MICRORNA 21 TARGETS B CELL LYMPHOMA 2 (BCL2) MRNA TO INCREASE

BETA CELL APOPTOSIS AND EXOSOMAL MICRORNA 21 COULD SERVE AS A

BIOMARKER OF DEVELOPING TYPE 1 DIABETES MELLITUS

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BETA CELL APOPTOSIS AND COULD SERVE AS A BIOMARKER OF

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The role of beta cell miR-21 in Type 1 Diabetes (T1D) pathophysiology has been controversial. Here, we sought to define the context of beta cell miR-21 upregulation in T1D and the phenotype of beta cell miR-21 overexpression through target identification. Furthermore, we sought to identify whether circulating extracellular vesicle (EV) beta cell-derived miR-21 may reflect inflammatory stress within the islet during T1D development.. Results suggest that beta cell miR-21 is increased in *in-vivo* models of T1D and cytokine-treated cells/islets. miR-21 overexpression decreased cell count and viability, and increased cleaved caspase-3 levels, suggesting increased cell death. *In silico* prediction tools identified the anti-apoptotic mRNA *B Cell Lymphoma 2 (BCL2)* as a conserved miR-21 target. Consistent with this, miR-21 overexpression decreased BCL2 transcript and protein expression, while miR-21 inhibition increased BCL2 protein levels and reduced cleaved caspase-3 levels following cytokine-treatment. miR-21-mediated cell death was abrogated in 828/33 cells, which constitutively overexpress BCL-2. Luciferase assays suggested a direct interaction between miR-21 and the BCL2 3'untranslated region. With miR-21 overexpression, PRP revealed a shift of *BCL-2* message toward monosome-associated fractions, indicating inhibition of *BCL2* translation. Finally, overexpression in dispersed human islets confirmed a reduction in *BCL2* transcripts and increased cleaved caspase 3 production. Analysis of EVs from human beta cells and islets exposed to cytokines revealed a 3-5-fold increase in miR-21.

Nanoparticle tracking analysis showed no changes in EV quantity in response to cytokines, implicating specific changes within EV cargo as responsible for the miR-21 increase. Circulating EVs from diabetic non-obese diabetic (NOD) mice displayed progressive increases in miR-21 that preceded diabetes onset. To validate relevance to human T1D, we assayed serum samples collected from 19 pediatric T1D subjects at the time of diagnosis and 16 healthy controls. Consistent with our NOD data, EV miR-21 was increased 5-fold in T1D samples. In conclusion, in contrast to the pro-survival role reported in other systems, our results demonstrate that miR-21 increases beta cell death via BCL2 transcript degradation and inhibition of BCL2 translation. Furthermore, we propose that EV miR-21 may be a promising marker of developing T1D.

Carmella Evans-Molina M.D., Ph.D., Chair

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LIST OF ABBREVIATIONS

T1D- Type 1 diabetes

miRNA-microRNA

mRNA- messenger RNA

miRNA 21- miR-21

NFKB- nuclear factor kappa B

EV- extracellular vesicle

BCL2- B Cell Lymphoma 2

MLD-STZ- multiple low dose streptozotocin

NOD- nonobese diabetic

INS-1- rat insulinoma cells

EndoC- EndoC B H1 cells

MIN6- mouse insulinoma cells

qRT-PCR- quantitative reverse transcription polymerase chain reaction

LNA- locked nucleic acid

UTR- untranslated region

PDCD4- Programmed Cell Death 4

PRP- polyribosomal profiling

GSIS- glucose stimulated insulin secretion

AOPI -acridium orange/ propidium iodide

NTA- nanoparticle tracking analysis

TEM- transmission electron microscopy

ANOVA- analysis of variance

CHAPTER ONE: INTRODUCTION/BACKGROUND

The Clinical Problem

The global and domestic incidence of Type 1 Diabetes (T1D) is rising at a rate of

nearly 3% per year, and contributes an annual cost of $14.4 billion to U.S. health

expenditures (1-4). Data from large clinical studies like the Diabetes Prevention Trial-1

suggest that subtle perturbations in beta cell function can be measured up to 18 months

prior to clinical presentation (5). After clinical presentation, patients often display

residual beta cell function that progressively declines over the course of the disease (6).

Whereas immunomodulatory therapies have shown some efficacy in attenuating T1D

progression when administered at the time of diagnosis, the long-term effectiveness of

these drugs is limited (7-10). These failures are related, in part, to ongoing autoimmunity,

but emerging data also suggest beta cells themselves may not be innocent bystanders in

human T1D development. Rather, metabolic stress pathways intrinsic to the beta cell,

which may be initially activated to provide mechanisms for increasing cell survival and

insulin production, could theoretically hasten the progression to apoptosis or enhance

beta cell responsiveness to immune infiltration and/or to immune-mediated destruction

(11, 12). Beta cells from rodent models and individuals with T1D demonstrate increased

stress markers compared to controls, and evidence exists to suggest these changes

precede the development of T1D (13-16). These findings suggest that limited efficacy of

immunotherapies in human T1D may also reflect inadequate treatment of T1D-related

beta cell dysfunction (17). Along these lines, there exists an urgent need to delineate

contributions of intrinsic beta cell dysfunction to T1D development, which may

ultimately pave the way for novel beta cell targeted therapies to be used in combination

with immunomodulatory approaches. Additionally, limited successes of conventional

therapies are likely also a consequence of irreparable damage to the functional beta cell

pool that has accumulated by the time of clinical presentation. Thus, strategies to define

biomarkers to identify and monitor abnormalities in beta cell health are also crucial goals

for T1D research. Such strategies would allow for earlier identification of incipient T1D,

prior to the development of hyperglycemia and widespread beta cell destruction, and

permit earlier administration and improved outcomes of novel and traditional T1D

therapies.

Beta cell inflammation and microRNAs (miRNAs)

An improved understanding of intrinsic stress pathways activated during T1D

development may pave the way for beta cell targeted therapies. One such pathway of

interest is the NF-κB signaling pathway, which is induced within the islet in response to

inflammatory cytokines, hyperglycemia, and oxidative stress (14, 18-20). Interestingly,

NF-κB signaling in the beta cell has been linked to both pro- and anti-apoptotic gene

expression, suggesting complex and multifaceted regulation of this signaling pathway

under inflammatory conditions (19, 21).

microRNAs (miRNAs) are short, noncoding RNAs that classically inhibit gene

expression by increasing messenger RNA (mRNA) degradation or directly inhibiting

mRNA translation (22). Multiple studies have identified miRNAs as key regulators of

beta cell development, glucose-stimulated insulin secretion, and beta cell dysfunction

(23-31). Global expression profiling performed in human and rodent islets subjected to

pro-inflammatory cytokine stress and viral infection as models of T1D demonstrates

marked changes in miRNA expression patterns (27, 28, 32). These findings suggest that

beta cell miRNAs may also contribute to activation of intrinsic beta cell stress pathways that act to augment or even initiate beta cell dysfunction and death during the development of T1D (11).

Tissue-derived miRNAs circulate and are stable in the blood, and differences within serum/plasma fractions have been described (33-35).   In particular, miRNAs are often enriched in EVs. These membrane bound nanoparticles, classified as exosomes, microvesicles, or apoptotic bodies, develop from exocytosis of multivesicular bodies (exosomes) or blebbing of the parent cell plasma membrane (microvesicles and apoptotic bodies) (36). EV molecular cargo, including RNAs, DNA, lipids, and protein, are determined by regulated processes and are altered by disease states (36-39). Specifically, beta cell EVs and their cargo have physiologic relevance to the T1D microenvironment (40-47). Islet EVs contain beta cell autoantigens and are able to increase antigen presentation and inflammatory cytokine production by antigen presenting cells, as well as stimulate endothelial cell angiogenesis (40-42). Importantly, cytokine treatment of beta cell lines or islets to mimic developing T1D induces physiologic changes in beta cell EV content that can impact recipient cells (40, 43-45). For example, treatment of beta cells with EVs from cytokine-treated parent beta cells can induce dose-dependent effects on recipient cell survival via transfer of EV microRNAs (43, 45). EVs from cytokine-treated islets also display enhanced immunostimulatory properties (40).

miRNA 21 (miR-21) is a Nuclear Factor-κb (NF-κb) dependent miRNA shown to be induced during the evolution of T1D (46, 48, 49). This miRNA has been well characterized in other cell types, and has been classically been labeled an "oncomiR" in cancer cells, due to inhibition of tumor suppressor genes, leading to pro-survival effects

(50-52). However, miR-21's role in the beta cell has been less clear, with conflicting data regarding pro-survival versus pro-apoptotic effects (46, 48, 53). Similarly, although elevations in circulating miR-21 have been reported in subjects with longstanding T1D, the specific etiology of these changes and differences during developing T1D have not been described (54, 55).

As illustrated in Figure 1, we hypothesized that miR-21 expressed by the beta cell in response to inflammatory stress impacts beta cell survival during T1D development. Furthermore, we proposed that differences in EV serum miR-21 levels may identify the activation of intrinsic beta cell stress pathways in emerging T1D and allow for the earlier clinical identification of T1D onset. Using human islets and cell line models of T1D, we sought to define the context of beta cell miR-21 upregulation and the phenotype of beta cell miR-21 overexpression by novel target identification. Our findings demonstrate an interaction between beta cell miR-21 and the antiapoptotic mRNA *B Cell Lymphoma 2* (*BCL2*), resulting in dual effects on *BCL2* transcript abundance as well as *BCL2* translation. In addition, we detected increased human islet EV miR-21 in response to cytokine-induced pro-inflammatory stress, and increased serum EV mir-21 in prediabetic NOD mice and children with recent onset T1D. These findings suggest that EV miR-21 may be a promising circulating biomarker of beta cell inflammation and developing T1D.

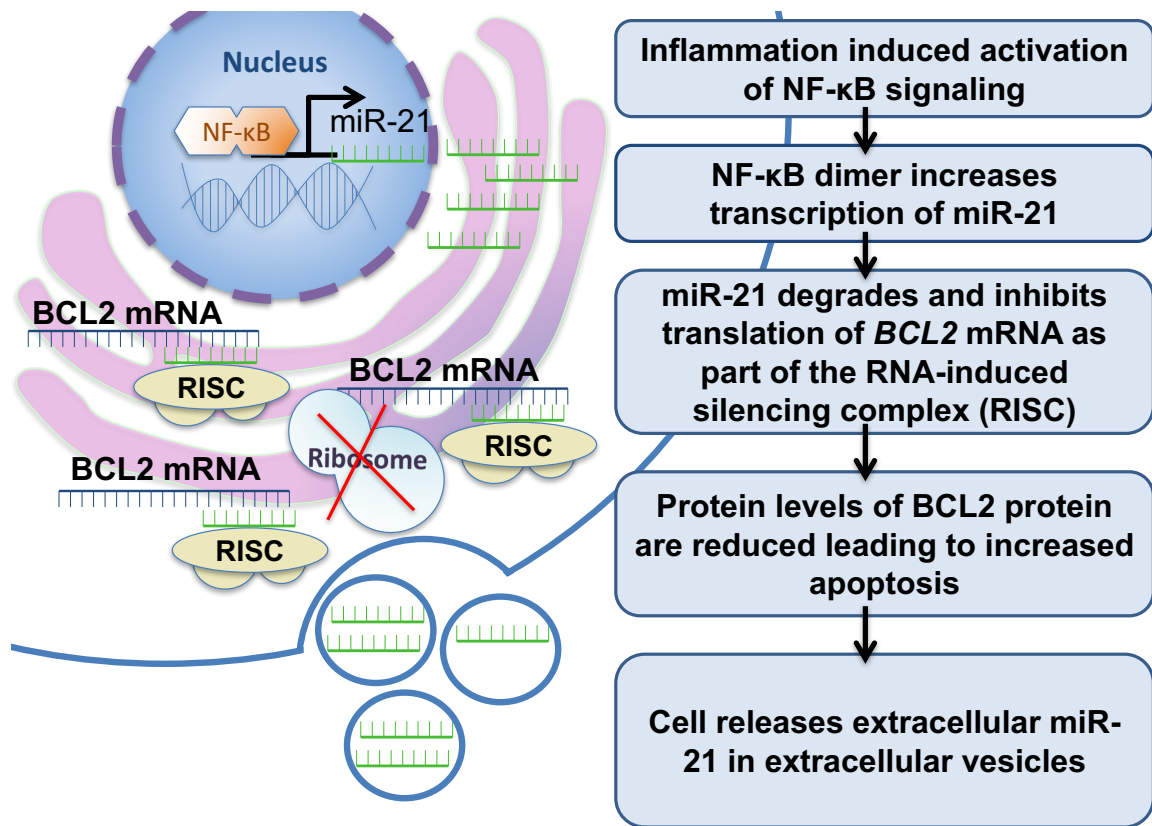**Figure 1. Proposed model of miR-21 production in response to cytokine stress in the microenvironment of T1D.** Inflammatory cytokines in the T1D microenvironment activate the NFκB signalling pathway, increasing miR-21 transcription. miR-21 binds to BCL2 transcripts resulting in transcript degradation and inhibiting translation of the antiapoptotic molecule. miR-21 is secreted extracellularly in extracellular vesicles.

CHAPTER TWO: METHODS AND RESULTS

Animals, Islets, and Cell Culture

Eight-week old male C57BL6/J mice (Jackson Laboratories, Bar Harbor, Maine) were treated with normal saline or multiple low dose streptozotocin (MLD-STZ) administered intraperitoneally at a dose of 35 mg/kg/day for 5 days to induce diabetes. Blood glucose was measured via tail vein nick using an Alphatrack glucometer (Abbott Laboratories, Abbott Park, IL). Pancreatic islets were isolated by collagenase digestion one week after STZ initiation as previously described (56). Eight-week old female nonobese diabetic (NOD, Jackson Labs) mice were followed weekly with serum collections and blood glucose measurements for onset of diabetes (blood glucose >11.1mmol/l), when islets were isolated for analysis. Non-diabetic NOD mice were followed until 20 weeks of age to rule out diabetes development, then euthanized for islet isolation. Blood collection for serum isolation and glucose measurements were done via tail vein nick. Blood glucose was measured using AlphaTRAK glucometer (Abbott Laboratories) following manufacturer's instructions. Serum samples were isolated using Microvette CB 300 system for capillary blood collection (Sarstedt). Islets were also isolated from age matched CD1 or NOR/Ltj mice (Jackson Labs). Animals were maintained within the Indiana University Laboratory Animal Resource Center under pathogen-free conditions and protocols approved by the institutional animal care and use committee, in accordance with the *Guide for the Care and Use of Laboratory Animals* (57).

Rat insulinoma (INS-1) (832/13) and (828/33) cells, Human EndoC-βH1 (EndoC), or mouse insulinoma (MIN6) beta cells, or cadaveric human islets from

nondiabetic donors (obtained from the Integrated Islet Distribution Program, exempt from Institutional Review Board approval) were cultured as described (58, 59) and treated with a cytokine mix consisting of 5ng/ml IL-1beta, 100ng/ml IFN-γ, and 10ng/ml TNF-α for 6, 24, or 48 hours. INS-1 cells were also treated with high glucose (25 mmol/l) or tunicamycin (300nmol/l) for 24 hours.

INS-1 (832/13) and (828/33) cells were seeded into a 12-well plate at a density of 4×10^5 cells/well and treated for 48 hours with miR-21 5p (accession # MI0000569) mimic used at a concentration of 45 pmol (Qiagen, Germany), or 100 pmol locked nucleic acid (LNA) inhibitor (Exiqon, Denmark), or negative controls (Qiagen, Exiqon) that had been complexed with 3 μl Lipofectamine 3000 and 100 μl Opti-Mem (Thermo Fisher, Grand Island, NY).

For human islet dispersion and transfection, after 4 hours of routine incubation at 37° C, islets were suspended in 4 ml of Accutase solution (Millipore, Billerica, MA) with 100 units of DNase I (Millipore) in a thermal mixer at 37 degrees, 1000 rpm for 10 minutes, followed by addition of 10ml of culture medium. Dispersed cells were collected by centrifugation at 200g for 3 minutes, then resuspended in culture medium and seeded into 12-well tissue culture plates (Falcon, Tewksbury, MA). Dispersed cells from 200 islets were transfected with 23 pmol of either negative control miRNA or miR-21 mimic using Lipofectamine 3000 reagent as described above. Cleaved caspase 3 ELISA (ThermoFisher) was performed on transfected human islets per the manufacturer's protocol.

Luciferase assays were performed using a Gaussia Luciferase/secreted alkaline phosphatase dual reporter system (Genecopoeia, Rockville, MD). Plasmids containing the

cloned wildtype human *BCL2* 3' untranslated region (UTR) or a mutated 3'UTR

(positions 710-716 or 720-726) downstream of a secreted gaussia luciferase reporter,

driven by an SV40 promoter and a secreted alkaline phosphatase reporter driven by a

CMV promoter were generated by Genecopoeia. INS-1 cells were seeded in a 12-well

plate and then treated with 1μg of plasmid DNA complexed with 3 μl of lipofectamine

3000 (in 100 μl Optimem) for 24 hours. Cells were then trypsinized and reseeded,

followed by transfection with a miR-21 mimic or negative control, as above, for 24

hours. A dual reporter luciferase assay kit (Genecopoeia) was used as per manufacturer's

instructions to quantify luciferase activity in 10 μl of media. Results were normalized to

secreted alkaline phosphatase activity measured using a SpectraMax M5 multiwell plate

reader (Molecular Devices, Sunnyvale, CA).

Cell counting was performed using 20 μl of trypsinized and resuspended INS-1

cells on a Cellometer (Nexcelom Bioscience, Lawrence, MA). Live/dead staining was

performed with Acridium Orange/ Propidium Iodide Dye (AOPI) (Nexcelom Bioscience)

mixed 1:1 with suspended cells after trypsinization. Fluorescence detection of reactive

oxygen species generation in INS-1 cells was performed using CellROX Deep Red

Reagent (ThermoFisher) per manufacturer's instructions. Cells were imaged on a LSM

700 fluorescent confocal laser scanning microscope (Zeiss, Germany), and fluorescence

intensity was assessed as mean signal per well using ZEN 2011 image processing

software (Zeiss).

RNA isolation and reverse transcription was performed using miRNeasy and

miScript II RT kits according to the manufacturer's instructions (Qiagen). Quantitative

reverse transcription polymerase chain reaction (qRT-PCR) was performed using the

miScript SYBR Green PCR Kit (Qiagen) and a Mastercycler ep realplex instrument (Eppendorf, Hauppage, NY). Relative RNA levels were established against the invariant small nuclear RNA RNU6-1 for miRNAs and beta-actin for mRNA species, using the comparative C_T method (60). Relative miRNA levels from EV isolates were established against *C. elegans* miR-39 mimic spike-in control (Qiagen).

Immunoblot analysis was performed as previously described (61). Briefly, equal concentrations of total protein were separated by SDS-PAGE and transferred to a polyvinylidene fluoride membrane, and incubated at 4 degrees overnight with primary antibodies. 1:750 dilutions of rabbit anti-B Cell Lymphoma 2 (BCL2), rabbit anti-Programmed Cell Death 4 (PDCD4), and rabbit anti-cleaved caspase-3 (Cell Signaling, Danvers, MA) and a 1:10,000 dilution of mouse anti-actin antibody (Millipore) were used. Bound primary antibodies were detected with donkey anti-mouse or donkey anti-rabbit antibodies (both at 1:10,000 dilutions, LICOR Biosciences, Lincoln, NE). Immunoreactivity was visualized using fluorometric scanning on an Odyssey imaging system and quantified by LI-COR software.

Polyribosomal profiling (PRP) experiments were performed as previously described (62). Briefly, INS-1 cell lysates were centrifuged through a 10-50% sucrose gradient for 2 hours at 40,000 rpm, and fractionated using a BioComp piston gradient fractionator. RNA absorbance at 254 nm was recorded using an inline UV monitor, and fractions were collected. Total RNA was reverse transcribed and qRT-PCR was performed using SYBR Green methodology as above.

Glucose stimulated insulin secretion (GSIS) was assayed as described (63). Supernatants were collected and assayed using a radioimmunometric assay for insulin

(Millipore). Values were normalized to total insulin content of the islet fraction.

Polyribosomal profiling (PRP) experiments were performed on INS-1 cell lysates as

previously described (62). Total RNA was reverse transcribed and qRT-PCR was

performed using SYBR Green methodology as above.

EV Isolation

Total EVs were isolated from culture media using ExoQuick TC reagent (System

Biosciences) and circulating EVs were isolated from 50 uL of serum using ExoQuick

reagent (System Biosciences) following manufacturer's guidelines. Sequential

centrifugation was utilized to separate EVs by size. The samples were centrifuged at

800g for 15 minutes to remove dead cells and cellular debris, after which the supernatant

was centrifuged at 2,000g to pellet large EVs/apoptotic bodies. 10,000g centrifugation of

the supernatant from the previous step was utilized to collect microvesicles, and finally

supernatant from the previous step was centrifuged at 100,000g to pellet the exosomes

(64). The remaining supernatant was also retained for analysis.

Isolation and relative purity of the EVs were confirmed by nanoparticle tracking

analysis (NTA), transmission electron microscopy (TEM), and western blot (WB).

Samples enriched for EVs of interest were analyzed for concentration and size

distribution with dynamic light scattering using a ZetaView instrument for NTA

(ParticleMetrix). Prior to measurement, the system settings were calibrated using 100nm

polystyrene particles. NTA measurement was recorded and analyzed at 11 positions per

sample with ZetaView Analyze software (ParticleMetrix). EV-enriched sample

preparations were fixed in EM-grade fixative containing 2% glutaraldehyde and 2%

paraformaldehyde in 0.1M buffered phosphate for at least 30 mins at 4°C. After fixation,

samples were placed on 200 mesh silicon monoxide formvar coated grids and stained

with NanoVan (Nanoprobes). Images were taken on an FEI Technai G2 Spirit TEM microscope. Fixed samples were processed and imaged by the University of Nebraska Medical Center EM Core.

Human Subjects.

This study was approved by the Indiana University Institutional Review Board. Informed consent was obtained from parents with assent from children. Random serum samples were obtained from pediatric subjects diagnosed with T1D by a pediatric endocrinologist within 3 days of T1D diagnosis. Samples were collected in serum separator tubes and serum was isolated by centrifugation and stored at -80° C. Exclusion criteria included diabetic ketoacidosis requiring an intensive care unit stay, diabetes other than T1D, history of prior chronic illness known to affect glucose metabolism, or use of medications known to affect glucose metabolism. Control serum from healthy pediatric subjects was obtained from a biorepository at Indiana University School of Medicine.

Statistics

Statistical analyses were performed using GraphPad Prism Version 6.00 (GraphPad Software, La Jolla, California). Student's t-tests were used for comparison between treatment and control groups. One-way analysis of variance (ANOVA) with Tukey's post-test for multiple comparisons was utilized when comparing >2 groups. For human data, Mann-Whitney U test, or Kruskal-Wallis analysis of variance with Dunn's posttest for analysis of more than 2 groups were used for nonparametric distributions. For all analyses, a p value of ≤ 0.05 was considered significant.

Results

Beta cell miR-21 production is induced in models of inflammation and T1D.

In order to determine whether inflammation present during the development of T1D was responsible for induction of beta cell miR-21, several models of T1D were utilized. Islets were isolated from two *in vivo* mouse models of T1D: mice treated with MLD-STZ (obtained one week after treatment initiation), and NOD mice (at the time of diabetes onset). Mice treated with MLD-STZ had increased islet miR-21 expression relative to saline-injected controls (**Figure 2A**), while islets from diabetic NOD mice demonstrated an even more pronounced increase in miR-21 expression compared to CD1 controls (**Figure 2B**). Next, INS-1 832/13 cells were treated with a cytokine mix for 6, 24, and 48 hours, and significantly increased miR-21 expression was observed at all timepoints (**Figure 2C-E**). To define whether this effect on miR-21 could be related to other components of the diabetic milieu, INS-1 cells were treated with 25mM glucose, to induce glucotoxicity and oxidative stress, or 300 nm tunicamycin for 24 hours, to induce endoplasmic reticulum (ER) stress (**Figure 2F-G**). Neither condition increased miR-21 expression, suggesting that miR-21 was upregulated specifically in response to pro-inflammatory signaling and not in response to hyperglycemia or ER stress.
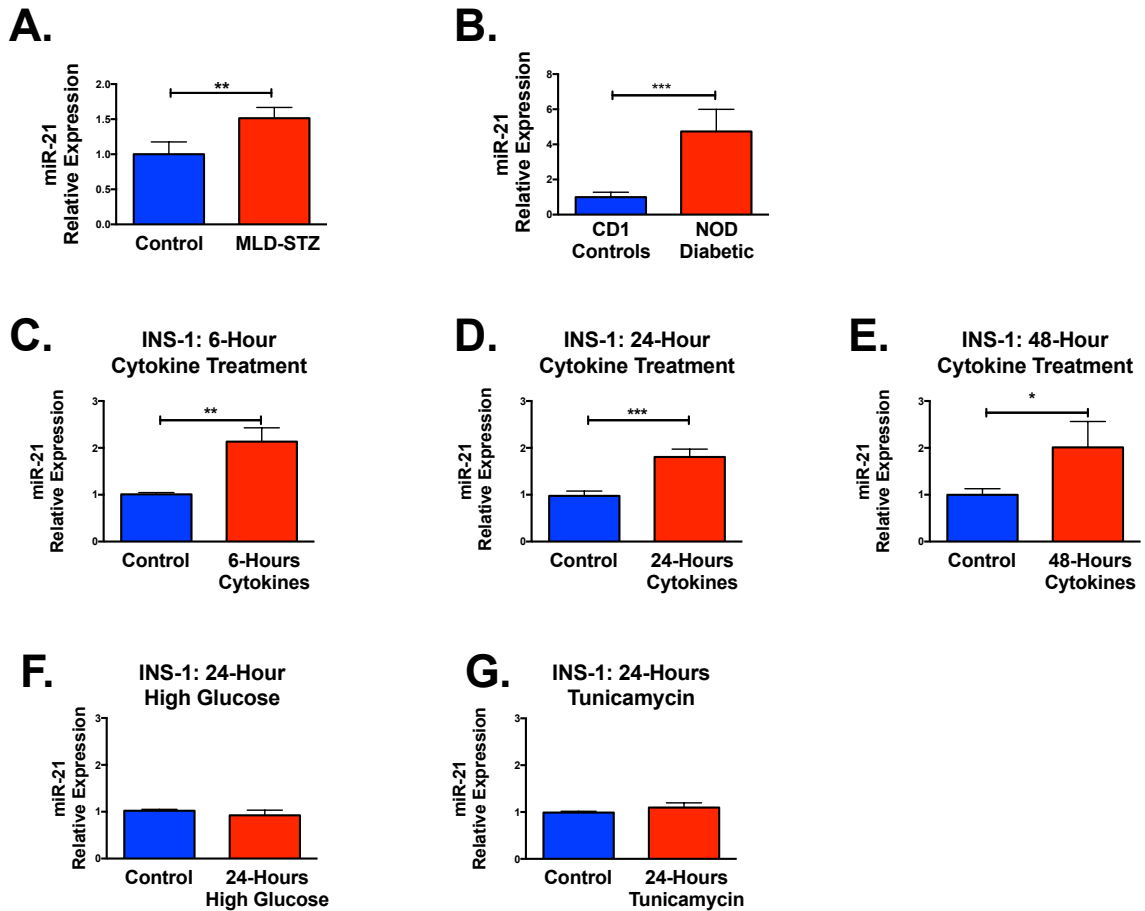
Figure 2. Beta cell miR-21 production is induced in models of inflammation and T1D. miR-21 expression was analyzed in: **A.** islets from mice treated with multiple low doses of STZ (MLD-STZ) or saline controls (n=5), or **B.** diabetic NOD mice (n=7) compared to CD1 controls (n=5), **C-E.** INS-1 beta cells were treated with an inflammatory cytokine mix of 5ng/mL IL-1beta, 100ng/mL IFN-γ, and 10ng/mL TNF-α for 6, 24, and 48 hours (n=5-9), **F.** INS-1 cells were treated with 24 hours of high glucose (25 mM) or **G.** tunicamycin (300nM) for 24 hours (n=5-6). *p≤0.05; **p≤0.01, and ***p ≤0.001

Beta cell effects of miR-21 overexpression

To define the phenotypic effects of increased miR-21 expression, INS-1 832/13 beta cells were transfected with an miR-21 mimic. After 48 hours, INS-1 cells with miR-21 overexpression were significantly less confluent than cells transfected with a negative control (**Figure 3A-B**), prompting live/dead staining with AOPI (**Figure 3A,C**). AOPI staining revealed a significant increase in the percentage of dead INS-1 cells in response to miR-21 overexpression. To ascertain whether activation of intrinsic death pathways was involved, immunoblot for cleaved capase-3 was performed (**Figure 3D**). A significant increase in cleaved caspase-3 protein levels confirmed a role for apoptosis in this phenotype. To understand the effect of miR-21 upregulation on beta cell function, GSIS was performed in INS-1 cells transfected with a miR-21 mimic. In contrast to cells transfected with the negative control miRNA, INS-1 cells overexpressing miR-21 showed a blunted insulin secretory response to stimulation with high glucose (**Figure 3E)**.
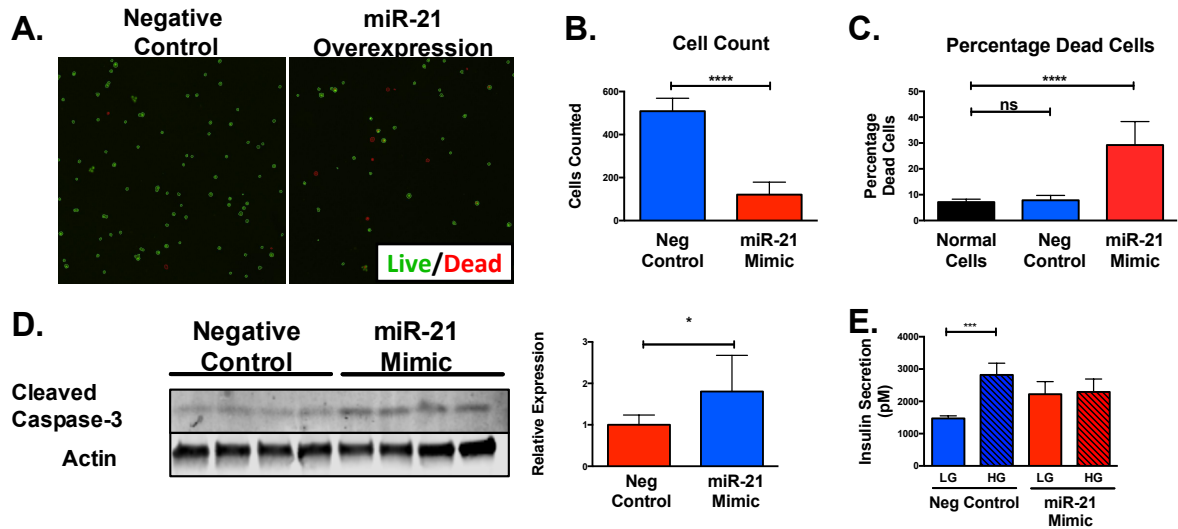
Figure 3. miR-21 overexpression increases beta cell apoptosis and decreases glucose-stimulated insulin secretion. INS-1 cells were transfected with a miR-21 mimic for 48 hours to increase miR-21 activity. **A.** Cells were stained with AOPI to assess viability, with green staining (AO) representing all cells and red staining (PI) representing compromised membranes. **B.** Total cells and **C.** Percentage dead cells were analyzed using a cell counter. **D.** Immunoblot for cleaved caspase-3 was performed to assay contribution of apoptosis to the increased cell death observed with miR-21 overexpression. **E.** After transfection with a miR-21 mimic, glucose stimulated insulin secretion of cells was assayed in response to low (2.5mM) and high (15 mM) glucose concentrations, and normalized to total insulin content of the cell lysate. (n=6-9 for all experiments); *p≤0.05; **p≤0.01, ***p ≤0.001, and ****p ≤0.0001

miR-21 reduces BCL2 levels via directly targeting the BCL2 3'UTR, resulting in reduced

mRNA levels and reduced translation of BCL2 transcripts.

Because the pro-apoptotic mRNA of the gene encoding Programmed Cell Death 4

(PDCD4) has previously been confirmed as a target of beta cell miR-21, immunoblot for

PDCD4 was performed. Our results confirmed reduced PDCD4 protein levels, consistent

with effective miR-21 overexpression (**Figure 4A**) (53).   However, our observation that

miR-21 overexpression increased beta cell death was at odds with a predicted pro-

survival effect of reduced PDCD4. Based on this, we turned to *in silico* prediction tools

to identify other potential miR-21 targets to explain the pro-apoptotic phenotype of beta

cell miR-21 overexpression (65, 66). We found that the 3'UTR of the mRNA encoding

the anti-apoptotic protein B Cell Lymphoma 2 (BCL2) was a predicted target for miR-21,

with a predicted binding site that was well conserved among vertebrate species (66, 67).

Figure 4B depicts predicted sites of interaction between the human BCL2 3'UTR and

miR-21.

Supporting this prediction, BCL2 protein levels were reduced in parallel with

increased miR-21 levels after 48 hours of cytokine treatment (**Figure 4C**). To verify this

relationship, qRT-PCR was performed to assess relative *BCL2* transcript quantity on INS-

1 832/13 cells transfected with a miR-21 mimic (**Figure 4D**). This analysis revealed

decreased transcript levels, consistent with mRNA degradation. In contrast, no

significant change in the abundance of transcripts of other BCL2 family members that

would be expected to impact apoptosis were detected (**Figure 4E**). To determine

whether a direct interaction occurred between miR-21 and the *BCL2* 3'UTR, INS-1 cells

were transfected with plasmids containing a luciferase reporter driven by the wild-type

BCL2 3'UTR, or *BCL2* 3'UTRs that had been mutated at 2 different sites included in the predicted miR-21 binding region, position 710-716 or position 720-726 (**Figure 4F**). Cells were then transfected with either the miR-21 mimic or a negative control. Consistent with a direct interaction of miR-21 and the *BCL2* 3'UTR, miR-21 overexpression reduced luciferase activity of INS-1 cells expressing the wild-type *BCL2* 3'UTR plasmid. However, no effect of miR-21 overexpression was present in INS-1 cells transfected with mutated 3'UTR plasmids.

To confirm a miR-21 mediated effect on protein levels, immunoblots for BCL2 protein were performed on INS-1 cells after miR-21 mimic transfection. This analysis demonstrated reduced levels of BCL2 protein with miR-21 overexpression (**Figure 4G**). To understand whether miR-21 mediated reductions in BCL2 protein occurred exclusively through mRNA degradation as shown in Figure 3D versus direct inhibition of translation, polyribosomal profiling experiments were performed to ascertain the translation status of *BCL2* mRNA. Although no global changes in mRNA translation were seen **(Figure 4H)**, INS-1 cells overexpressing miR-21 revealed a shift of *BCL2* transcripts toward monosome-associated fractions, and away from the polysome-associated fractions, indicating a reduction in active *BCL2* translation (**Figure 4I**). These results suggest that both mRNA degradation, as well as translational inhibition contribute to miR-21-mediated reductions in BCL2 protein levels.

A.

Negative Control   miR-21 Mimic

PDCD4

Actin

B.

Position 705 of BCL2 3'UTR 5' …agGAAAGUAUUUUUUUAAGCUa…
                   : | | : | : | | : | | | | | | |
Hsa miR-21-5p   agUUGUAGUCAGACUAUUCGAu…

C.

Control   48 Hours Cytokines

BCL2

Actin

D.

E.

Neg Control   miR-21 Mimic

BCL-xL   MCL-1   BAX   PUMA   Dp5   BID

F.

Wild Type *BCL2* 3'UTR

Mutated *BCL2* 3'UTR Position 710-716

Mutated *BCL2* 3'UTR Position 720-726

G.

Negative Control   miR-21 Mimic
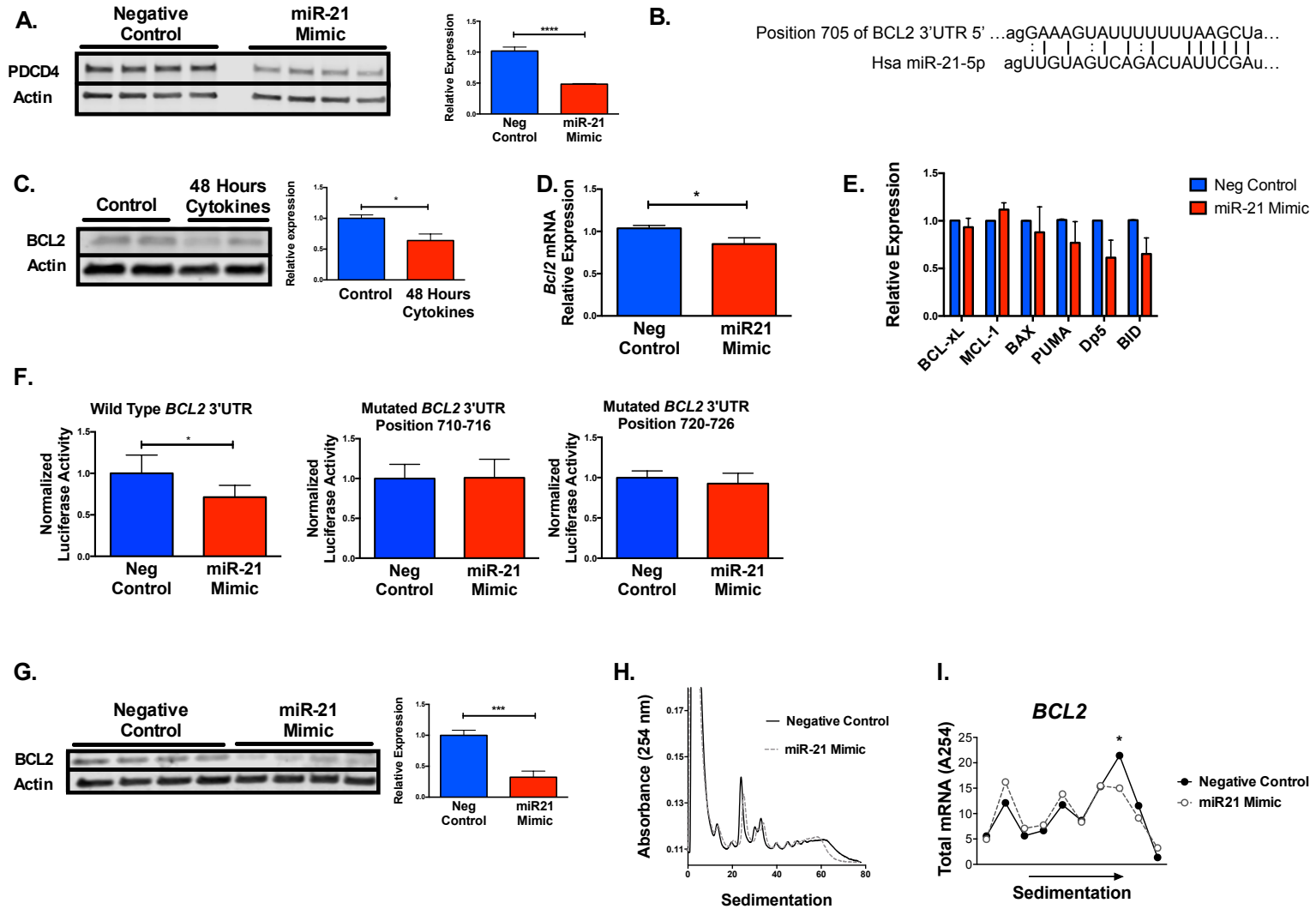
BCL2

Actin

H.

I.

BCL2

Figure 4. miR-21 reduces BCL2 levels via directly targeting the BCL2 3'UTR, resulting in reduced mRNA levels and reduced translation of BCL2 transcripts. A. After transfection of miR-21 mimic, overexpression was verified by performing immunoblot for the known beta cell miR-21 target, PDCD4 (n=4). **B.** Predicted binding sites of miR-21 within *BCL2* 3'UTR. **C.** BCL2 protein levels are reduced by treatment with inflammatory cytokines (n=6). **D.** *BCL2* mRNA levels after mimic transfection (n=8). **E.** Expression of other BCL2 Family mRNAs after mimic transfection. **F.** Luciferase assay was performed on cells expressing wild type BCL2 3'UTR or BCL2 3' UTR mutated at position 710-716 or 720-726, then transfected with a miR-21 mimic (n=4). **G.** Immunoblot after mimic transfection revealed a reduction in BCL2 protein levels (n=8). **H-I:** Polyribosomal profiling was performed to ascertain effects of miR-21 overexpression on *BCL2* mRNA translation (n=3). **H.** A representative global profile is pictured. **I.** Aggregated data from 3 experiments revealing a left shift of BCL2 mRNA away from polysome-associated fractions, with a significantly decreased percentage of transcripts in fraction 8, indicating a reduction in active translation. *p≤0.05; **p≤0.01, ***p ≤0.001, and ****p ≤0.0001

Validation of effects of miR-21 overexpression in human islets

To validate relevancy of our findings in a human model, human islets were treated

with a mix of pro-inflammatory cytokines for 24 and 48 hours. Similar to results

obtained in rodent beta cell lines and islets, we observed a significant increase in miR-21

expression at the 48-hour time point (**Figure 5A -B**). To confirm a direct role of miR-21

on BCL2 and apoptosis in human islets, we transfected miR-21 mimics into dispersed

islets. Consistent with our findings above, we observed that miR-21 overexpression led

to an increase in cleaved caspase 3 (measured using ELISA) and a reduction in *BCL2*

expression **(Figure 5C -D)**.
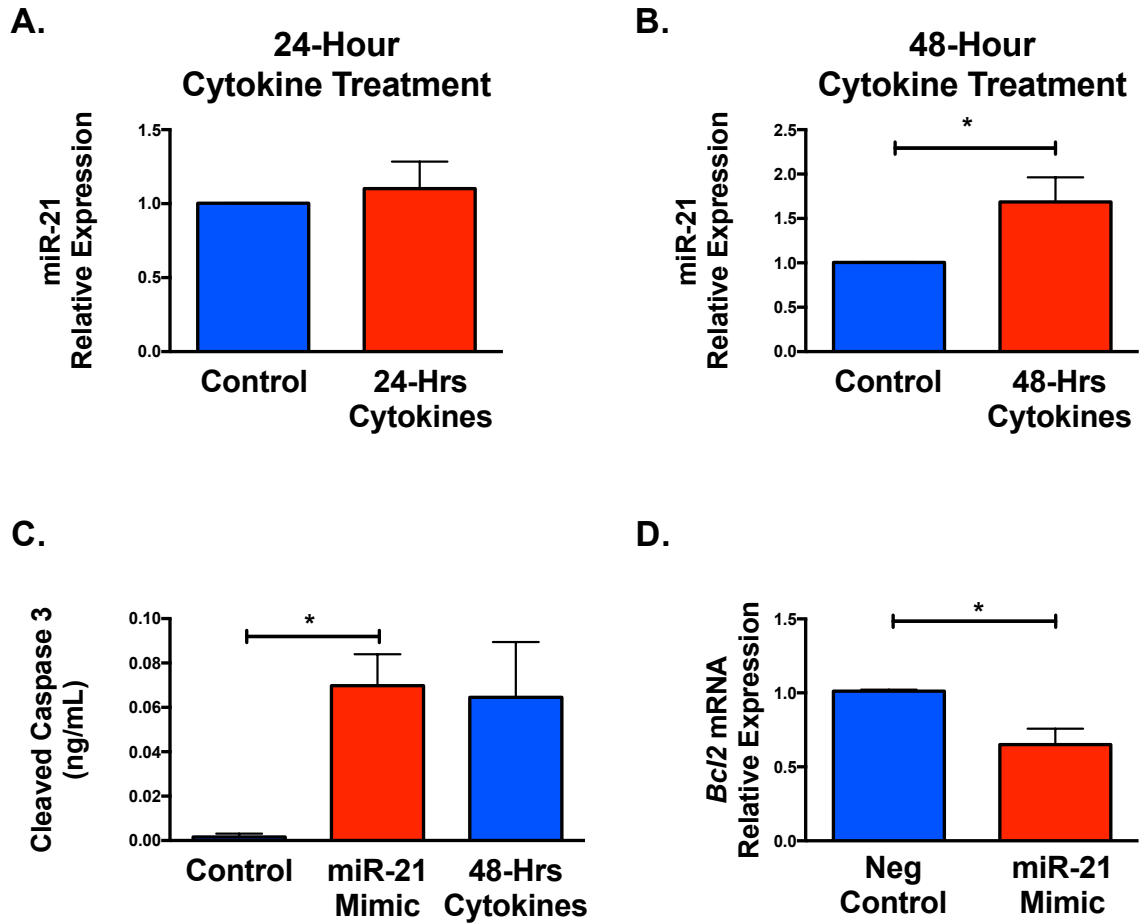
Figure 5. Human islet miR-21 is increased by inflammatory cytokine treatment, and increases apoptosis in association with reduced *BCL2* expression. A-B. miR-21 expression was quantified after human islets were treated with an inflammatory cytokine mix of 5ng/mL IL-1beta, 100ng/mL IFN-γ, and 10ng/mL TNF-α for 24 and 48 hours. **C.** Cleaved caspase 3 expression was quantified using ELISA in dispersed human islets after miR-21 mimic transfection. 48-hour treatment with cytokine mix is also included as a positive control. (n=3) **D.** *BCL2* transcripts were quantified after mimic transfection. (n=4) *p≤0.05;

Effects of beta cell miR-21 inhibition

To define effects of miR-21 inhibition, INS-1 cells were transfected with a miR-21 inhibitor for 48 hours (**Figure 6A-B**). At baseline, a trend towards increased BCL2 was present (**Figure 6A**), while PDCD4 levels were unchanged (**Figure 6B**). To determine whether miR-21 inhibition had a protective effect against proinflammatory cytokines, INS-1 cells were treated for 16 hours with a proinflammatory cytokine mix. Interestingly, in combination with cytokine treatment, miR-21 inhibition reduced cleaved caspase-3 levels compared to negative controls (**Figure 6C**). Here, there was a trend towards increased BCL2 levels in cells pretreated with the miR-21 inhibitor (**Figure 6D**). Again, no differences were present in PDCD4 levels (**Figure 6E**).

In order to ascertain the contribution of BCL2 inhibition on miR-21's pro-death effects, we transfected INS-1 828/33 cells, which constitutively overexpress BCL2, with a miR-21 mimic (59). BCL-2 levels were decreased by the miR-21 mimic. However, BCL-2 expression remained higher than levels observed in the wild-type INS-1 cells **(Figure 6F).** Consistent with this, the percentage of dead cells (assessed by AOPI staining, quantified in **Figure 6G**) was significantly lower in BCL-2 overexpressing INS-1 cells compared to wild-type INS-1 cells transfected with the mimic.

A.

B.

C.

D.

E.

F.

G.
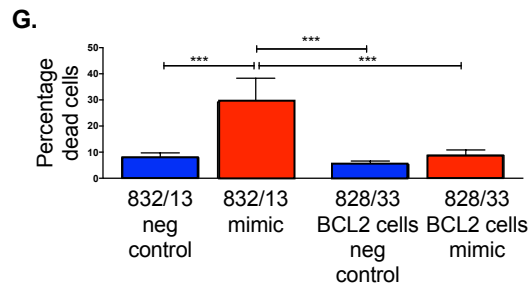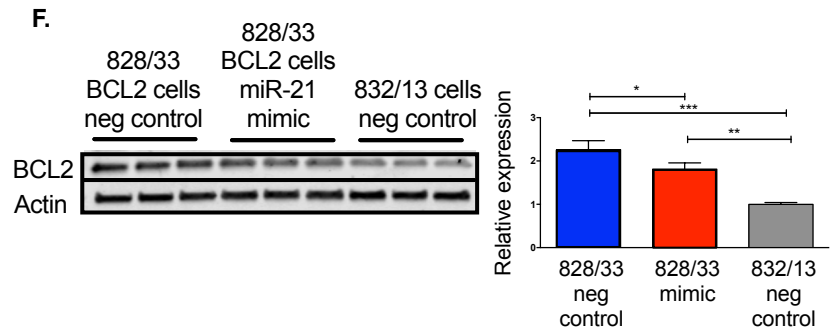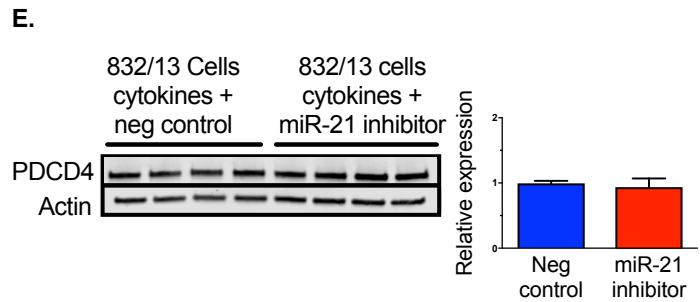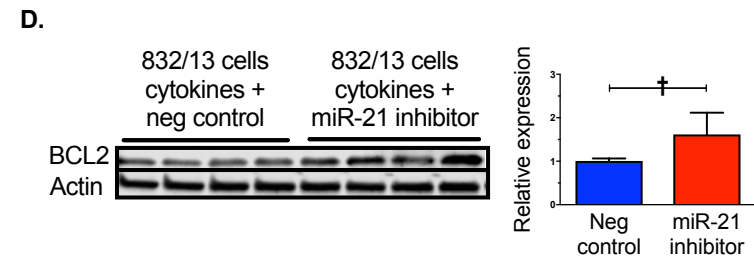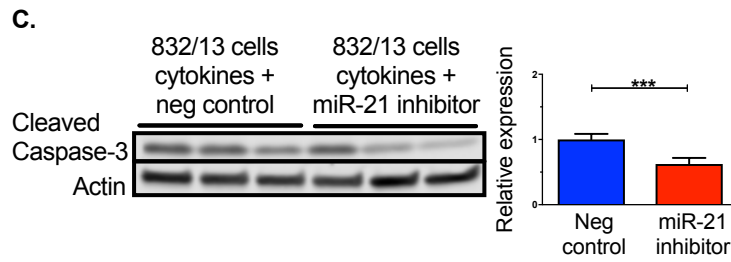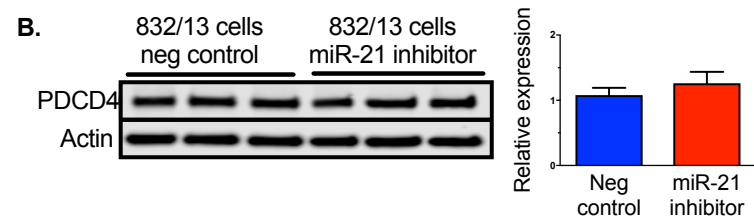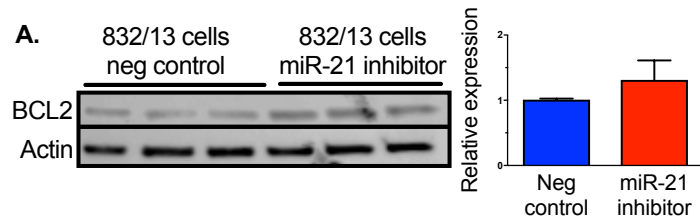
Figure 6 miR-21 inhibition reduces cytokine-induced cleaved caspase-3 production, while BCL2 overexpression reduces miR-21 mediated apoptosis. A-B. miR-21 LNA inhibitor was transfected in INS-1 832/13 (wild-type) beta cells for 48 hours to inhibit miR-21 activity. Immunoblots for: **A.** BCL2 and **B.** PDCD4 were performed.   **C-E.** After miR-21 inhibitor treatment, INS-1 832/13 cells were treated with 16 hours of cytokine mix and immunoblots were performed for **C.** Cleaved Caspase-3, **D.** BCL2, and **E.** PDCD4. **F-G**. INS-1 828/33 cells, which constitutively overexpress BCL2, were transfected with a miR-21 mimic for 48 hours. **F.** Immunoblot for BCL2. **G.** Percentage dead cells. (n=3-5); *p≤0.05; **p≤0.01, and ***p ≤0.001

Beta Cell EV miR-21 Release is Increased After Exposure to Inflammatory Cytokines

To determine whether developing T1D increases beta cell EV miR-21, we also quantified miR-21 in EVs from INS-1 cells and human islets after treatment with 24 hours of the inflammatory cytokine mix **(Figure 7A,C).** Here, cytokine exposure induced a 3-6 fold increase in total EV miR-21. Interestingly, the levels of increase in EV miR-21 from the EndoC human beta cells and from human islets were of higher magnitude than the increases in miR-21 expression, suggesting specific trafficking of miR-21 to the EVs.

To understand whether increased levels of EV miR-21 may be related to possible increased release of EVs under inflammatory conditions, we performed nanoparticle tracking analysis (NTA) to determine EV quantity and size distribution. No significant differences in either the size distribution or particle concentration were detected in INS-1 cells or in human islets after 24 hours of cytokine treatment **(Figure 7B-C)**. These data suggest that the cytokine-induced increase in beta cell EV miR-21 is due to specific increases within the vesicle cargo, and not an effect of an increased net release of EVs.
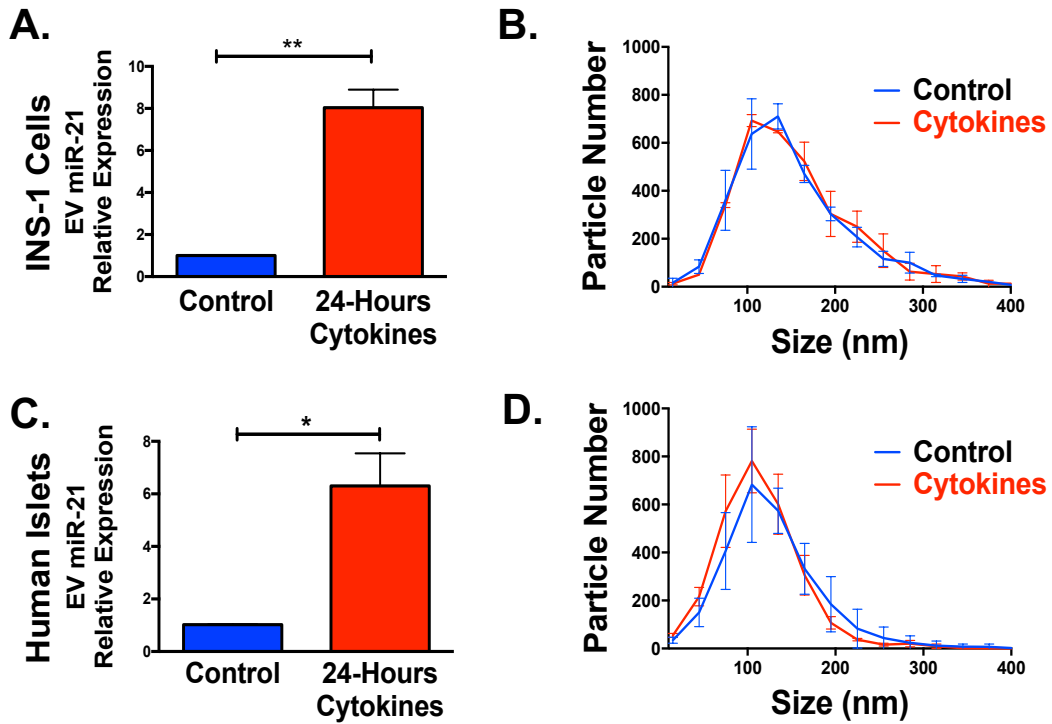
Figure 7. Inflammatory cytokine exposure increases beta cell EV miR-21. INS-1 cells and human islets were treated with cytokine mix of IL-1beta, INFγ, and TNFα for 24h. **A,C.** miR-21 levels were assessed by qRT-PCR in EVs from INS-1 cells and from human islets. (n=3-6). NTA was performed to profile EV particle concentration and size distribution from cytokine treated **B.** INS-1 cells, and **D.** human islets. (n=3 each) Results displayed as mean ±SD; *p ≤ 0.05, **p ≤ 0.01

Cytokine-induced increases in beta cell EV mir-21 are predominantly due to beta cell exosome miR-21

We next endeavored to determine whether cytokine-induced increases in beta cell EV miR-21 may be specific to a particular EV subtype. To this end, we utilized sequential ultracentrifugation to separate EVs by size, allowing for isolation of larger EVs (apoptotic bodies), medium sized EVs (microvesicles), and smaller vesicles (exosomes) (68, 69). Western blot, and TEM were performed to validate isolations (**Figure 8C-D**). q-RTPCR of each fraction revealed that in INS-1 beta cells and in human islets, miR-21 was consistently increased in the apoptotic bodies and exosomes (**Figure 8A-B**). NTA analysis of centrifugation fractions revealed much higher number of exosomes than either microvesicles or the apoptotic bodies (Figure **8D-E**). Interestingly, the apoptotic bodies were present at very low concentration, despite being the earliest recovered fractions collected by the sequential centrifugation. This finding suggests that apoptotic bodies, and possibly other large EVs, are responsible for only a minor fraction of the total observed increase in beta cell EV miR-21, with the exosomes being the primary type of vesicle carrying increased levels of miR-21.
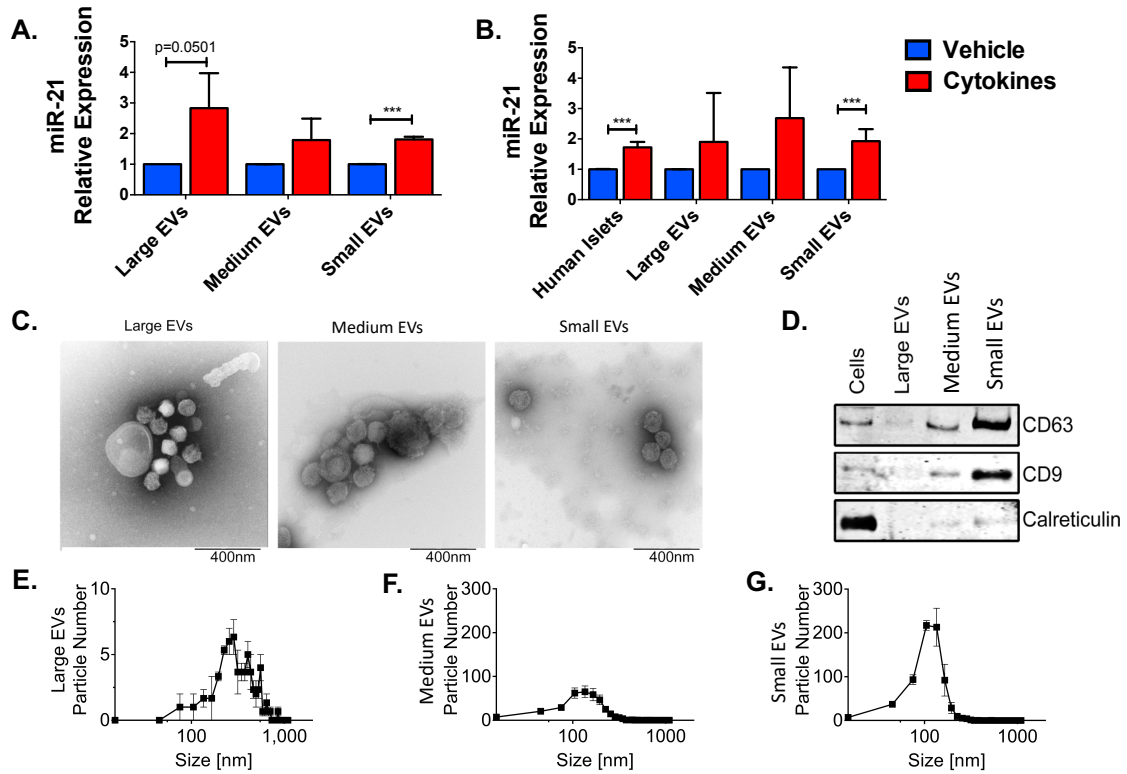
Figure 8. Increased EV miR-21 is specific to exosomes. **A-B**. Media from cytokine-treated INS-1 cells (n=3) and human islets (n=5) was separated by sequential centrifugation into large EV (apoptotic bodies), medium EV (microvesicle), and small EV (exosome) fractions. **C.** TEM and **D.** immunoblot of EndoC cell derived EVs were used to validate EV isolation by serial ultracentrifugation; **E-G.** NTA analysis was performed on each EV fraction. TEM images display representative data from three independent experiments. Results displayed as mean ±SD; *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, by 2-tailed *t* test.

Progressive elevations in circulating EV miR-21 precede the onset of diabetes in NOD Mice

Based on increased beta cell EV mir-21 release in response to cytokine exposure *ex vivo*, we predicted that circulating EV miR-21 would also be increased during *in vivo* development of T1D. To test this prediction, we utilized the NOD mouse model of T1D. First, we tested islets from female diabetic NOD mice at the time of diabetes onset, as compared to NOR controls (**Figure 9A**). We found a 2.5-fold increased expression of miR-21 in the islets of diabetic animals. We then isolated EVs from terminal serum of diabetic mice, and detected a ~10 fold increase in the levels of miR-21 compared to NOR controls (**Figure 9B**). Next, to assess changes in circulating EV miR-21 during T1D development, we collected weekly serial serum samples from diabetic NOD mice starting at 8 weeks of age until the time of T1D onset (**Figure 9C**). Here, we observed an age-dependent increase in miR-21 in the circulating EVs of prediabetic NOD mice starting at 12 weeks of age (**Figure 9D**). To establish relationships to the development of T1D we normalized these values to those of age-matched NOR controls and plotted the data based on relative time to T1D onset (**Figure 9E**). NTA of these circulating EVs revealed no significant variations in the EV concentration relative to age or prior to the onset of T1D (**Figure 9F-G**). These data confirm that progressive increases in serum EV miR-21 predate hyperglycemia during developing diabetes in this mouse model of T1D.
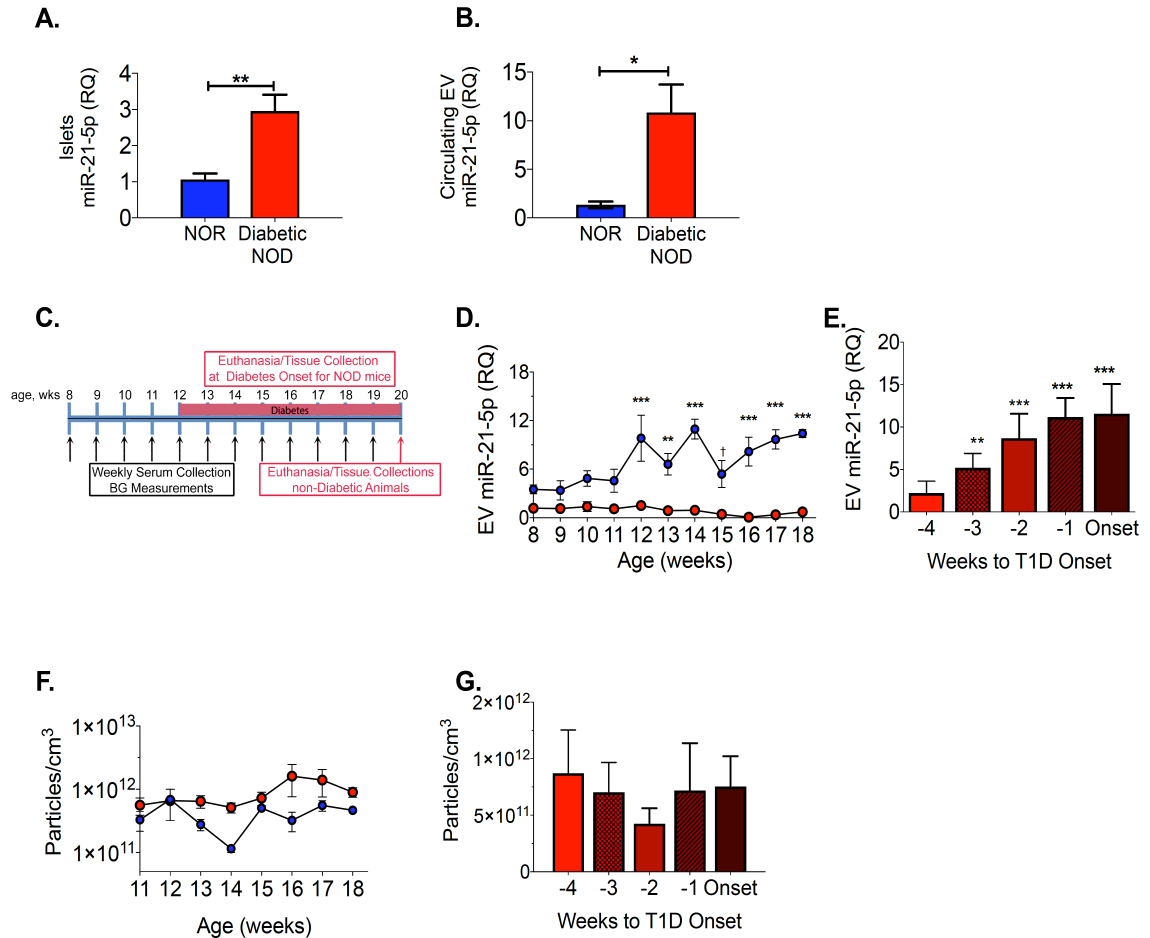
Figure 9. Elevations in circulating EV miR-21 precede onset of T1D in NOD mice.

qRT-PCR was performed to quantify relative levels of EV miR-21 in the **A.** islets and

the **B.** terminal serum of diabetic NOD mice compared to NOR controls. **C.** Longitudinal

weekly serum collections and blood glucose assessments of NOD (n=7-9) and control

NOR (n=5) mice were performed, starting at 8 weeks of age and until either development

of diabetes, or until 20 weeks of age. **D:** qRT-PCR was performed to quantify serum EV

miR-21 in NOD mice relative to controls by age (n=3-9/group). **E:** NOD serum EV miR-

21 relative to age-matched NOR controls was also analyzed with relationship to diabetes

onset (defined as first glucose >200 mg/dL). NTA of serum EVs in NOD mice showed

no significant changes with relation to age **(F)** or diabetes onset **(G)** (n=6). Results

displayed as mean ± SD; *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 by 2-tailed Student's *t* test,

1-way ANOVA with Holm-Sidak's multiple comparisons test, or 2-way ANOVA with

Sidak's multiple comparisons test. RQ=relative quantity.

miR-21 is Increased in the Circulating EVs from New-Onset T1D Patients

To establish relevance to human disease, we analyzed clinical serum samples from 19 pediatric subjects with new onset T1D and 16 healthy pediatric control subjects. Demographic characteristics are presented in Table 1; no significant differences in age, gender, or BMI percentile for age were present between groups. We quantified total serum miR-21, total serum EVs and the miR-21 from circulating EVs. In contrast to reports from subjects with longstanding T1D, total serum miR-21 was decreased in recent onset T1D patients compared to controls (**Figure 10A**). However, consistent with our *in vitro* and NOD mouse findings, levels of serum EV miR-21 in were 5-fold increased in samples from T1D subjects (**Figure 10B**).

We also performed NTA on serum samples to assess differences in the overall numbers and size distribution of circulating EVs that revealed a reduction total circulating EVs in T1D samples, specifically in smaller EVs (**Figure 10C**). We measured correlations between serum EV miR-21 and age, BMI Z-score, and HbA1c (for T1D subjects only). In control subjects, younger age correlated with higher EV mir-21 levels (r_s=-0.506, p=0.474). However, this relationship was not present in T1D subjects. We did not detect any correlations between BMI percentile or HbA1c and EV miR-21 levels, but because our samples were collected at the time of T1D diagnoses, all subjects had uncontrolled T1D. In addition, no differences were detected based on separation by gender.

We also asked whether our observed differences in serum and EV miRNAs would apply to a different miRNA biomarker of diabetes. For this analysis, we chose to measure miR-375, which has been proposed as a circulating biomarker of beta cell death

(**Figure 10D-E**) (70, 71). By contrast to miR-21, in participants with type 1 diabetes,

miR-375 was similarly increased in both serum and in serum EVs. We next examined

associations between the relative levels of serum EV miR-21 and miR-375 among

participants with diabetes (**Figure 10F**). Although there was overlap in some individuals,

the levels of the two microRNAs were not correlated overall, with examples of

individuals with predominant elevations in either serum EV miR-21 or serum EV miR-

375.

Table 1. Demographic characteristics of human subject groups.

Variable	Healthy Control (n= 16)	T1D (n=19)	*P* value
Age (years)	10.5 (9, 12)	10.5 (8,12)	0.8579
Male sex (%)	62.5	62.5	1.00
BMI percentile	84 (72.5, 89.5)	52.5 (32.5, 87.5)	0.1343
HbA1C (%/mmol/mol)	n/a	11.3 (10.25,12.33) / 100 (89,111)	n/a

Results are displayed as median (Interquartile range). T1D- Type 1 diabetes, BMI- body mass index, HbA1c-Hemoglobin A1c
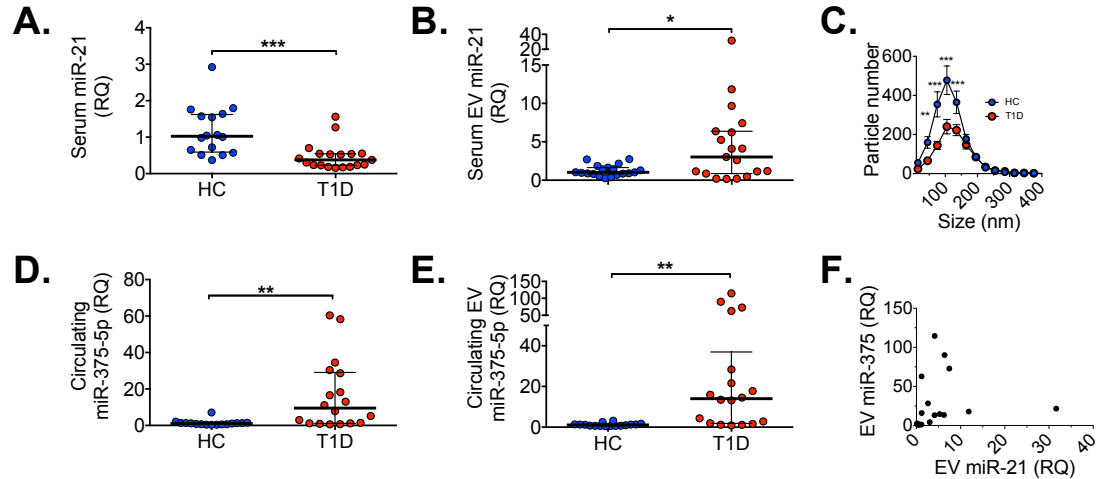
Figure 10. Circulating EV miR-21 is elevated in persons with new-onset T1D. Serum samples from healthy pediatric controls (HC) and new-onset pediatric T1D subjects (T1D) were assessed for relative levels of circulating miR-21 in **A.** whole serum and in **B.** circulating EVs by qRT-PCR (n=16-19/group). **C.** NTA was performed to quantify serum EV concentration and size distribution. **D.** Relative levels of total serum miR-375-5p and **E.** circulating EV miR-375-5p were also quantified. **F.** Relationship between serum EV miR-21-5p and EV miR-375-5p in samples from individuals with type 1 diabetes. Results shown as median ± IQR except Figure 10C (mean ± standard deviation); *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.

CHAPTER THREE: CONCLUSIONS

Previous studies have demonstrated increased beta cell miR-21 expression in response to cytokines and inflammation, specifically via activation of NFκB signaling (46, 49, 53). Consistent with other cell types, beta cell miR-21 has been confirmed to target the tumor suppressor PDCD4 (49, 53). Knockout of islet PDCD4 protected against beta cell death and diabetes development in NOD mice, and after treatment with high dose STZ, suggesting that miR-21 mediated inhibition of PDCD4 could potentially have a pro-survival effect (53). However, others have shown that miR-21 overexpression via transfection demonstrated no improvement in beta cell survival (46). More recent work reported increased beta cell apoptosis after stable lentiviral miR-21 overexpression. However, the authors did not observe concurrent reductions in PDCD4 with miR-21 overexpression, or identify a mechanism explaining potential pro-apoptotic effects of miR-21 (48), leaving a number of unresolved controversies regarding the effect of miR-21 on beta cell survival and function.

Consistent with other studies, we found that beta cell miR-21 was induced by multiple *in vitro* and *in vivo* models of inflammation and T1D, including cytokine treatment of human islets, islets from mice treated with MLD-STZ, and NOD mouse islets. Moreover, we ruled out contributions from ER stress or hyperglycemia associated with diabetes development. The elevation of miR-21 in non-diabetic NOD islets is an interesting finding, and consistent with inflammatory induction of miR-21 expression, as NOD-Severe Combined Immunodeficiency (NOD-SCID) mice exhibit islet macrophage and dendritic cell infiltration, increased TNF-α signaling, and intrinsic islet dysfunction, despite the absence of hyperglycemia (14, 72). Of note, and consistent with islet miR-21

expression, we were unable to detect differences in islet *BCL2* mRNA between diabetic and nondiabetic NOD mice (data not shown).

We endeavored to first clarify the effects of miR-21 on beta cell survival and secondly to identify a beta cell miR-21 target to explain these effects. In INS-1 beta cells, our results revealed that miR-21 overexpression, induced using a miR-21 mimic, led to a clear increase in cell death, despite reductions in PDCD4 protein levels. Correspondingly, inhibition of miR-21 was able to reduce cleaved caspase-3 expression after cytokine treatment. Differences in our data and previous work may have several explanations, but are mostly likely due to differences in method, dose, and effectiveness of miR-21 manipulation in target cells. However, we verified that our system resulted in effective miR-21 overexpression by ensuring significant suppression of PDCD4, a verified beta cell miR-21 target. Furthermore, identification of a target that explains the pro-apototic phenotype lends credibility to our system and findings.

Based on *in silico* prediction tools, we identified the *BCL2* 3'UTR as a highly-conserved miR-21 binding site. BCL2, a member of the antiapoptotic BCL2-like-protein subgroup of the BCL2 protein family, is involved in regulation of mitochondrial outer membrane permeabilization and apoptosis (73). When bound to BH3-only "activator" proteins, BCL2-like proteins are able to render activator proteins latent. However, when the interaction between BCL2-like proteins and activator proteins is disrupted, activator proteins are released in to the cytoplasm, where they induce a conformational change and activation of pro-apoptotic Bax-like proteins. This ultimately leads to pore formation in the outer mitochondrial membrane and release of pro-apoptotic proteins into the cytosol (73, 74). Because of BCL2's pro-survival properties, miR-21 mediated reductions in

BCL2 mRNA stability and suppression of *BCL2* translation could explain the pro-apoptotic phenotype we observed in beta cells (75, 76).

Although *BCL2*'s 3'UTR has a predicted miR-21 binding site, reduced BCL2 levels have not been typically identified as an effect of miR-21. To the contrary, in other cell types, miR-21 overexpression has been associated with increased BCL2 protein levels, (77-79). Treatment of breast cancer cells with estrogen receptor agonists was associated with reductions of miR-21 expression and increased BCL2 mRNA and protein levels, suggesting a potential inhibitory interaction. However, these results could also be explained by a direct effect of estrogen agonists on BCL2 expression (80). In contrast, our data support a direct interaction between miR-21 and the *BCL2* 3'UTR in beta cells, and provide direct evidence of miR-21's effect on BCL2 mRNA levels as well as translation. Moreover, we confirmed the link between miR-21 overexpression, reductions in BCL2, and increased apoptosis in human islets. This novel report of miR-21 inhibition of BCL2 may explain the unusual pro-apoptotic phenotype of miR-21 seen in the beta cell. Indeed, overexpression of BCL2 was able to reduce miR-21-mediated death in INS-1 cells.

There are several limitations to our study of miR-21's effects on BCL2. BCL2 overexpression has previously been shown to protect against cytokine-induced apoptosis in beta cell lines and islets *ex vivo*. However, in vivo BCL2 overexpression delayed but was unable to fully prevent diabetes development in NOD mice (75, 81, 82). While these data suggest that beta cell BCL2 overexpression may be partially protective, diabetes prevention in this model may require combinatorial approaches with a second intervention, such as immune modulation. Similarly, as previous work has suggested that

loss of BCL2 may not be sufficient to induce apoptosis in isolation, key methodological differences in time frame and/or method of BCL2 reduction may explain our observed effects (83, 84).

Lastly, BCL2 inhibition was recently shown to increase beta cell mitochondrial metabolism and glucose stimulated insulin secretion (83). Because miR-21 induced reductions in BCL2 might be expected to increase GSIS, our findings of decreased GSIS with miR-21 overexpression are likely not directly due to reduced BCL2 levels (83). Our results are most likely related to the progression of apoptosis and cell death present in a high percentage of cells, with subsequent degranulation and alterations in function. Alternatively, down-regulation of other suggested miR-21 target mRNAs, such as *Pclo,* which encodes a protein important for cyclic AMP potentiation of insulin secretion, may lead to effects on insulin secretion independent of miR-21's effects on cell survival (49). Further investigation of other miR-21 target mRNAs is needed to fully understand the *in-vivo* relevance of changes in beta cell miR-21 expression.

Our findings also demonstrate that beta cell EV miR-21 cargo is increased in response to treatment with inflammatory cytokines. This increase was predominantly due to cytokine-induced effects on beta cell exosome miR-21. We also report the novel observation that circulating EV miR-21 may be a biomarker of developing T1D, in that progressive elevations in serum EV miR-21 preceded hyperglycemia in NOD mice, and were present in pediatric subjects with new onset T1D.

miRNA profiling in beta cells has demonstrated that the exosome contents differ from the cytoplasmic contents of cells of origin (85). Our data point to an enrichment in the cytokine effect on miR-21 within beta cell exosomes, relative to intracellular miR-21.

Based on our data that miR-21 increases β cell apoptosis, it is tempting to speculate that exosome miR-21 may be involved in the proapoptotic effect of exosomal miRNA transfer from cytokine treated cells (86). Future studies will define the pathophysiologic significance of EV miR-21 transfer to recipient beta cells in the progression to T1D.

We observed important differences between circulating total miR-21 and EV miR-21 levels. Our findings of decreased serum miR-21 among T1D subjects are in contrast to several published reports of increased circulating miR-21 in patients with more established T1D (54, 55). However, reports describing total circulating miRNAs in new onset T1D or prediabetic patients, which would be more clinically consistent with our subject population, have not described differences in miR-21-5p (87-89).

Although long-term incubation of islets with cytokines has been reported to increase exosome release, we did not observe a change in beta cell or islet EV quantity after 24 hours of cytokine treatment (40). Lack of an increase in total EV count from islets or in serum of T1D subjects suggests that our observed increases in EV miR-21 are related to increases in miR-21 content per EV. Our findings of parallel levels of miR-375 in total serum and serum EVs suggest that differential miRNA concentrations in serum vs. serum EVs is not universal. Differences among individuals with type 1 diabetes suggest that increases in these EV miRNAs could reflect different biological processes relating to type 1 diabetes pathophysiology, with differences depending on the mechanism of release of the miRNA being studied. For example, miR-375 may be non-selectively released by dying beta cells as both EV-independent circulating miRNA and in EVs, while miRNAs reflecting other components of the diabetic islet microenvironment could be selectively packaged into EVs. These findings confirm the

importance of defining circulating EV miRNA content in addition to total circulating miRNA.

Although we did observe variation among human subjects with T1D, there was a clear subset of type 1 diabetes subjects that displayed elevations in EV miR-21, with half of our diabetes samples above the range for control subjects. This variation likely reflects the heterogeneous nature of T1D that has been observed in other clinical cohorts (90-92). We did not observe a relationship with glycemic control; however, this may reflect the timing of sample collection (at clinical diagnoses, when all subjects had uncontrolled diabetes).

Because miR-21 is a widely abundant miRNA, release from multiple tissue sources represents another likely source of variation in our study and is a significant limitation of work to define the biomarker potential of circulating miRNAs in general. However, our observations of consistent increases in EV miR-21 after cytokine exposure and before diabetes onset in the NOD mouse suggest that beta cell EV miR-21 is feasibly a major contributor to our observed signal in serum. Emerging strategies to isolate islet or beta cell-derived EVs from the circulation are likely to increase the specificity of this assay and allow for earlier and more precise detection of developing T1D (93). Alternatively, identification of exosome subpopulations responsible for increased exosome miR-21, or a panel of circulating exosome miRNAs impacted during T1D development may provide a more robust signal (68, 69, 94).

EV isolation methodology was an important consideration in our study. As there is no consensus on the ideal method for EV enrichment from biological samples, recommendations based on the position statement from the International Society for

Extracellular Vesicles suggest the choice of methodology to be guided by "(a) the specific scientific question asked and (b) on the downstream applications used" (95, 96). Because ultracentrifugation is the most commonly utilized and accepted EV isolation method, we chose to perform ultracentrifugation to characterize EV subtypes from cell lines and islets (97, 98). However, this method typically requires large sample volumes, limiting feasibility in clinical studies (99). Based on this, we chose to use a precipitation-based method to isolate total EVs for serum analyses in mice and children, which has also been demonstrated as an acceptable methodology for analysis of RNAs in circulating EVs (100-102). Our validation of EV isolation by NTA, immunoblot, and TEM suggests successful EV isolation using both methodologies.

In conclusion, our results suggest a novel role for miR-21 in the beta cell through induction of apoptosis via *BCL2* mRNA degradation and inhibition of *BCL2* mRNA translation. These findings provide important insight into the role of inflammation-induced elevations in beta cell miR-21 during diabetes development. Our work also suggests that beta cells exposed to inflammatory stress release exosomes containing increased miR-21 cargo into the circulation, and that these EVs could serve as a biomarker of developing T1D. Future work will utilize *in vivo* beta cell-specific models to study the effects of miR-21 over- and under-expression in order to define whether modulation of beta cell miR-21 levels may represent a potential target for strategies aimed at reducing beta cell death in diabetes. Other future work will determine whether transfer of exosomal miR-21 to surrounding beta cells contributes to type 1 diabetes development, identify targets that allow for isolation of beta cell-specific EVs, and define other beta cell EV miRNAs affected during developing T1D. Prospective evaluations of

human subjects are also indicated to determine whether glycemic control impacts

circulating EV mir-21, and whether levels are predictive of differences in T1D clinical

outcomes, such as honeymoon duration or loss of endogenous C-peptide secretion.

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CURRICULUM VITAE

Emily K. Sims

EDUCATION/EMPLOYMENT:

POST-GRADUATE

Indiana University-IUPUI Indianapolis, IN	M.S. Translational Science	Aug 2014-May 2018

FELLOWSHIP

Indiana University Indianapolis, IN	Pediatric Endocrinology	July 2010-Aug. 2013

RESIDENCY

Indiana University Indianapolis, IN	Pediatrics	July 2008- July 2010

INTERNSHIP

Indiana University Indianapolis, IN	Pediatrics	July 2007- June 2008

GRADUATE

University of Alabama at Birmingham Birmingham, AL	Doctor of Medicine	July 2003-May 2007

UNDERGRADUATE

Auburn University Auburn, AL	B.S. Biomedical Sciences *(summa cum laude)*	Sept 1999- May 2003

HOSPITAL APPOINTMENTS :

Research Assistant Professor, Riley Hospital for Children, Indianapolis, IN- August 5, 2013- June 31, 2015

Assistant Professor of Pediatrics, Riley Hospital for Children, Indianapolis, IN- July 1, 2015- current

PROFESSIONAL ORGANIZATIONS:

American Academy of Pediatrics	Member	2007-present
Endocrine Society	Member	2011-present
Pediatric Endocrine Society	Member	2011-present

American Diabetes Association	Member	2014-present
Society for Pediatric Research	Elected Member	2016-present

PROFESSIONAL HONORS AND AWARDS:

Alumni Academic Scholarship to Auburn University	1999
Clinical Research Award, Medical Student Research Day	2004
Gold Humanism Honors Society Class of 2007	2006
Alpha Omega Alpha Honors Society Class of 2007	2007
Selected for Endocrine Society Early Investigator's Workshop (San Francisco, CA)	2011
Presidential Poster Presentation at Pediatric Endocrine Society (Denver, CO)	2011
Pediatric Endocrine Society Travel Grant (Denver, CO)	2011
Riley Hospital Red Shoes Award for family centered care	2011
Indiana University Pediatrics Department Morris Green Research Scholar	2012
Endocrine Society Travel Grant (Houston, TX)	2012
Selected to attend Endocrine Society Trainee Day (Houston, TX)	2012
Selected for Pediatric Endocrine Society Fellow Spring Retreat (Cape Cod, MA)	2012
Pediatric Endocrine Society Travel Grant (Boston, MA)	2012
National Institutes of Health Loan Repayment Program (2 competitive renewals)	2013-2018
Selected to attend European Society of Pediatric Endocrinology Summer School (Lake Maggiore, Italy)	2013
Presidential Poster Presenter at PES Conference (Washington D.C.)	2013
Indiana Clinical and Translational Sciences Institute Poster/Abstract Award	2014

Selected for Indiana University's Leadership in Academic Medicine Faculty Development Program	2014
American Society for Clinical Investigation Young Physician-Scientist Award	2014
Indiana University Purdue University Indianapolis Prestigious External Award Recognition Recipient	2015
Midwest Islet Club Poster Award (Chicago, IL)	2015
Pediatric Endocrine Society Clinical Scholar Award	2015
Selected for Indiana University Empower Program	2016
Central Society for Clinical and Translational Research K Award Grant (Chicago, IL)	2016
TrialNet Junior Investigator Award	2017
Central Society for Clinical and Translational Research Early Career Development Award	2017
Endocrine Society Early Investigators Award	2017

RESEARCH/ CREATIVE ACTIVITY:

AWARD GRANTS/ FELLOWSHIPS
Ongoing
Biomedical Research Grant   Period: 12/01/17- 11/30/18
Identification of β-Cell Specific Extracellular Vesicles
Role: PI
$50,000
Goal: Define tools to isolate circulating β cell derived extracellular vesicles.

Juvenile Diabetes Research Foundation   Period: 9/2017-8/2019
 2-SRA-2017-498-M-B
Identification of β Cell Dysfunction in Relatives of Individuals with Type 1 Diabetes Mellitus
Role: Primary PI
$957,617.94
Goal: Define the presence or absence of β Cell dysfunction in family members of individuals with type 1 diabetes.

IU Postdoc Challenge Award   Period: 5/2017-5/2018

*Identification of β-Cell-derived Extracellular Vesicle
Surface Peptides*
Role: Mentor
PI: Alexander Lakhter
$5,000
Goal: Identify β Cell specific EV surface peptides using proteomics

Central Society for Clinical and Translational Research   Period:5/17-5/2018
(CSCTR) Early Career Development Award
Role: PI
$10,000
Goal: The goal of this award is to provide research support to outstanding early career
investigators.

Wells Seed Funding   Period: 2/17-2/2018
*Effects of DNA Damage and p53 Activation on β Cell
mRNA Translation*
Role: PI
$25,000
Goal: The goal of this funding is to use RNA Sequencing to identify transcripts impacted
by β Cell DNA Damage

IU Enhanced Mentoring Program with Opportunities for   Period: 12/2016-12/2017
Ways to Excel in Research (EMPOWER)
Role: PI
$10,000
Goal: The Goal of this award is to provide research support for early stage investigators
to submit applications for external funding.

IU Showalter Research Trust Fund   Period: 7/2016- 12/2017
*Novel Circulating Extracellular Vesicle microRNAs
Identifying Development of Type 1 Diabetes*
Role: PI
$60,000
Goal: The goal of this project is to Identify novel extracellular vesicle microRNAs
that may be altered during the development of Type 1 Diabetes.

IU Health Values Funds for Research   Period: 01/2016-6/2018
*miR-21 Leads to β Cell Death and is a Potential
Biomarker of Type 1 Diabetes*
Role: PI
$100,000
Goal: Develop a β cell specific miR-21 knock out mouse and identify whether elevations
in circulating miR-21 can predict developing Type 1 Diabetes.

NIH/NIDDK K08DK103983   Period: 09/12/2014-08/2019

β-cell Derived miR-21 as an IntrinsicProtective
Response and Biomarker in Type 1 Diabetes
Role: PI
$147,557
Goal: Career Development Award to support salary as a bridge to independence.

NIH/NIDDK Loan Repayment Program   Period: 07/2013-06/2018
Role: PI
Goal: Repays $70,000 of student loan debt over 2 years.

Past
Center for Diabetes and Metabolic Diseases   Period: 07/2015-06/2016
Pilot and Feasibility Award (Supported by NIDDK
Grant Number P30DK097512)
Circulating Extracellular Vesicle miRNAs as a
Biomarker of Type 1 Diabetes
Role: PI
$45,000
Goal: Identify novel circulating EV miRNAs that may predict T1D Development.

Pediatric Endocrine Society Clinical Scholar Award   Period: 07/2015-06/2016
β-cell Derived miR-21 as an IntrinsicProtective
Response and Biomarker in Type 1 Diabetes
Role: PI
$12,500
Goal: Define role of miR-21 in inhibition of the pro-death proteins Programmed Cell
Death 4 (PDCD4) and Phosphatase and Tensin Homolog deleted on Chromosome 10
(PTEN).

Indiana Clinical and Translational Sciences   Period: 01/2014-09/2014
Institute- KL2TR001106 and UL1TR001108
β-cell Derived miR-21 as an IntrinsicProtective
Response and Biomarker in Type 1 Diabetes
Role: Awardee
(PI: A. Shekhar)
Goal: Provides 75% salary support plus $7200 for expenses as a bridge to extramural
funding for young investigators.

T32 Training Grant: 5T32DK065549-08   Period: 07/2011-08/2013
Role: Trainee
(PI: Eugster, Erica)
Goal: the goal of this training grant is to support the training of pediatric endocrine
fellows as researchers. My primary project during this period was a large-scale cohort
study comparing differences between two mouse strains in the compensatory response of

the β cell to diet induced obesity, results of which were recently published in the *American Journal of Physiology: Endocrinology and Metabolism.*

Morris Green Scholar Research Fellowship   Period: 08/2012-08/2013
Role: PI
Goal: The goal of this program is to support the development of physician-scientists. The fellowship program provided $12000 in supplemental salary support as well as $3000 in travel funds to attend national meetings.

PRINT AND/OR ELECTRONIC PUBLICATIONS
Original Research Articles

1. Wall TC, **Senicz E,** Evans HH, Woolley A, Hardin JM. Hearing screening practices among a national sample of primary care pediatricians. *Clinical Pediatrics*. 2006; 45(6):559-566.

2. **Sims EK***, Eugster EA, Nebesio TD. Detours on the road to diagnosis of Graves Disease. *Clinical Pediatrics*. 2012; 51 (2): 160-4. ***Corresponding Author**

3. **Sims EK***, Addo OY, Gollenberg AL, Himes JH, Hediger ML, Lee PA. Inhibin B and luteinizing hormone levels in girls aged 6-11 years from NHANES III, 1988-1994. *Clinical Endocrinology.* 2012;77(4):555-63. ***Corresponding Author**

4. **Sims EK***, Garnett S, Guzman F, Sultan C, Eugster E, on behalf of the fulvestrant McCune-Albright study group. Fulvestrant Treatment of Precocious Puberty in Girls with McCune-Albright Syndrome. *International Journal of Pediatric Endocrinology*. 2012; 2012(1):26. ***Corresponding Author**

5. Chaudry ZZ, Morris DL, Moss DR, **Sims EK**, Kono T, Evans-Molina C. Streptozotocin is Equally Diabetogenic Whether Administered to Fed or Fasted Mice. *Lab Animals.* 2013; 47 (4): 257-65.

6. **Sims EK,** Hatanaka M, Morris DL, Tersey SA, Kono T, Chaudry ZZ, Day KH, Moss DR, Stull ND, Mirmira RG, Evans-Molina C. Divergent Compensatory Responses to High Fat Diet Between C57BL6/J and C57BLKS/J Inbred Mouse Strains. *Am J Physiol Endocrinol Metab.* 2013 Dec; 305 (12): E1495-511.

7. Kono TM, **Sims EK**, Moss DR, Yamamoto W, Ahn G, Diamond J, Tong X, Day KH, Territo PR, Hanenberg H, Traktuev DO, March KL, Evans-Molina C. Human adipose-derived stromal/stem cells protects against STZ-induced hyperglycemia: analysis of hASC-derived paracrine effectors. *Stem Cells.* 2014; 32 (7): 1831-42.

8. Hatanaka M, Maier B, **Sims EK,** Templin AT, Kulkarni RN, Evans-Molina C, Mirmira RG. Palmitate Induces mRNA Translation and Increases ER Protein Load in Islet β cells via Activation of the Mammalian Target of Rapamycin Pathway. *Diabetes*. 2014;63(10):3404-15.

9. **Sims EK**, Chaudhry Z, Watkins R, Syed F, Blum J, Ouyang F, Perkins S, Mirmira RG, Sosenko J, DiMeglio LA, and Evans-Molina C. Elevations in the Fasting Serum Proinsulin:C-peptide Ratio Precede the Onset of Type 1 Diabetes. *Diabetes Care*. 2016; 39 (9): 1519-26.

10. **Sims EK***, Lakhter AJ, Anderson-Baucum E, Kono T, Tong X, and Evans-Molina C. MicroRNA 21 Targets BCL2 mRNA to Increase Apoptosis in Rat and Human Beta Cells. *Diabetologia*. 2017; 60(6):1057-1065. *** Corresponding author**

11. Hatanaka M, Anderson-Baucum E, Lakhter A, Kono T, Maier B, Tersey S, Tanizawa Y, Evans-Molina C, Mirmira RG, and **Sims EK***. Chronic high fat feeding restricts islet mRNA translation initiation independently of ER stress via DNA damage and p53 activation. *Scientific Reports*. 2017; 7(1):3758. ***Corresponding author**

12. Lakhter AJ, Pratt, RE, Moore, RE, Doucette KK, Maier BF, DiMeglio LA, and **Sims EK**. Beta cell extracellular vesicle miR-21-5p cdrgo is increased in response to inflammatory cytokines and serves as a biomarker of type 1 diabetes. In Press at *Diabetologia*. . ***Corresponding author**

Review Articles, Printed Commentaries, and Case Reports:
1. Fuqua JS, **Sims EK**. Contraceptive Quandaries: Contraception Decisions in the Pediatric Endocrinology Office. *Journal of Clinical Endocrinology and Metabolism*. 2012; 97(1): 73-6.

2. **Sims E**, Evans-Molina C. Stem cells as a tool to improve outcomes of islet transplantation. *Journal of Transplantation.* 2012; 2012:736491.

3. **Sims EK,** Evans-Molina C. Urinary Biomarkers for the Early Diagnosis of Retinopathy and Nephropathy in Type 1 Diabetes Mellitus: A "Steady Stream" of Information Using Proteomics. *Translational Research.* 2014; 163 (3): 183-7.

4. **Sims EK,** Evans-Molina C. Commentary on: Macrophage Migration Inhibitory Factor in Acute Lung Injury: Expression, Biomarker, and Associations. Published in Laurence J. Centennial Celebration of Translational Research: the Journal of Laboratory and Clinical Medicine. *Translational Research*. 2015; 165 (1):1-6.

5. Lakhter AJ, **Sims EK*.** Emerging Roles for Extracellular Vesicles in Diabetes and Related Metabolic Disorders. *Molecular Endocrinology.* 2015; 29 (11):1535-48. *** Corresponding author**

6. Mirmira RG, **Sims EK**, Syed F, Evans-Molina C. Biomarkers of β-Cell Stress and Death in Type 1 Diabetes. *Curr Diab Rep.* 2016; 16(10):95.

7. **Sims EK.** Chewing the Fat: A Metabolic Role for Ldb1 Beyond the Pancreas? *Endocrinology*. 2017; 158 (5): 1113-1115. *** Corresponding author**

INVITED TALKS

"Research Proposal: ASCs in Treatment of Diabetes Mellitus"	Endocrine Society Trainee Day San Francisco,CA	2011
"Precocious Puberty in McCune-Albright Syndrome"	Indiana University Endocrinology Grand Rounds	2011
"Bariatric Surgery in Morbidly Obese Adolescents: Con"	Pediatric Endocrine Society Summer School Cape Cod, MA	2012
"Stems Cells as a Tool to Enhance Islet Transplants"	Indiana University Endocrine Grand Rounds	2012
"Growth Hormone Treatment in Prader Willi Syndrome"	European Society for Pediatric Endocrinology Summer School Lake Maggiore, Italy	2013
"Extracellular MicroRNAs in Diabetes Mellitus"	Indiana University Endocrine Grand Rounds	2013
"β-cell Derived miR-21 as an Intrinsic Protective Response and Biomarker in Type 1 Diabetes"	Indiana University Wells Center Center for Diabetes Research Seminar	2014
"β-cell Derived miR-21 as a Proapoptotic Response and Biomarker in Type 1 Diabetes"	Indiana University Diabetes, Endocrine, and Metabolism Research Seminar	2015
"β-cell Derived miR-21 as a Proapoptotic Response and Biomarker in Type 1 Diabetes"	Indiana University Pediatric Grand Rounds	2015
"Type 1 Diabetes in Pediatrics"	Deaconess Hospital Grand Rounds Evansville, IN	2016
"Elevations in Serum Proinsulin:C-peptide Ratio Predate Onset of Type 1 Diabetes"	TrialNet Steering Meeting Bethesda, MD	2016
"Recruitment in the Pathway to Prevention"	TrialNet Steering Meeting Denver, CO	2017
"Biomarkers of Beta Cell Dysfunction in	TrialNet Affiliate Meeting	2017

Type 1 Diabetes"	Chicago, IL	
"Genetic Risk in Type 1 Diabetes"	TrialNet Steering Meeting Reston, VA	2017
"miRNAs in Diabetes Risk" (future)	Endocrine Society Meeting Chicago, IL	2018
"Biomarkers of Beta Cell Dysfunction in Type 1 Diabetes (future)	Central Society Society for Clinical and Translational Research Chicago, IL	2018

INVITED PRESENTATIONS- REFEREED:
Regional
Lakhter A, Syed F, Maier B, Mirmira RG, Evans-Molina C, and **Sims EK**. "Elevated Levels of miR-21 in Circulating Extracellular Vesicles Predate Onset of Type 1 Diabetes Mellitus" (Oral Abstract) Midwest Islet Club meeting 2016, Indianapolis, IN.

National
Sims EK, Restrepo I, Tong X, Kono T, Mirmira R, and Evans-Molina C. "β-cell Derived miR-21 as a Proapoptotic Response and Biomarker in Type 1 Diabetes" (Oral Abstract) American Diabetes Association Meeting, 2015, Boston MA.

Sims EK, Ouyang F, Perkins S, DiMeglio LA, Blum J, Mirmira RG, and Evans-Molina C. "Elevations in Serum Proinsulin:C-peptide Ratio Predate Onset of Type 1 Diabetes" (Oral Abstract) Pediatric Endocrine Society meeting 2016, Baltimore, MD.

Sims EK, Ouyang F, Perkins S, DiMeglio LA, Blum J, Mirmira RG, and Evans-Molina C. "Elevations in Serum Proinsulin:C-peptide Ratio Predate Onset of Type 1 Diabetes" (Guided Audio Poster) American Diabetes Association meeting 2016, New Orleans, LA.

Sims EK, Ouyang F, Perkins S, DiMeglio LA, Blum J, Mirmira RG, Mastracci T, and Evans-Molina C. Elevations in Serum Proinsulin:C-peptide Ratios in Type 1 Diabetes: Before Diabetes Onset AND in Longstanding Disease" (Oral Abstract) Human Islet Research Network meeting 2016, Bethesda, MD.

Sims EK, Nyalwidhe J, Davis A, Haataja L, Ouyang F, Perkins S, DiMeglio LA, Blum J, Morris M, Mirmira RG, Nadler J, Mastracci T, Arvan P, Greenbaum C, and Evans-Molina C. Circulating Biomarkes of Beta Cell Dysfunction in Type 1 Diabetes" (Oral Abstract) Network for Pancreatic Organ Donors with Diabetes (nPOD) meeting 2017, Fort Lauderdale, FL.

Lakhter, A, Pratt, R, Syed F, Maier B, Mirmira RG, Evans-Molina C, and **Sims EK.** "Elevations in EV miR-21 as a Biomarker of Developing T1D" (Oral Abstract) Human Islet Research Network 2017; Bethesda, MD.

Lakhter, A, Pratt, R, Syed F, Maier B, Mirmira RG, Evans-Molina C, and **Sims EK.**
"Elevations in EV miR-21 as a Biomarker of Developing T1D" (Oral Abstract)
Endocrine Society Meeting 2017; Orlando, FL.

Sims EK, Tersey S, Nelson J, Mirmira R, and Evans-Molina C. "Persistent Elevations in
Beta Cell Death in Longstanding Type 1 Diabetes" International Meeting of Pediatric
Endocrinology, 2017, Washington, DC.

International
Lakhter, A, Pratt, R, Syed F, Maier B, Mirmira RG, Evans-Molina C, and **Sims EK**.
Increase in miR-21 in Circulating Vesicles Precedes the Onset Of Type 1 Diabetes"
(Guided Audio Poster) International Society for Extracellular Vesicles Meeting, 2017,
Toronto, Canada

POSTER PRESENTATIONS- REFEREED
Local
Sims EK, Eugster EA, Nebesio TD**.** "Detours on the road to diagnosis of Graves
Disease" Pediatric Scholars Day. 2010.

Sims EK, Garnett S, Guzman F, Sultan C, Eugster E, on behalf of the fulvestrant
McCune-Albright study group. "Fulvestrant Treatment of Precocious Puberty in Girls
with MAS" Pediatric Scholars Day. 2011.

Sims EK, Hatanaka M, Morris DL, Tersey SA, Kono T, Chaudry Z, Day K, Moss DR,
Stull ND, Mirmira RG, Evans-Molina C. "Variations in Susceptibility to Diet-Induced
Obesity Between C57BL6/J and C57BLKs/J Inbred Mouse Strains" Pediatric Scholars
Day. 2013. ***Selected for Poster discussion session**

Sims EK, Restrepo I, Tong X, Kono T, Mirmira R, Evans-Molina C "β-cell Derived
miR-21 as an Intrinsic Protective Response and Biomarker in Type 1 Diabetes" Indiana
CTSI Meeting. 2014.
*Selected for Abstract/Poster Award

Lakhter A, Syed F, Maier B, Mirmira RG, Evans-Molina C, and **Sims EK**. "Elevated
Levels of miR-21 in Circulating Extracellular Vesicles Predate Onset of Type 1 Diabetes
Mellitus" Center for Diabetes and Metabolic Diseases Research Symposium. 2016.

Pratt, R, Lakhter A, Syed F, Maier B, Mirmira RG, Evans-Molina C, and **Sims EK**.
"Elevated Circulating Extracellular Vesicle miR-21 as a Noninvasive Marker of Insulitis
Type 1 Diabetes Mellitus" Summer Research Program in Academic Medicine
Symposium. 2016.

Park, G, Liu Z, Mirmira RG, Mather K, Gupta S, and **Sims EK**. Analysis of Beta Cell
Death and Dysfunction in HIV Positive Individuals" Summer Research Program in
Academic Medicine Symposium. 2016.

Doucette K, Dumas M, Lakhter A, Anderson-Baucum E, Sims EK. "Effects of miR-21 on β cell insulin regulator, Spry2 in INS-1 Cells" CDMD Research Symposium. 2017.

Neymann A, Nelson J, Tersey S, Mirmira R, Evans-Molina C, Sims EK. "Persistent Elevations in β- cell Death Among Subjects with Longstanding Type 1 Diabetes " CDMD Research Symposium. 2017.

Lakhter A, Syed F, Maier B, Mirmira RG, Evans-Molina C, and **Sims EK**. "Elevated Levels of miR-21 in Circulating Extracellular Vesicles Predate Onset of Type 1 Diabetes Mellitus" Center for Diabetes and Metabolic Diseases Research Symposium. 2017.
*Selected for poster award

Regional
Sims EK, Hatanaka M, Tersey SA, Kono T, Moss DR, Stull ND, Mirmira RG, Evans-Molina C."Strain related differences in the response to diet-induced obesity and PPAR gamma agonists" Midwest Islet Club.   2012, Pittsburgh, PA

Sims EK, Hatanaka M, Tersey SA, Kono T, Moss DR, Stull ND, Mirmira RG, Evans-Molina C."Strain related differences in the response to diet-induced obesity and PPAR gamma agonists" Midwest Islet Club.   2013, Ann Arbor, MI

Sims EK, Restrepo I, Tong X, Kono T, Mirmira R, Evans-Molina C. "β-cell Derived miR-21 as an Intrinsic Protective Response and Biomarker in Type 1 Diabetes" Midwest Islet Club. 2014, Birmingham, AL

Sims EK, Restrepo I, Tong X, Kono T Mirmira R, Evans-Molina C. "β-cell Derived miR-21 as a Proapoptotic Response and Biomarker in Type 1 Diabetes" Midwest Islet Club. 2015, Chicago, IL
*Selected for poster award

Sims EK, Lakhter A, Anderson-Baucum E, Restrepo I, Tong X, Kono T, Evans-Molina C. "miR-21 Increases Beta Cell Apoptosis Via Inhibition of the Antiapoptotic Protein B Cell Lymphoma 2, and Could Serve as a Biomarker Of Type 1 Diabetes Mellitus" Central Society for Clinical and Translational   Research. 2016, Chicago, IL
* Selected for CSCTR K Award Grant

Pratt, R, Lakhter A, Syed F, Maier B, Mirmira RG, Evans-Molina C, and **Sims EK**. "Elevated Circulating Extracellular Vesicle miR-21 as a Noninvasive Marker of Insulitis Type 1 Diabetes Mellitus" NIDDK Medical Student Summer Research Program in Diabetes and Obesity. 2016, Nashville, TN

National
Sims EK, Eugster EA, Nebesio TD "Detours on the road to diagnosis of Graves Disease" 8th Joint Meeting of LWPES/ESPE. 2009, New York, NY

Sims EK, Garnett S, Guzman F, Sultan C, Eugster E, on behalf of the fulvestrant McCune-Albright study group. "Fulvestrant Treatment of Precocious Puberty in Girls with MAS" Pediatric Endocrine Society Meeting. 2011,Denver, CO
*Selected for Presidential Poster Presentation

Sims EK, Hatanaka M, Tersey SA, Kono T, Moss DR, Stull ND, Mirmira RG, Evans-Molina C."Strain related differences in the response to diet-induced obesity and PPAR gamma agonists" Endocrine Society. 2012, Houston, TX.

Sims EK, Hersch J, Haddad N, Imel EA. "Multilocus hypomethylation resulting in Beckwith Wiedemann Syndrome and Pseudohypoparathyroidism 1B in a 7 year-old girl" Pediatric Endocrine Society Meeting. 2013, Washington D.C.
*Selected for Presidential Poster Presentation

Sims EK, Hatanaka M, Tersey SA, Kono T, Moss DR, Stull ND, Mirmira RG, Evans-Molina C."Strain related differences in the response to diet-induced obesity and PPAR gamma agonists" Endocrine Society Meeting. 2013, San Francisco

Sims EK, Ehsan Z, Howenstine M, Bozic M, Nebesio TD. "Diabetes as the Initial Manifestation of Possible CFTR-Related Dysfunction in an Adolescent Male" Pediatric Endocrine Society Meeting. 2014, Vancouver

Sims EK, Restrepo I, Tong X, Kono T, Mirmira R, and Evans-Molina C. "β-cell Derived miR-21 as an Intrinsic Protective Response and Biomarker in Type 1 Diabetes" American Society for Clinical Investigation. 2014, Chicago, IL

Sims EK, Boulware D, Evans-Molina C, Greenbaum C, Rodriguez H, Krischer J, DiMeglio L and the T1D Trial NetStudy Group. "Who is Not Returning? Recruitment of Subjects to the TrialNet Pathway to Prevention" American Diabetes Association. 2015, Boston, MA

Sims EK, Restrepo I, Tong X, Kono T, Mirmira R, and Evans-Molina C. "β-cell Derived miR-21 as a Proapoptotic Response and Biomarker in Type 1 Diabetes"International Society for Extracellular Vesicles. 2015, Bethesda, MD

Lakhter A, Syed F, Maier B, Mirmira RG, Evans-Molina C, and **Sims EK**. "Elevated Levels of miR-21 in Circulating Extracellular Vesicles Predate Onset of Type 1 Diabetes Mellitus" American Diabetes Association. 2016, New Orleans LA.

Sims EK, DiMeglio L, Nelson J, Tersey S, Mirmira R, Evans-Molina C. "Circulating Unmethylated and Methylated Preproinsulin DNA in Longstanding Type 1 Diabetes" Human Islet Research Network Meeting. 2016, Washington D.C.

Sims EK, Lakhter A, Anderson-Baucum E, Restrepo I, Tong X, Kono T, Evans-Molina C. "miR-21 Increases Beta Cell Apoptosis Via Inhibition of the Antiapoptotic Protein B Cell Lymphoma 2 (BCL2)" Pediatric Endocrine Society Meeting. 2016, Baltimore, MD.

Sims EK, Nyalwidhe J, Davis A, Haataja L, Ouyang F, Perkins S, DiMeglio LA, Blum J, Morris M, Mirmira RG, Nadler J, Mastracci T, Arvan P, Greenbaum C, and Evans-Molina C. Dysfunctional Proinsulin Processing in Longstanding Type 1 Diabetes" Endocrine Society and Levine Symposium. 2017, Orlando, FL.

Hatanaka M, Anderson-Baucum E, Lakhter A, Kono T, Maier B, Tersey S, Tanizawa Y, Evans-Molina C, Mirmira RG, and **Sims EK***. "Chronic High Fat Feeding Restricts Islet mRNA Translation Initiation Independently of ER Stress Via DNA Damage and p53 Activation" Endocrine Society and Levine Symposium. 2017, Orlando, FL.

Lakhter, A, Pratt, R, Syed F, Maier B, Mirmira RG, Evans-Molina C, and **Sims EK.** "Elevations in EV miR-21 as a Biomarker of Developing T1D" Levine Symposium 2017; Orlando, FL.

Lakhter, A, Pratt, R, Syed F, Maier B, Mirmira RG, Evans-Molina C, and **Sims EK.** "Elevations in EV miR-21 as a Biomarker of Developing T1D" Extracellular RNA Communication Consortium Meeting 2017; Washington D.C.

TEACHING ACTIVITIES:

Type 1 Diabetes- Pediatric Resident Lecture	2013- 2017
Growth- Medical Student Clinical Rotation Lecture Series	2014-2015
Hypothyroidism Pediatric Endocrine Fellows Lecture	2014
Hormone Stimulation Testing- Pediatric Endocrine Fellows Lecture	2014-2017
Putting Together a Specific Aims Page- Morris Green Research Scholars Lecture	2015-2016
Pituitary Dysfunction- Pediatric Endocrine Fellow Lecture	2016
Type 1 Diabetes- Medical Student Clinical Rotation Lecture Series	2016-2017
Basic Science Techniques- Pediatric Endocrine Fellow Lecture	2017-2018

PROFESSIONAL SERVICE:
Institutional:

Wells Intern Summer Research Day	oral presentation judge	2015
Center for Diabetes and Metabolic Diseases Annual Symposium	poster judge	2016
Committee for Pediatric Endocrine Grand Rounds Cochair		2016-2019

Indiana University Clinical and Translational Sciences Institute- Pilot Funding for Research Use of Core Facilities	grant reviewer	2017

Regional:

American Diabetes Association	Volunteer at diabetes camp	2012-2016
Central Society for Clinical and Translational Research	abstract reviewer	2013-2017
American Diabetes Association	speaker at research day and ask the expert events	2014, 2015
Midwest Islet Club	Session Moderator, abstract reviewer	2016, 2017

National:

National Association for Rare Disorders	editor for patient report on on MAS	2014-2016
NIH NIDDK Special Emphasis Panel ZDK1-GRB-S(M1)	ad hoc reviewer	2015
Michigan Diabetes Research Center Pilot and Feasibility Study Grant Program	ad hoc reviewer	2017
Pediatric Endocrine Society	abstract reviewer	2017
Endocrine Society	Peds Endocrine associate editor Medscape/Endocrine Society Consults	2017

Invited Scientific Journal Peer Review
Journal of Diabetes and its Complications
American Journal of Physiology: *Endocrinology and Metabolism*
Molecular Endocrinology
Journal of Endocrinology and Diabetes Mellitus
Journal of Pediatric Surgery
Diabetes Technology and Therapeutics
Orphanet Journal of Rare Diseases
Endocrine Practice
Pediatrics

Scientific Reports
Endocrinology
Acta Diabetologica
Journal of Clinical Investigation
Diabetes
Diabetes Care
Diabetologia