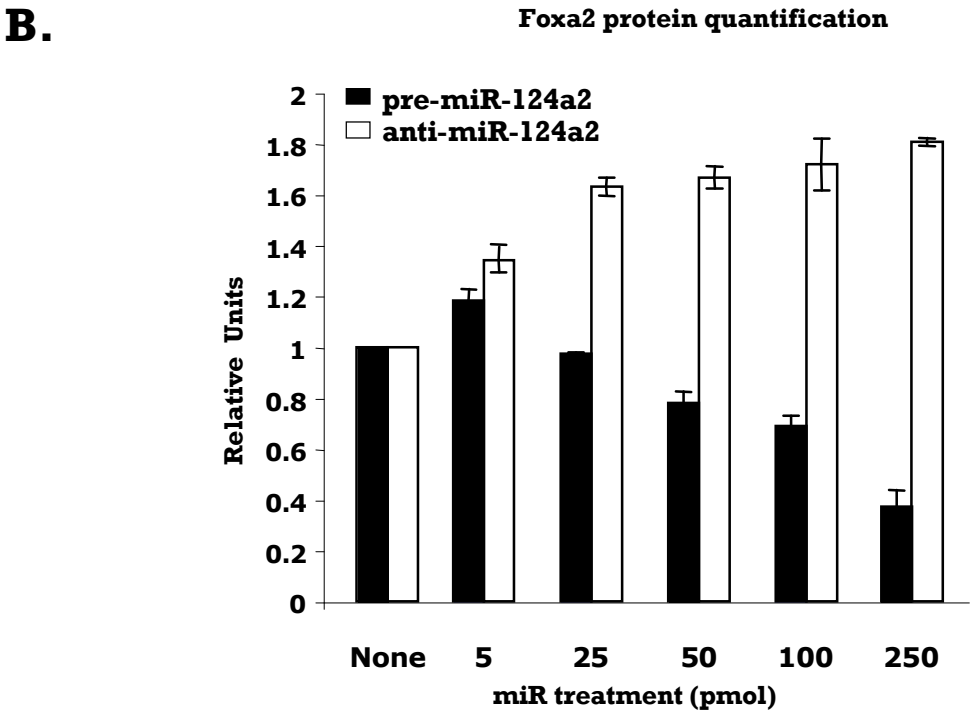


Figure 2





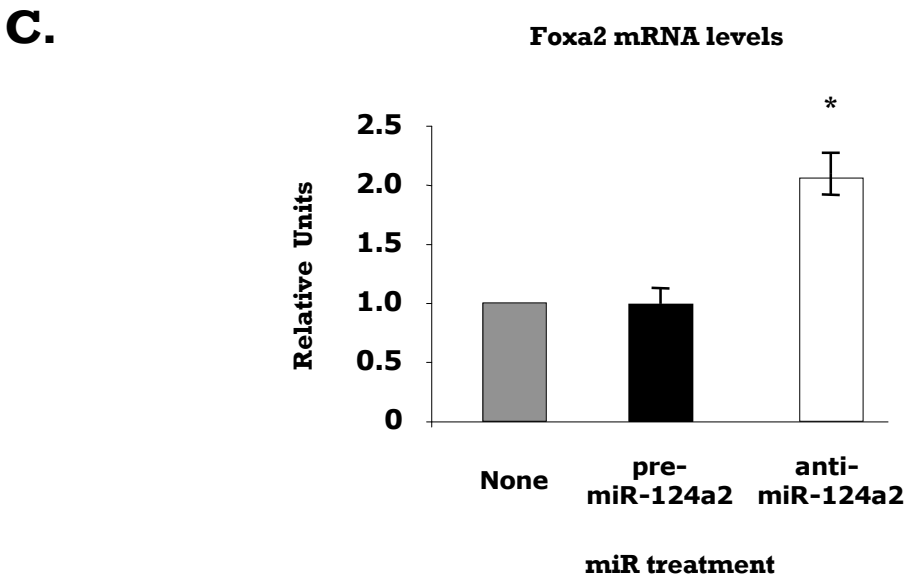


Figure 3

A. **miRNA-124a-recognition element (miR-124a-RE) found in Foxa2 transcript**

```
3' ACCGUAAGUGGCGCACGGAAUU 5'   Human miRNA 124a
   |||  ||: |:|||||||:|||       Energy: -22.2 kCal/mol
5' UGG--UUU-CUGCGUGCUUUAU 3'   Human transcript FOXA2
   |||  ||| ||||||||||||||      Conservation: 100.0%
5' UGG--UUU-CUGCGUGCUUUAU 3'   Mouse transcript FOXA2
   |||  ||: |:|||||||:|||       Energy: -22.2 kCal/mol
3' ACCGUAAGUGGCGCACGGAAUU 5'   Mouse miRNA 124a
```

B.

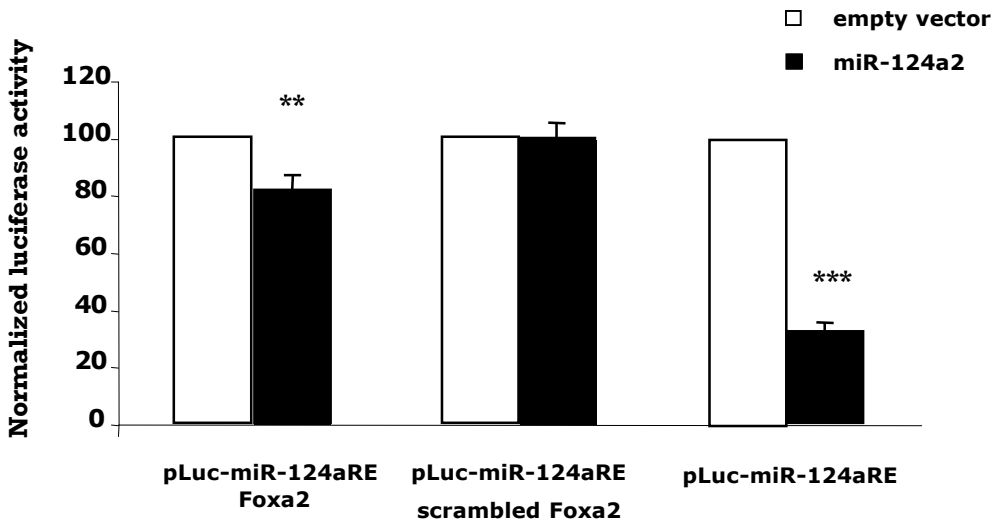


Figure 4

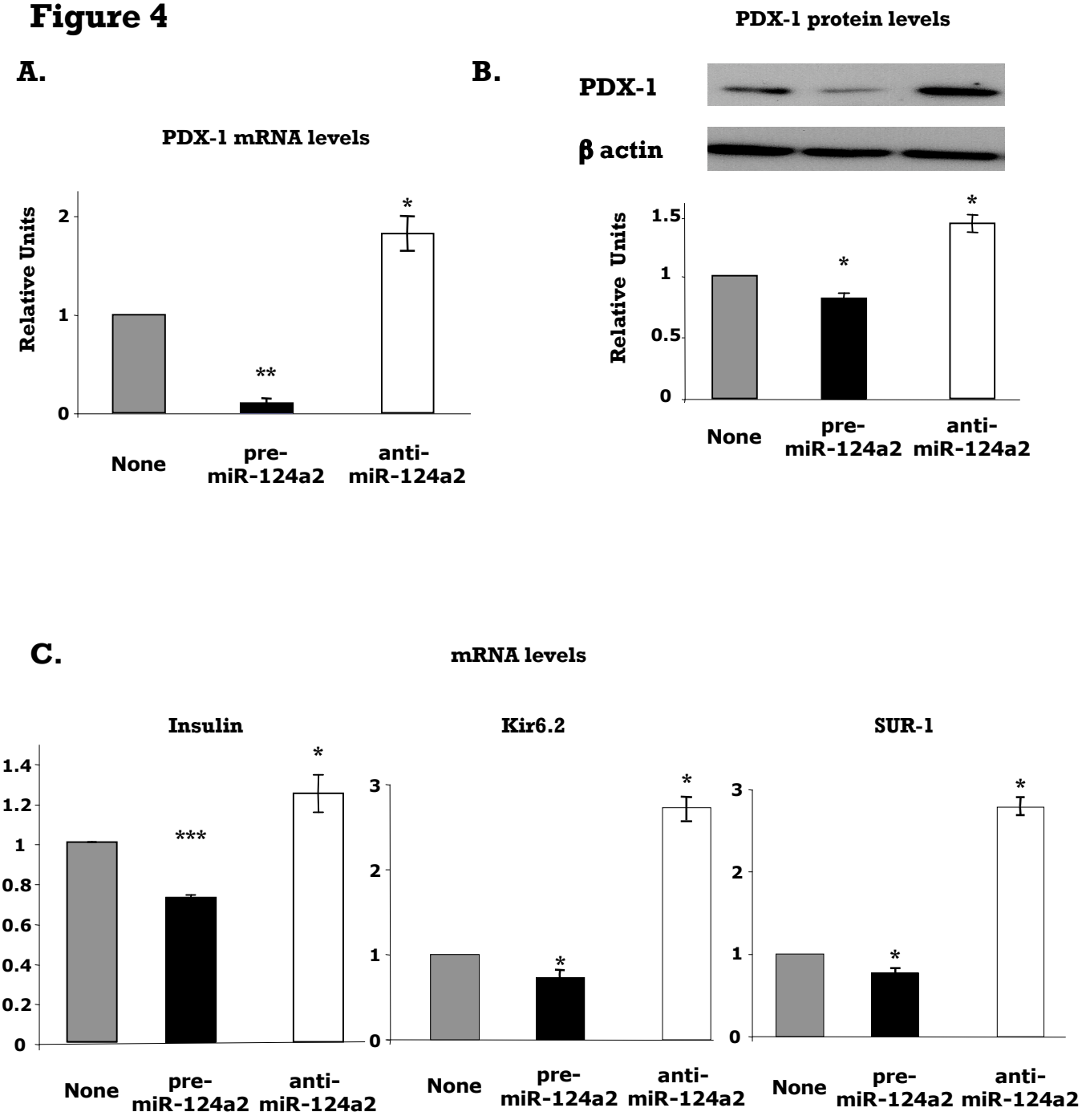


Figure 5

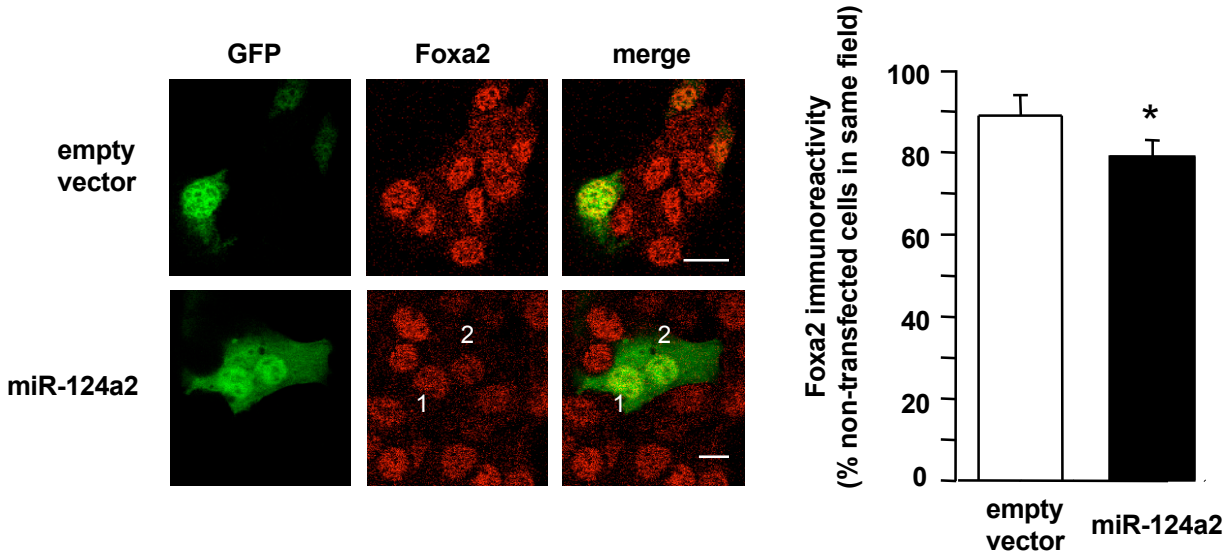


Figure 6

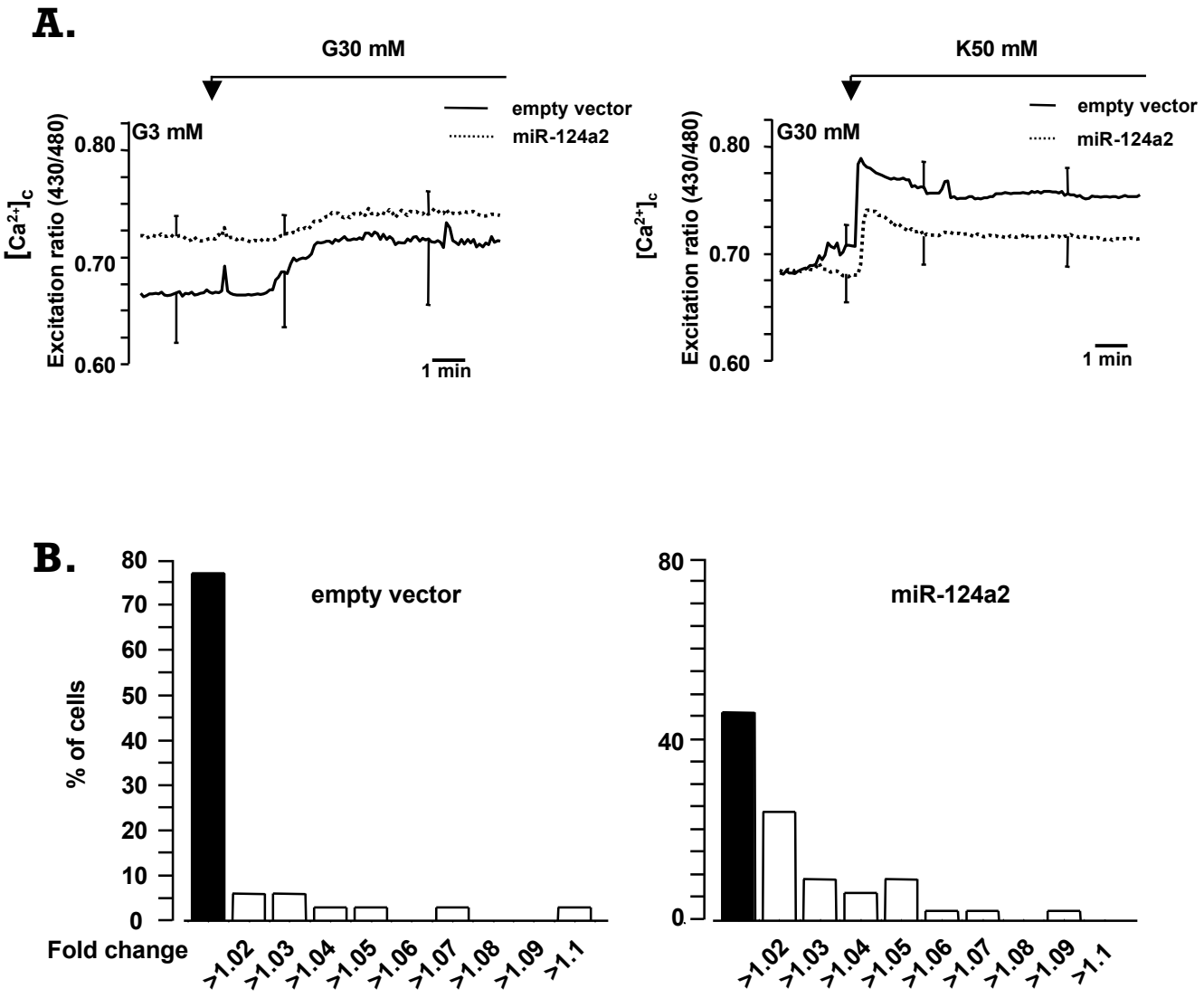
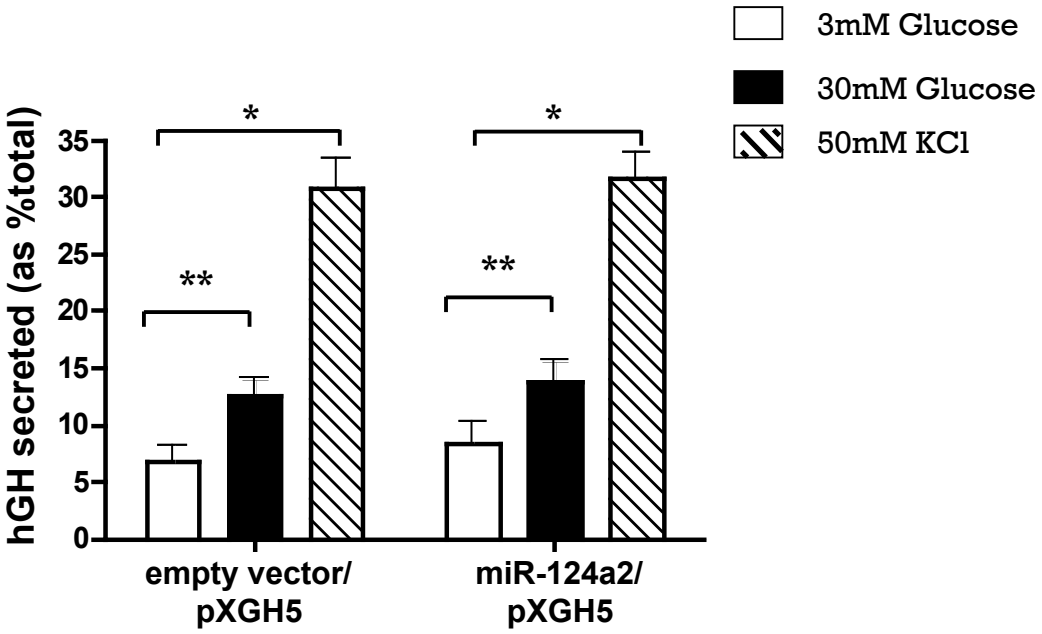


Figure 7

A.



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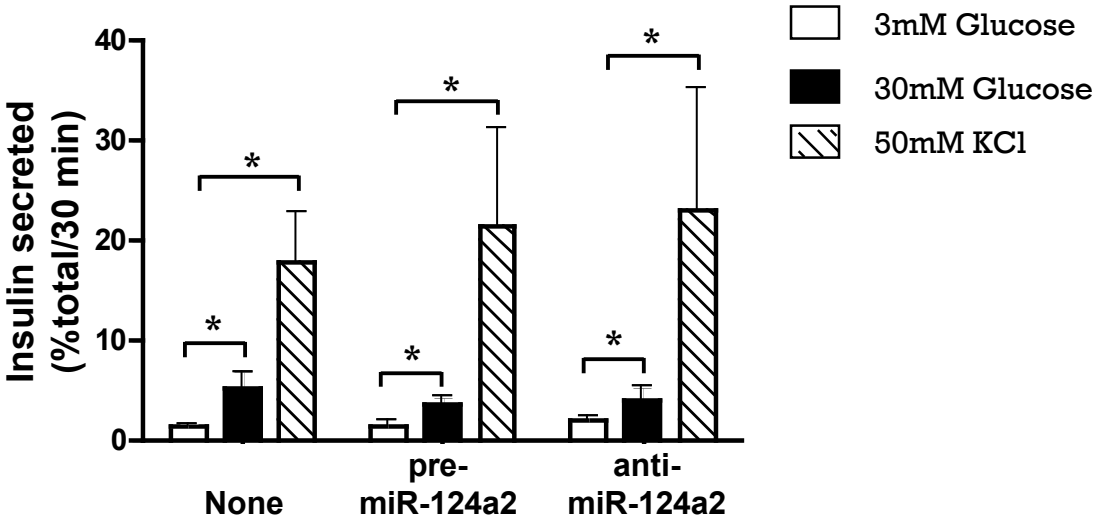

Figure 8

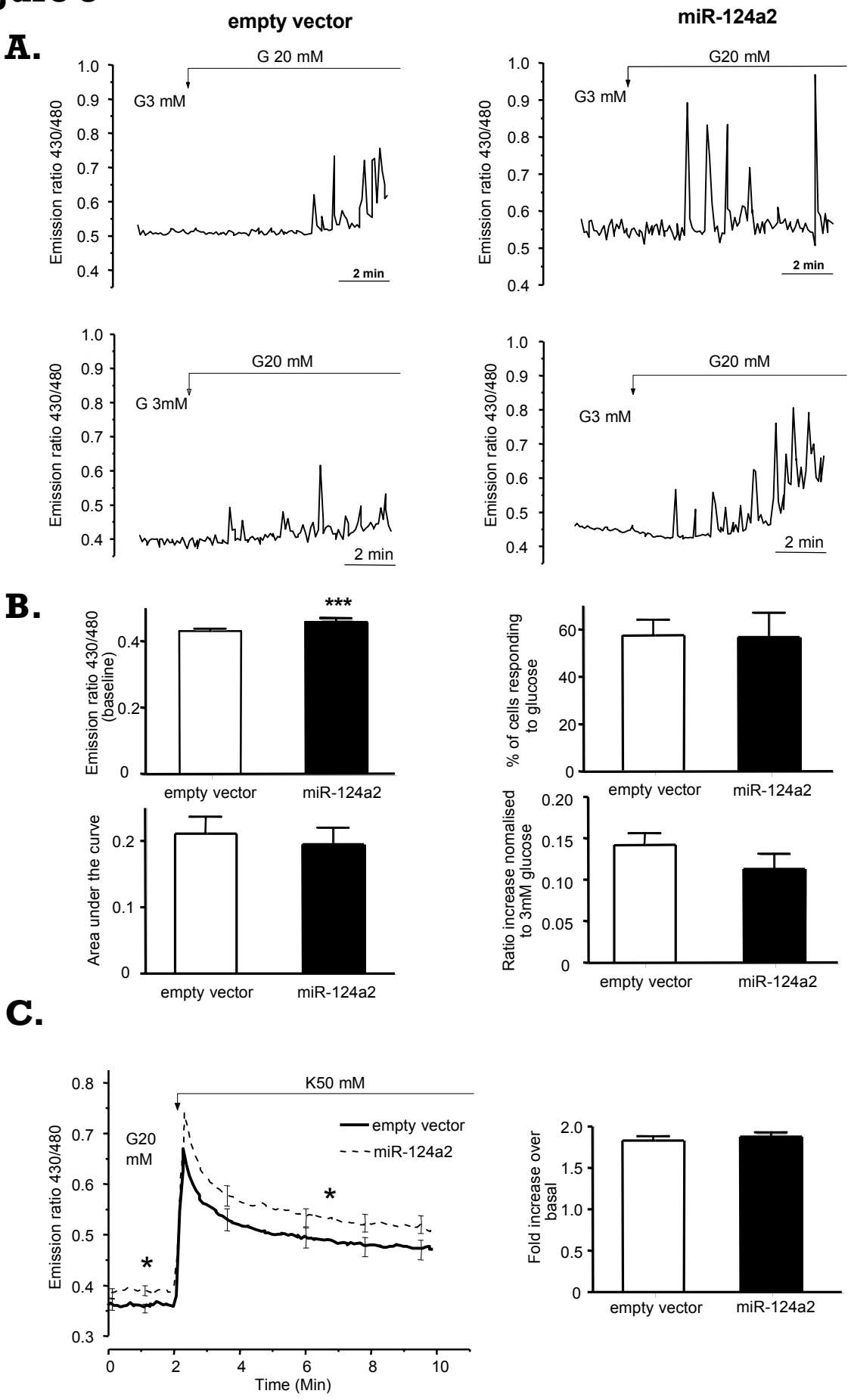

Figure 9