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Differential impact of glucose administered intravenously and orally on circulating miR-375 levels in human subjects

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Oral glucose impacts circulating miR-375 in humans

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The authors declare no competing interests.

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Abstract

Background

To date, numerous nucleic acid species have been detected in the systemic circulation including microRNAs (miRNAs); however their functional role in this compartment remains unclear.

Objective

The aim of this study was to determine whether systemic levels of miRNAs abundant in blood, including the neuroendocrine tissue-enriched miR-375, are altered in response to a glucose challenge.

Design

Twelve healthy males were recruited for an acute cross-over study which consisted of two tests each following an eight-hour fasting period. An oral glucose tolerance test (OGTT) was performed and blood samples were collected over a 3-hour period. Following a period of at least one week, the same participants were administered an isoglycemic intravenous glucose infusion (IIGI) with the same blood collection protocol.

Results

The glucose response curve following the IIGI mimicked that obtained after the OGTT, but as expected systemic insulin levels were lower during the IIGI compared to the OGTT ($P<0.05$). MiR-375 levels in circulation were increased only in response to an OGTT and not during an IIGI. In addition, the response to the OGTT also coincided with the transient increase of circulating glucagon-like peptide-1 (GLP-1), glucagon-like peptide-2 (GLP-2), and glucose-dependent insulinotropic polypeptide (GIP).

Conclusions

The present findings show levels of miR-375 increase following administration of an OGTT and in light of its enrichment in cells of the gut, suggest that the gastrointestinal tract may play a significant role to the abundance and function of this microRNA in the blood.

Précis

Here we show using a clinical cohort the impact of glucose administered orally on miR-375 in the blood. This result suggests a role for the gut in regulating miR-375 levels in systemic circulation.

Abbreviations:

IIGI	Isoglycemic intravenous glucose infusion
GLP-1	Glucagon-like peptide-1
GLP-2	Glucagon-like peptide-2
GIP	Glucose-dependent insulinotropic polypeptide
miRNA	microRNA
OGTT	Oral glucose tolerance test

Introduction

It is now widely accepted that miRNAs are present in the circulation; however their precise function in this compartment is not completely understood (1)(2)(3). MiR-375 is an abundant miRNA that has been identified in several tissues including pancreatic islets, the pituitary, adrenal glands, and the gastrointestinal tract (4). Whereas miR-375 has been established as a regulator of pancreatic β-cell function, its expression profile beyond this one cell type suggests a prominent role in other metabolic processes (5)(6)(7). Previous reports have identified miR-375 in the blood indicating that endocrine cells may contribute to its presence in circulation (1). As studies also now show alterations in systemic levels of miRNAs during disease states, it is still unclear how the kinetics of the miRNAs in circulation reflect changes in metabolism and physiology (8). Whether the miRNAs are actively released in response to changes in systemic glucose or other factors present in blood or whether they are constitutively released has also not been described.

In response to dietary intake, several hormones including insulin and the incretins, glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP), are released into the blood to facilitate several physiological processes and ultimately to lower blood glucose levels by promoting insulin release (9). GLP-1 is released by the intestinal endocrine L-cells and performs an array of functions in addition to its direct action on the β-cell. GLP-1 acts to slow gastric emptying while indirectly inhibiting glucagon secretion via the release of somatostatin. Meanwhile, GIP present in the K-cells of the small intestine, will facilitate energy storage via direct action on adipose tissue, and promote bone formation by increasing osteoblast proliferation (9). Studies primarily in rodent models have begun to explore the role of GLP-1 and GIP in other tissues including the brain and together these observations highlight the complex nature of incretin action in the context of energy homeostasis.

In this study we sought to determine whether miRNAs are released into the systemic circulation following a glucose challenge in human subjects and whether the presence of specific miRNAs coincide with established factors regulating glucose homeostasis. To date, extensive miRNA profiling has been performed on human tissues including blood. Here we show the systemic level of miR-375 is increased following oral ingestion of glucose implicating a role for the gut in the release of this small RNA into

circulation. We also observe that miR-375 is the most highly abundant and enriched miRNA in Sox9-High cells of the intestine, and robustly expressed and enriched in Sox9-Low cells, suggesting that the enteroendocrine cell population, and possibly the proliferating cells of the intestinal crypt, may contribute to systemic levels of this miRNA.

Materials and Methods

Human Patient Samples

As previously reported, approval was obtained from the Danish Data Protection Agency (2007-58-0010) and the Ethics Committee of the Central Denmark Region (1-16-02-377-13) and informed consent was obtained from all patients for being included in the study (10). Twelve healthy Caucasian males, aged 20 to 50 years, participated in the study which was registered at ClinicalTrials.gov (NCT02213276). During the OGTT and IGII experiments, blood samples were collected at 0, 15, 30, 60, 120, and 180 minutes from receiving the glucose load which lasted 5 minutes. For the 3-hour fasting control experiment, blood samples were collected after 0, 1, 2 and 3 hours. Blood samples for plasma analysis of insulin, GIP, GLP-1 and GLP-2 were prepared and quantified at the Department of Clinical Biochemistry at Aarhus University Hospital accredited in accordance with ISO15189 (10). After collection, blood samples were centrifuged at 2000 x g for 10 minutes and then stored at -80°C until analysis. Plasma insulin was measured by ELISA (cat. no. K6219; Dako, Glostrup, Denmark). All samples were extracted in a final concentration of 70% ethanol before GIP and GLP-1 measurements and 75% before GLP-2 measurements. Total GIP was measured using a radioimmunoassay with a C-terminally directed antibody (antiserum. no. 80867), which reacts fully with intact GIP and N-terminally truncated forms (11). The standard was human GIP (Bachem, cat no. H-5645) and the tracer was ^{125}I-labeled human GIP (Perkin Elmer, cat no. Nex402). Total GLP-1 was measured using a radioimmunoassay with an antibody (antiserum. no. 89390) specific for the C-terminal of the GLP-1 molecule and reacting equally with intact GLP-1 and the primary (N-terminally truncated) metabolite (12). Intact GLP-2 was measured using a radioimmunoassay as originally described (13). The antiserum (code no. 92160) is directed against the N-terminus of GLP-2 and

therefore measures only fully processed GLP-2 of intestinal origin. For standards, we used recombinant human GLP-2 and the tracer was ^{125}I-labeled rat GLP-2 with an Asp33 -> Tyr33 substitution. Sensitivity for all the radioimmunoassays was below 5 pmol/l, and intra-assay coefficient of variation below 10 %.

Gene Expression Analysis

Total RNA was isolated from plasma using Trizol LS (Invitrogen) with glycogen as a carrier. MiRNAs were quantified by TaqMan Assays using the TaqMan MicroRNA Reverse Transcription Kit and miRNA specific primer sets (Thermo Scientific). Plasma miR-375, miR-148a, and miR-27b levels were normalized to miR-16 expression in each respective sample.

Small RNA Sequencing Analysis

Raw miRNA counts were retrieved from Supplementary Table 4 of Kang et al. 2017 (14). The publically available data was downloaded from Sequence Read Archive FTP site and NCBI dbGaP on 12/08/2015 resulting in 823 data sets from human tissue samples from and 395 data sets originating from serum and CSF. The precise details of the analysis can be retrieved from the Material and Methods section of Kang et al. 2017 (14).

Tissue samples were normalized to estimated number of miRNA molecules per cell and we assumed the total amount of miRNA per cell to be ~100,000 (15). Counts for individual miRNA were divided by the sum of all miRNA reads in the sample and multiplied with the assumed total miRNA abundance. The same normalization was applied to body fluid samples but as the total abundance of miRNA in those fluids is unknown, the values do not represent copies per cell and are indicated as 'arbitrary units'. In total, 1787 miRNA were detected and analyzed; independent sample sizes were blood cells (n=197), liver cells (n=93), brain cells (n=102), serum samples (n=212), exosome samples (n=37) and cerebrospinal fluid (CSF) (n=180). In summary, the number of times a given miRNA is detected in sequencing depends on its abundance and the overall sequencing depth (16).

For small RNA sequencing from gut tissue of Sox9-EGFP mice, IEC isolation and FACS was performed with the UNC Flow Cytometry Core Facility as described previously (17). RNA was isolated from sorted subpopulations using the Total RNA Purification Kit (Norgen Biotek), and small RNA libraries were prepared with a range of

~60ng to ~850ng of total RNA using the CleanTag Small RNA Ligation Kit (TriLink Biotechnologies, CA) at the sequencing facility at the University of Texas Health Sciences Center, San Antonio. Libraries were sequenced on the Illumina HiSeq2500 platform, yielding an average of ~40 million total reads per sample. Raw sequencing data and miRNA quantification tables for all samples can be accessed through the NCBI GEO accession number GSE XXXX.

Animal Studies.

All animal studies were approved by the Institutional Animal Care and Use Committee at the University of North Carolina, where the mouse experiments were performed. Chow-fed male Sox9-EGFP mice were housed in cages with ¼-inch Bed-O'cobs laboratory animal bedding. At 24-28 weeks of age, the mice were anesthetized using isofluorane, then euthanized by cervical dislocation.

Statistical analysis

Comparisons between data sets with two groups were evaluated using an unpaired Student's t-test. Repeated measures ANOVA was performed using GraphPad Prism Software 6.07 to compare levels of miR-375, glucose, insulin and gastrointestinal hormones between OGTT, IIGI and the 3-hour fasting control (Supplemental Table 1). Post hoc statistics were performed using Sidak's multiple comparison test. A *P*-value of less than or equal to 0.05 was considered statistically significant.

Results

Quantification of miR-375 in human blood

Surveying miRNA abundances in >800 human samples from six different tissues and body fluids we observed high abundances of miR-16 in all analyzed tissues including blood cells, liver, brain, serum, exosome and CSF samples (Figure 1a and 1b). In contrast, miR-375 was only abundant in serum, exosomes, liver and CSF, while it was moderately expressed in the brain and virtually absent in blood cells. This lack of expression in blood cells suggests that miR-375 may be exported into the bloodstream by adjoining tissues. In contrast, miR-124 was found to be only moderately expressed in the brain and was at low levels in all other analyzed tissues (Figure 1b). This further underlines the differential tissue expression pattern and function of miRNAs.

Systemic miR-375 increases during OGTT

In light of its abundance in circulation, we quantified systemic miR-375 levels in the plasma of healthy human subjects in response to either an OGTT or an isoglycemic IIGI. Blood glucose levels were comparable between the OGTT and IIGI experiments (Figure 2a). The plasma miR-375 level significantly increased during the OGTT where it reached a maximum after 60 and 120 min (~4.8 and ~5.3-fold increase, respectively) and returned to baseline level after 180 min. In contrast, during the IIGI expression of this microRNA remained indistinguishable from steady state levels (Figure 2a, Supplemental Figure 1a, and Supplemental Table 1).

The oral glucose administration resulted in a pronounced increase in circulating gut hormones GIP, GLP-1, GLP-2 (at time 30 minutes, ~3.9, ~2.2, and ~3.5-fold increase, respectively) as well as plasma insulin (~8.5-fold) as recently published (Figure 2b-f) (10). During the intravenous glucose infusion plasma levels of GIP, GLP-1 and GLP-2 remained at baseline levels, while insulin showed only a small increase (Figure 2c-f). Together these results indicate the temporal rise in miR-375 correlates with increased levels of the gut hormones and this is further supported with both area-under-curve calculations and analyzing miR-375 levels using analysis of covariance (ANCOVA), as plotted in relation to GIP or GLP-1 (Supplemental Figure 1b-e). Meanwhile, we also addressed the expression of two additional miRNAs abundant in human serum, miR-148a amd 27b, and observed no changes in systemic levels during the OGTT or IIGI (Suppementary Figure 2a and 2b) (14).

MiR-375 is enriched in enteroendocrine cells

We next performed small RNA-sequencing on intestinal epithelial subpopulations isolated from 24-28-week-old male Sox9-EGFP reporter mice to identify cell types enriched for miR-375 (Figure 3) (18)(19). As described previously, FACS sorting resulted in four independent cell populations: the Sox9-High group is enriched for enteroendocrine cells and reserve/quiscent intestinal stem cells (Figure 3a), Sox9-Low is enriched for actively-cycling intestinal stem cells (Figure 3b), Sox9-Sublow is enriched for transit amplifying cells (Figure 3c), and Sox9-Neg is enriched for enterocytes (Figure 3d) (17). Together, these results show that miR-375 is the most highly expressed and

highly enriched miRNA in Sox9-High cells and is also robustly expressed and enriched in Sox9-Low cells (though not as high as in Sox9-High cells).

Discussion

Our knowledge is incomplete with regards to the dynamics and function of the majority of circulating factors in human blood. It has long been known that administration of glucose orally versus intravenously differentially affects the release of insulin due to the role of the incretins in islet cell function; however the full extent to the interplay of these endocrine factors in the context of glucose and energy metabolism remains to be determined (20)(21). In the present study, we observed the presence of miRNA sequences known to be enriched in tissues central to the regulation of glucose and energy homeostasis including miR-375. Interestingly, higher levels of miR-375 were observed in the plasma of human subjects following an oral glucose challenge in contrast to an intravenous glucose infusion. Levels of miR-375 peaked 30 minutes after the peaks of insulin and incretins, establishing that systemic increase of miR-375 is temporally associated the release of these hormones. The differential response of miR-375 in blood may yet identify an additional physiologic function of the gut or its secreted incretin hormones. Consistent with previous observations, both methods of glucose administration achieved similar blood glucose levels while the insulin secretory response was diminished during the IIGI (22)(23). The robust increase in plasma insulin levels quantified during the OGTT coincided with a concomitant increase in plasma GLP-1, GLP-2, and GIP. Hence, these results present correlative evidence that the gut may play either a direct or indirect role in the regulation of the circulating levels of miR-375 in response to oral glucose.

It is also unclear whether our results are related to previous studies measuring miR-375 in blood after pharmacological destruction of beta cell mass; previous studies have reported an alteration in the cellular distribution within the gastrointestinal tract after administration of streptozotocin to mice (24)(25)(26). Latreille *et al.* reported that the pancreatic β-cell, the cell type expressing the highest levels of miR-375, contributes to ~1% of the systemic miR-375 levels, indicating that the amount of miRNAs released by the β-cell is either negligible in comparison to other tissues or they are rapidly taken

up or they are degraded once in circulation (25). It is also possible that several endocrine cell types release miR-375 in response to incretin signaling in line with their role as paracrine effectors and insulin secretagogues.

Our observations here showing a rise in miR-375 levels in blood following the oral intake of glucose suggests several possibilities regarding the role of the gut with regard to the presence miRNAs in circulation. First, circulating levels of this miRNA may either originate from the gut or respond to signaling molecules released by this tissue. Our profiling results from sorted cells of the gut confirm miR-375 to be highly abundant in subpopulations enriched for both enteroendocrine cells and intestinal stem cells (and also present albeit at lower levels in transit-amplifying cells and enterocytes) (27). In light of the heterogeneity of L-cells, another possibility is that L-cells of the upper gut act primarily in nutrient sensing while in the lower gut, these cells contribute to the release of miR-375 (28). The highest levels of miR-375 were measured between 30 and 120 minutes post-administration; therefore the presence of this miRNA in the secretory granules storing either insulin or GLP-1 is unlikely. It is unclear how many additional miRNAs the differential impact of oral glucose versus intravenous on systemic levels will be shown to affect as abundant sequences such as miR-148a and miR-27b were observed not altered.

A recent study by Kahn and colleagues has shown evidence that circulating miRNAs are derived from adipose tissue and are able to target genes of the liver (29). Using a tissue-specific knockout of the miRNA-processing enzyme Dicer, they observed a decrease in circulating miRNAs present in exosomes and that over-expression of miR-99b was able to suppress activity of a reporter construct containing the 3'UTR of the gene Fgf21, a circulating factor expressed by the liver and other tissues (29). MiR-375 is also detected in liver and while expression of miR-375 in this tissue is several magnitudes lower than the levels measured in the pancreatic islet, miR-122 is detected in blood suggesting the hepatocyte is also a site of miRNA export (6)(30)(31). However, it would be prudent for future investigations to determine whether the measured amounts of miR-375 are endogenous expression from hepatocytes. Given the volume of blood transported throughout the liver, it is likely a significant fraction of miR-375 expression quantified in this tissue is derived from its presence in the systemic circulation. Therefore the

functional relevance of miR-375 in the liver at steady state conditions is unclear at this time. Given the dozens of other more abundant miRNA sequences present, most notably miR-122, the function of low-expressed miRNAs is not known based on the absence of strong evidence showing target regulation in vivo. While dysregulation of miR-375 target genes in the liver cannot be ruled out, none of the targets of this miRNA that were experimentally-validated using knockout tissues, have been studied in the context of glucose or lipid metabolism in the liver (4). The mild hyperglycemia observed in miR-375 knockout mice appears to result from increased glucagon levels stemming from increased alpha cell mass in the islet (4).

In summary, our observations on miR-375 in circulation continue to reflect the exceedingly complex and dynamic state of human blood following glucose uptake. In light of the temporal relationship between miR-375 and incretin hormones, future studies should explore the physiological relevance of miRNA in circulation and whether functional overlap exists between non-coding RNA and signaling hormones.

Declarations

Ethics Approval and Consent to Participate

As previously reported, approval was obtained from the Danish Data Protection Agency (2007-58-0010) and the Ethics Committee of the Central Denmark Region (1-16-02-377-13) and informed consent was obtained from all patients for being included in the study (10).

Consent for publication

Not applicable.

Availability of data and material

All primary data supporting the findings of this study are available on reasonable request.

Author's contributions

X.Y., Z.W., S.R., M.T., T.R., S.G.T., B.C.E.P., M.K., C.O., J.F., P.S., M.F., J.S., S.G., and M.P. contributed to the conception and design of the study, and wrote the manuscript. All authors approved the final version of this manuscript.

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Figure Legends

Figure 1. Abundances of miR-375, miR-16 and miR-124 in three human tissues and three body fluids. (**a**) Abundances of the three miRNAs in blood cells, liver and brain. The vertical axis shows estimated miRNA molecules per cell, logarithmic scale. (**b**) Abundances in serum, exosome and cerebrospinal fluid. The vertical axis indicates arbitrary units for cell-free samples. In total, 197 blood cell, 93 liver, 102 brain, 212 serum, 37 exosome and 180 cerebrospinal samples were profiled.

Figure 2. Measurements of miR-375, glucose, insulin, GIP, GLP-1, and GLP-2 in the plasma of healthy human individuals during an oral glucose tolerance test (OGTT), intravenous isoglycemic glucose infusion (IIGI), and a 3-hour fasting control (open circles (OGTT), filled squares (IIGI), and open squares (control)). (**a**) Plasma miR-375 levels during the OGTT (open circles), the IIGI (filled squares), and during the control (open squares). (**b**) Plasma glucose concentrations during the OGTT (open circles), the IIGI (filled squares), and during the control (open squares). (**c**) Plasma insulin concentrations during the OGTT (open circles), the IIGI (filled squares), and during the control (open squares). (**d**) Plasma GIP concentrations during the OGTT (open circles), the IIGI (filled squares)), and during the control (open squares). (**e**) Plasma GLP-1 concentrations during the OGTT (open circle), the IIGI (filled squares), and during the control (open squares). (**f**) Plasma GLP-2 concentrations during the OGTT (open circle), the IIGI (filled squares), and during the control (open squares). Results are presented as mean ± SEM. Data on plasma glucose, insulin, GLP-1, GLP-2, and GIP have recently been published and are reproduced with permission (10). The summary of statistical analyses for these datasets is compiled in Supplemental Table 1.

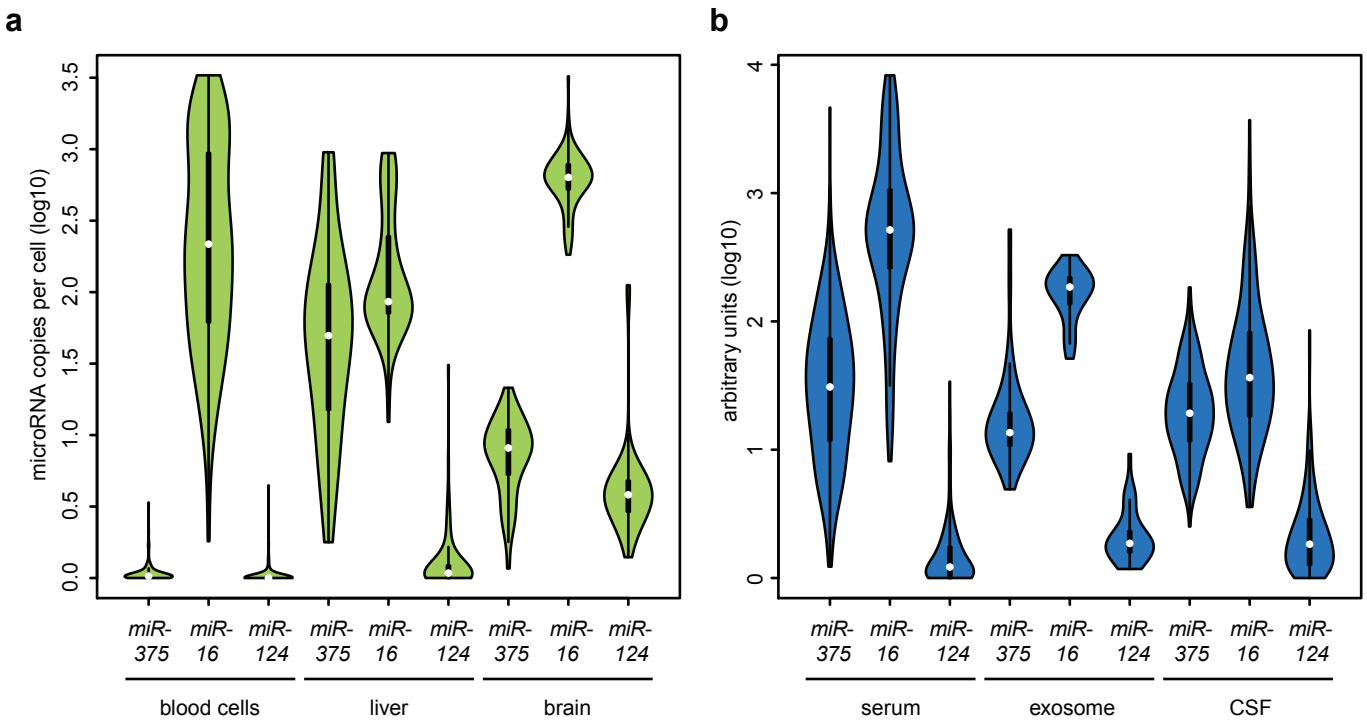
Figure 3. MiR-375 is highly expressed and significantly enriched in independent intestinal epithelial subpopulations. (**a**) Bar graph showing normalized levels of expression for miR-375 (y-axis) from small RNA-sequencing across four different intestinal epithelial subpopulations (x-axis) in 24-week-old male Sox9-EGFP reporter mice (n=3). Sox9-High (enriched for enteroendocrine cells), Sox9-Low (enriched for intestinal stem cells), Sox9-Sublow (enriched for transit-amplifying cells), and Sox9-Neg (enriched for enterocytes). RPMMM, reads per million mapped to microRNAs. (**b**) Scatter plot showing average expression of microRNAs from small RNA-sequencing in Sox9-High cells (y-axis) and fold-enrichment in Sox9-High cells relative to unsorted intestinal epithelial cells (x-axis) from 24-week-old male Sox9-EGFP reporter mice (n=3). Only microRNAs with average RPMMM > 1000 are displayed. Each data point represents a microRNA, miR-375 is labeled. (**c**) Scatter plot showing average expression of microRNAs from small RNA-sequencing in Sox9-Low cells (y-axis) and fold-enrichment in Sox9-Low cells relative to unsorted intestinal epithelial cells (x-axis) from 24-week-old male Sox9-EGFP reporter mice (n=3). Only microRNAs with average RPMMM > 1000 are displayed. Each data point represents one single microRNA, miR-375 is labeled. (**d**) Scatter plot showing average expression of microRNAs from small RNA-sequencing in Sox9-Sublow cells (y-axis) and fold-enrichment in Sox9-Sublow cells relative to unsorted intestinal epithelial cells (x-axis) from 24-week-old male Sox9-EGFP reporter mice (n=3). Only microRNAs with average RPMMM > 1000 are displayed. Each data point represents a microRNA, miR-375 is labeled. (**e**) Scatter plot showing average expression of microRNAs from small RNA-sequencing in Sox9-Neg cells (y-axis) and fold-enrichment in Sox9-Neg cells relative to unsorted intestinal epithelial cells (x-axis) from 24-week-old male Sox9-EGFP reporter mice (n=3). Only microRNAs with average RPMMM > 1000 are displayed. Each data point represents a microRNA.

Supplemental Figure Legends

Supplemental Figure 1. Analysis of plasma levels of miR-375, GIP, and GLP-1, during an oral glucose tolerance test (OGTT), an intravenous isoglycemic glucose infusion (IIGI), and during the fasting control (Control). (**a**) Comparison of area under the curve

analyses for miR-375 during the OGTT, IIGI, and fasting control. (**b**) Comparison of area under the curve analyses for GIP during the OGTT, IIGI, and fasting control. (**c**) Comparison of area under the curve analyses for GLP-1 during the OGTT, IIGI, and fasting control. (**d**) Plasma levels of total GIP plotted against plasma levels of miR-375 from each individual patient during the OGTT and IIGI. (**e**) Plasma levels of total GLP-1 plotted against plasma levels of miR-375 from each individual patient during the OGTT and IIGI. The summary of statistical analyses for these datasets is compiled in Supplemental Table 1.

Supplemental Figure 2. Quantification of miR-148a and miR-27b in the plasma of healthy human individuals during an oral glucose tolerance test (OGTT), an intravenous isoglycemic glucose infusion (IIGI), or during a 3-hour fasting control. (**a**) Plasma miR-148a levels during the OGTT (open circles), the IIGI (filled squares), and the fasting control (open squares). (**b**) Plasma miR-27b levels during the OGTT (open circles), the IIGI (filled squares), and the fasting control (open squares). Results are presented as mean ± SEM.



Yan et al., 2017
Figure 1

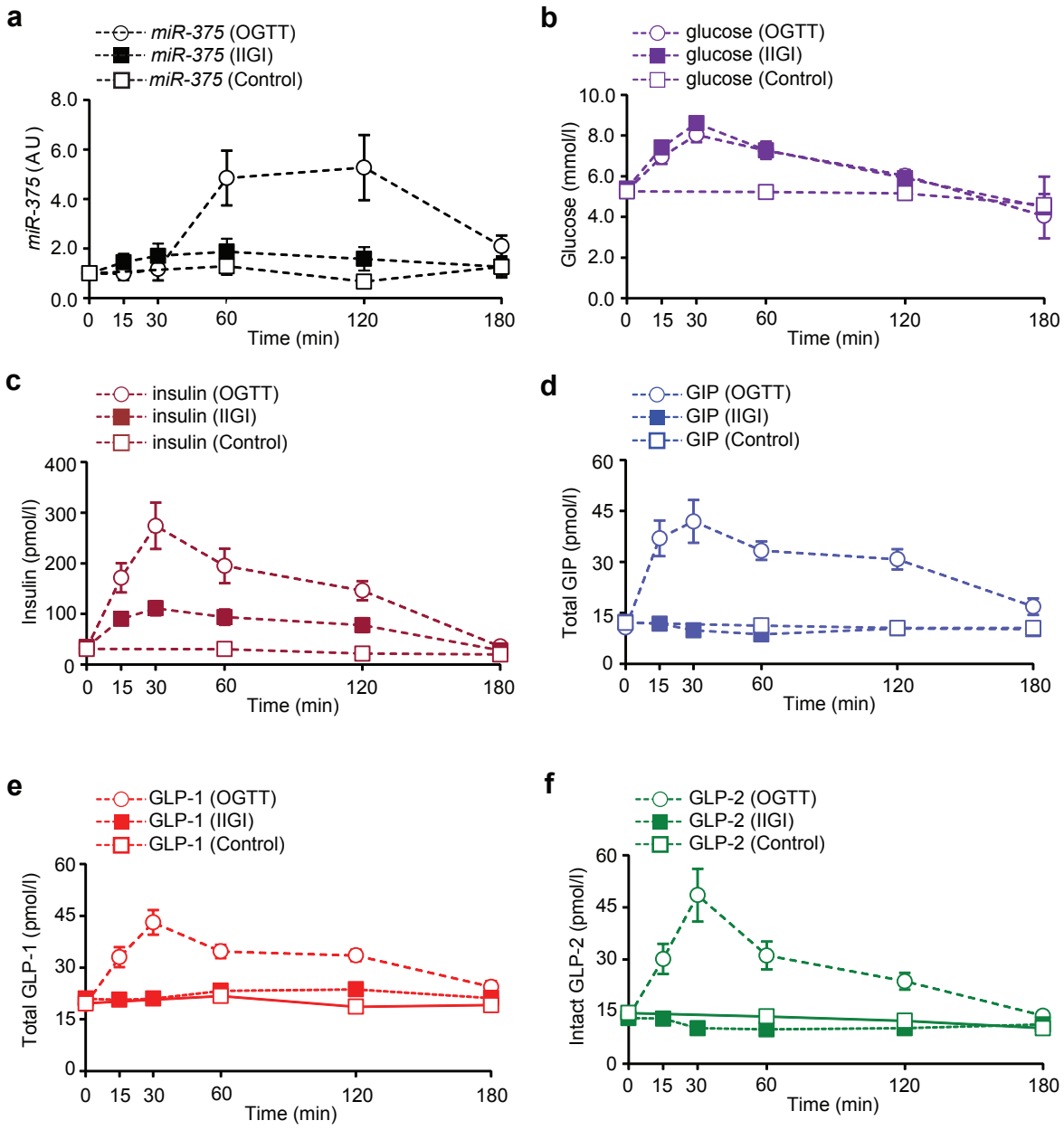


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Figure 2

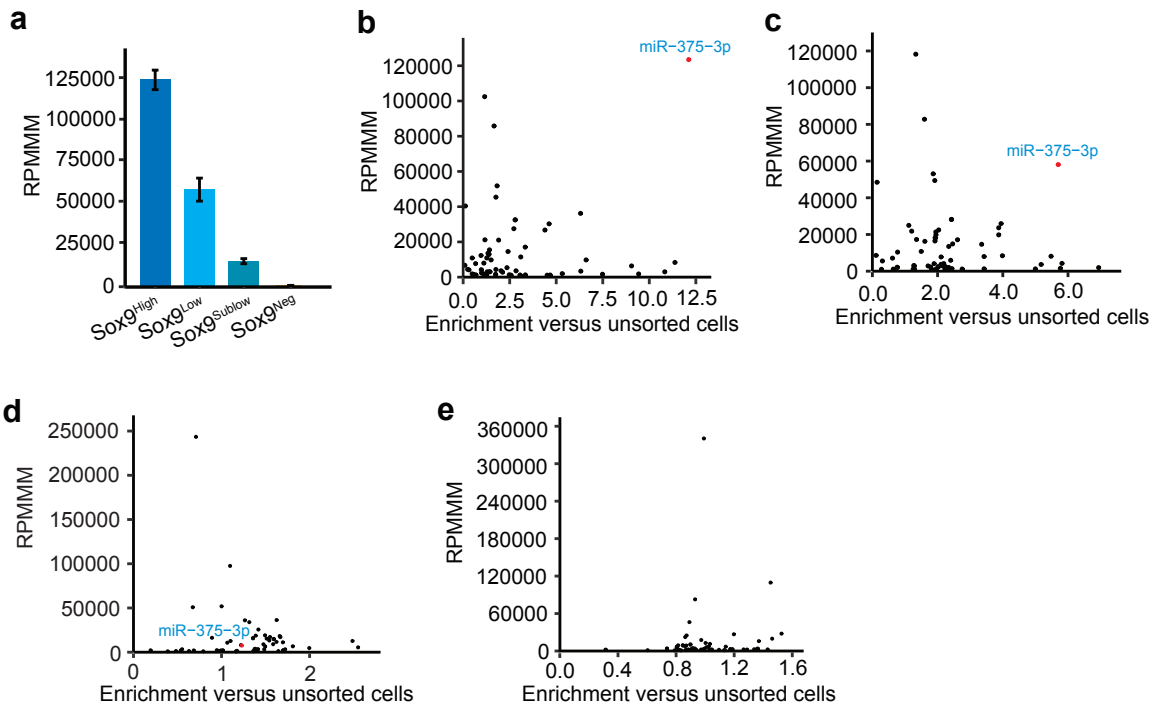

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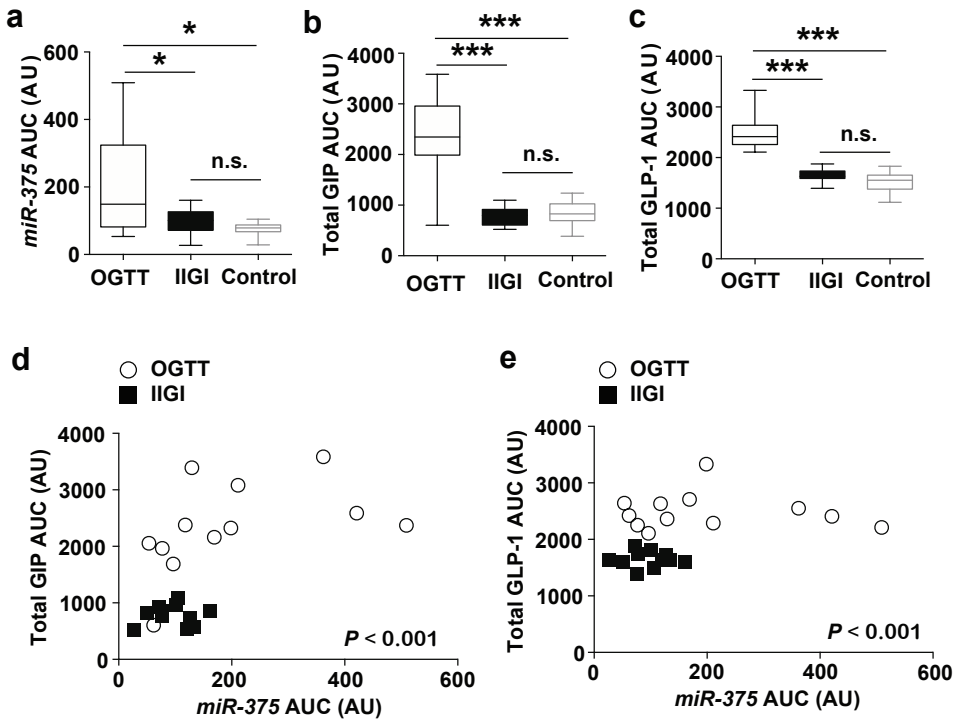
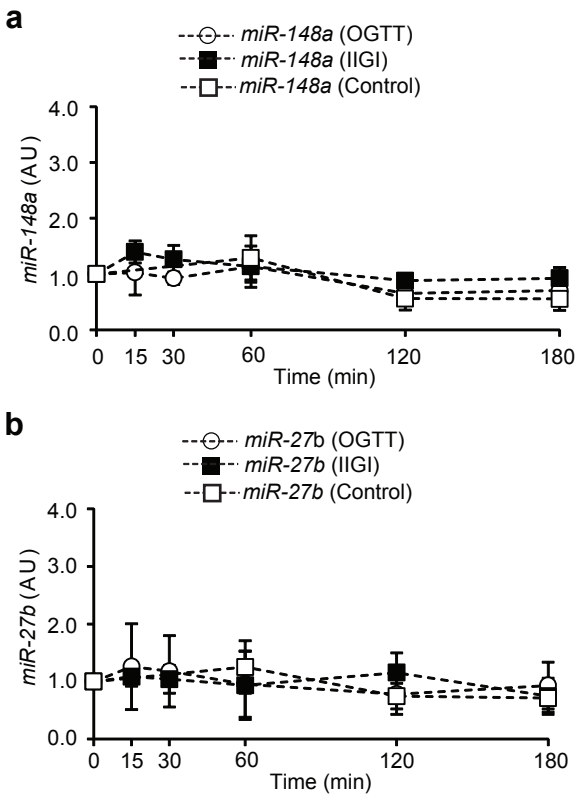
Figure 3



Yan et al., 2017
Supplementary Figure 1



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Supplementary Figure 2