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MicroRNAs miR-23a-3p, miR-23b-3p and miR-149-5p regulate the expression of pro-apoptotic BH3-only proteins DP5 and PUMA in human pancreatic beta cells.

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ABSTRACT

Type 1 Diabetes is an autoimmune disease leading to beta cell destruction. MicroRNAs (miRNAs) are small non-coding RNAs that control gene expression and organ formation. They participate in the pathogenesis of several autoimmune diseases, but the nature of miRNAs contributing to beta cell death in T1D and their target genes remain to be clarified.

We performed a miRNA expression profile on human islet preparations exposed to the cytokines IL-1β+IFN-γ. Confirmation of miRNAs and target genes modification in human beta cells was performed by real-time qPCR. Single stranded miRNAs inhibitors were used to block selected endogenous miRNAs. Cell death was measured by Hoechst/propidium iodide staining and activation of caspase-3.

Fifty-seven miRNAs were detected as modulated by cytokines. Three of them, namely miR-23a-3p, miR-23b-3p and miR-149-5p, were down-regulated by cytokines and selected for further studies. These miRNAs were found to regulate the expression of the pro-apoptotic Bcl-2 proteins DP5 and PUMA and consequent human beta cell apoptosis.

These results identify a novel cross-talk between a key family of miRNAs and pro-apoptotic Bcl-2 proteins in human pancreatic beta cells, broadening our understanding of cytokine-induced beta cell apoptosis in early T1D.

INTRODUCTION

Type 1 Diabetes (T1D) is a multifactorial autoimmune disease characterized by selective pancreatic beta cell destruction in the course of islet inflammation (insulitis), which is triggered by a complex "dialog" between the immune system and the target beta cells [1]. Many of the key steps of this dialog are regulated by candidate genes for T1D [2-4], in cross talk with environmental cues such as viral infections [5-7]. The inflammatory process is mediated by T cells (mostly CD8+ and, to a less extent, CD4+ lymphocytes) and macrophages [8-10]. These invading immune cells contribute to selective beta cells destruction via both cell-to-cell contact and through the local release of pro-inflammatory cytokines such as IL-1β, IFN-γ, TNF-α and IL-17A [1, 11, 12].

MicroRNAs (miRNAs) are a family of endogenous small non-coding RNAs with around 22 nucleotides in length. They bind to the 3' untranslated region (3'UTR) of target genes and inhibit gene expression by degrading and/or preventing translation of their target messenger RNAs [13] . MiRNAs play a crucial role in organ formation during embryogenesis, including pancreas development and beta cell differentiation [14]. Moreover, they display an important role in maintaining functional beta cell mass [15-17] and endocrine cell identity [18, 19] during adult life.

Several recent studies have indicated a role for miRNAs in the regulation of autoimmunity progression and diabetes development [20-23], including the regulation of inflammatory cytokine-mediated beta-cell dysfunction and death [24-26]. Additionally, there may be a link between miRNAs and regulation of T1D candidate genes [27] and beta cell responses to viral infection [28]. The ultimate mechanisms by which these miRNAs and their target genes regulate human beta cell dysfunction and death remain, however, to be clarified. Particularly, it remains unclear whether miRNAs, individually or as families, regulate the activity of the pro-apoptotic Bcl-2 family members that execute pancreatic beta cell death [1, 7]. Against

this background, we presently aimed to identify novel cytokine-modulated miRNAs in human pancreatic islets and, departing from these findings, to elucidate the pro-apoptotic pathways regulated by these miRNAs in the human beta cells. Our findings identified a novel family of miRNAs that regulate two key proteins involved in human beta cell apoptosis, namely DP5 and PUMA.

RESEARCH DESIGN AND METHODS

Culture of human islet cells and the human beta cell line EndoC-βH1. Human islets from 13 non diabetic donors were isolated in Pisa using collagenase digestion and density gradient purification [29] . The donors (7 men and 6 women) were 71 ± 3 years old and had a BMI of 25 ± 1 [kg/m^2] (Supplementary Table 1). Islets beta cell percentual content, as evaluated by immunofluorescence for insulin using a specific anti-insulin antibody (Supplementary Table 2) was 54 ± 3%. The islets were cultured at 6.1 mmol/l glucose as previously described [2, 30]. The human beta cell line EndoC-βH1 (kindly provided by Dr R. Scharfmann, University of Paris, France) [31] was cultured as previolsly described [12, 32].

Cell treatment. Both human islet cells and the EndoC-βH1 cells were exposed to the following cytokine concentrations, based on previous dose-response experiments performed by our group [30, 32, 33]: recombinant human IL-1β (R&D Systems, Abingdon, UK), 50 U/ml; recombinant human IFN-γ (Peprotech, London, UK), 1000 U/ml.

Taqman microRNA array profiling. Total RNA was isolated with the miRNeasy micro kit (Qiagen, Venlo, Netherlands). DNase digestion was performed using RNase-Free DNase kit (Qiagen) following the manufacturer's instructions. The quality of the extracted RNA was evaluated using a Bio Drop instrument (Isogen Life Science, Temse, Belgium). MiRNA expression profiling was performed using TaqMan Array Human MicroRNA Cards Panel A v2.1 (Life Technologies, Paysley, UK) which allowed us to evaluate the expression of 384 miRNAs. MiRNAs were reverse-transcribed using Megaplex RT primers Human Pool A v2.1. A total of 500 ng of RNA was used for each reaction, which included 1.33 µl of 10X Megaplex RT primers, 0.33 µl of 100 mM dNTPs, 1.33 µl of 10X RT buffer, 1.50 µl of 25mM $MgCl_2$, 0.17 µl of 20U/µl RNAse Inhibitor, 2.50 µl of 50U/µl Multiscribe Reverse Transcriptase and 0.33 µl H2O (all from Life Technologies). The product of this reaction was then incubated for 40 cycles at 16°Cx2min, 42°Cx1min, 50°Cx1sec and then at 85°Cx5min.

Then, 9 µl of synthesized cDNA were loaded in TaqMan Array Human MicroRNA cards following the manufacturer instructions. A ViiA7 Real Time PCR instrument was used to perform TaqMan Array Cards reaction runs (Applied Biosystems-Life Technologies).

RNA interference. The short interfering RNAs (siRNAs) and single-stranded miRNA inhibitors used in this study are described in Supplementary Table 3. The siRNAs targeting DP5 and PUMA have been previously validated, including comparison against a second siRNA causing similar biological effects [34]. The optimal concentration of siRNAs used for cell transfections (30 nmol/l) was previously established by our group [30, 35]. Single-stranded miRCURY LNA inhibitors (Exiqon, Vedbaek, Denmark) that specifically block endogenous miRNAs were used at a concentration of 120 nmol/l based our own dose response experiments (data not shown) and as described [25]. Allstar Negative Control siRNA (siCTRL) (Qiagen) was used as negative control in all experiments. This siCTRL does not affect beta cell gene expression or insulin release as compared with non-transfected cells [35]. Transient transfection was performed with Lipofectamine RNAiMAX lipid reagent (Invitrogen-Life Thecnologies) following the manufacturer's instruction. After 8h transfection, cells were cultured for a 48h recovery period before exposure to cytokines.

Assessment of cell viability. The percentage of viable, apoptotic and necrotic cells was determined by staining with the DNA-binding dyes propidium iodide (5 µg/ml; Sigma-Aldrich, Bornem, Belgium) and Hoechst dye 33342 (5 µg/ml; Sigma-Aldrich) as described [30, 36]. A minimum of 600 cells were counted for each experimental condition by two independent observers, with one of them unaware of sample identity. The agreement between findings obtained by the two observers was always >90%. In some cases, apoptosis was also confirmed by caspase 3 cleavage by Western blot (see below).

Measurement of miRNA and mRNA expression. 40 ng and 150 ng of total RNA was respectively reverse-transcribed for miRNA and mRNA detection. cDNA was generated with

the miScript II RT Kit (Qiagen) following the manufacturer's instruction for miRNAs detection or as previously described [9] for mRNA espression. 1 ng of cDNA was used as a template for Real-Time PCR reaction using the miScript SYBR Green PCR kit and appropriate miScript Primer Assay for individual miRNAs of interest (Qiagen), following the manufacturer's instruction. A Rotor-Gene Q (Qiagen) was used for both human islets and human EndoC-βH1 cells samples. All samples were run in duplicate and miRNA expression was normalized to the expression level of two small nucleolar RNAs (RNU6 and SNORD61) or miR-375-3p (an islet-specific miRNA whose expression is not modified by cytokine tratment; see Results below). MiRNA expression was determined using Relative Quantification (RQ) method ($RQ = 2^{-\Delta ct}$). 15 ng of cDNA was used as a template for real-time PCR for mRNA amplification using iQ SYBR Green Supermix on a LightCycler instrument (Roche-Diagnostic, Vilvoorde, Belgium) and on a Rotor-Gene Q (Qiagen), respectively for human islets and human EndoC-βH1 cells. The concentration of the gene of interest was calculated as copies per µl using the standad curve method [37] and gene expression values were corrected by the housekeeping gene β-actin, whose expression in human islets is not affected by cytokine treatment [30, 38]. The primers used in this study are provided in Supplementary Table 4.

Western blot. For Western blot, cells were washed with cold PBS and lysed using Laemmli Sample Buffer. Total proteine were extracted and resolved by 8-14% SDS-PAGE, transferred to a nitrocellulose membrane, and immunoblotted with the specific antibodies for the protein of interest (Supplementary Table 1) as described [30]. The densitometric values were normalized by the housekeeping proteins β-actin or α-tubulin.

Immunofluorescence. Double immunofluorescence for insulin and cleaved caspase-3 was performed on human dispersed islets after miR-23a-3p inhibition and cytokine treatment as previously described [39].

Luciferase assay. HeLa cells were transfected with dual luciferase reporter plasmids pEZXMT04-DP5-3'UTR (HmiT088427-MT06) together with a precursor expressing vector pEZX-miR-23a (HmiR0298MR04) (Genecopoeia, Rockville, USA). pEZX-miR scramble vector (CmiR-0001-MR04) was used as control. 125 ng of total DNA was transfected at a ratio of 1:50 (3'UTR:miRNA). HeLa cells were harvested 48h post-transfection and assayed using Dual Luciferase Reporter assay (Promega, Fitchburg, USA). Luciferase activity was measured using GLOMAX 20/20 luminometer (Promega).

Ethic statements. Human islet collection and handling were approved by the local Ethical Committee in Pisa, Italy.

Statistical analysis. Data are presented as mean values ± SEM or plotted as box plots, indicating lower quartile, median, and higher quartile, with whiskers representing the range of the remaining data points when the number of experiments is ≥ 4 for each conditions. Alternatively, when the number of experiments is < 4 data are represented as points indicating individual experiments. Comparisons were performed by two-tailed paired Student's *t*-test or by analysis of variance (ANOVA) followed by paired Student's *t*-test with Bonferroni correction, as indicated. A p value <0.05 was considered as statistically significant.

RESULTS

Cytokine treatment modifies miRNAs expression in human beta cells. To identify relevant miRNAs involved in cytokine-induced beta cell apoptosis and dysfunction, three independent human islets preparations were left untreated or treated for 48h in the presence of pro-inflammatory cytokines IL-1β+IFN-γ. A global miRNA expression profile was determined by TaqMan Human MicroRNA Array Cards (Figure 1 A). Data were exported using VIIA7 RUO software and then analysed using Expression Suite software v1.0.3 (Life Technologies). Only miRNAs with Ct<35, where the Ct is the threshold cycle to detect fluorescence for specific miRNAs, and with a high efficiency amplification plot were taken into consideration for subsequent analysis. 231 of 384 miRNAs analysed were considered as present in our samples. All raw data were normalized using three small nucleolar RNAs included in the card (RNU6, RNU44 and RNU48). Twenty-two miRNAs were found up-regulated in cytokine-exposed human islets (considering a cut-off value of fold change ≥ 2 *vs* untreated islets), whereas 35 miRNAs were found down-regulated after cytokine treatment (considering a cut-off value of fold change ≤ 0.75 *vs* untreated islets) (supplementary Table 5). Comparison of this list of cytokine-modulated miRNAs against available data on miRNA expression profile performed in purified human alpha and beta cells [19], indicates that 12 modified miRNAs, namely miR-101-5p, miR-129-5p, miR-145-5p, miR-149-5p, miR-154-5p, miR-193b-3p, miR-23b-3p, miR-27a-3p, miR-27b-3p, miR-361-5p, miR-494-5p, miR-654-3p, are enriched in beta cells, whereas 3 modified miRNAs, namely miR-146a-5p, miR-221-3p and miR-708-5p, are more expressed in alpha cells. Using the miRWalk database [40], the miRecords target prediction program [41] and based on the available literature [42], we initially selected for subsequent studies two miRNAs, namely miR-23a-3p and miR-23b-3p, targeting key genes potentially involved in beta cell death. Cytokine-induced down-regulation of miR-23a-3p and miR-23b-3p was confirmed in the same set of samples by

quantitative real-time PCR, using custom-designed primers (Figures 1B and 1C). Moreover, downregulation of additional miRNAs potentially involved in cell death, i.e. miR-149-5p, miR-221-3p and miR-27a-3p was also evaluated (Supplementary Figures 1B-D). Of note, there was no modification in the expression of miR-375-3p, a well-established islet-specific miRNA involved in pancreas development and maintenance of beta cell identity [17] (Supplementary figure 1A), while miR-146a-5p and miR-155-5p (Supplementary Figures 1E and 1F), two well-established pro-inflammatory miRNAs [24, 43] were up-regulated. Similar results were observed in additional human islets preparations (independent experiments from the array screening) (Supplementary Figure 2) and in insulin-producing human EndoC-βH1 cells (Supplementary Figure 3). There were, however, some differences between human islets and human EndoC-βH1 cells regarding miRNAs modulation. Specifically, the observed downregulation of miR-27a-3p in human islets (Supplementary Figure 1D and Supplementary Figure 2D) was not confirmed in EndoC-βH1 cells (supplementary Figure 3D), and there was a very low expression (Ct > 35 or undetectable) of miR-155-5p in EndoC-βH1 cells. These small differences observed between human islets and human EndoC-βH1 cells may be due at least in part to the cell heterogeneity within the pancreatic islet. Thus, comparison between the miRNA expression profile of whole human islets against FACS-enriched beta-cell population [44], indicate a higher miR-27a-3p and miR-155-5p expression in human islets as compared to enriched beta-cells [44], while there is a good similarity for most of the other miRNAs.

MiR-23a-3p and miR-23b-3p regulate the expression of pro-apoptotic Bcl-2 family members in human beta cells. We next investigated whether predicted target genes involved in beta cell death were regulated, by cytokine treatment, in an opposite manner as compared to miR-23a-3p and miR-23b-3p. The Ct values of expression of miR-23 family members under basal condition in human islets and human EndoC-βH1 cells were

respectively 18.3 ± 0.1 and 24.1 ± 0.1 for miR-23a-3p and 18.3 ± 0.1 and 19 ± 0.1 for miR-23b-3p (n=7). A decrease in miR-23a-3p and miR-23b-3p expression in human islets (Figures 2A and 2B) and in human EndoC-βH1 cells (Figures 3A and 3B) was paralleled by increased mRNA expression of PUMA and NOXA (Figures 2D and 2E) in human islets and DP5, PUMA, NOXA, BAX, BIM-total and BIM-small (Figures 3 C-H) in human EndoC-βH1 cells. These results support the hypothesis that pro-apoptotic Bcl-2 family members are regulated, directly or indirectly, by miR-23a-3p and miR-23b-3p in beta cells.

To elucidate the molecular mechanisms by which miR-23a-3p and miR-23b-3p regulate beta cell survival, single stranded miRNAs inhibitors were used to block these endogenous miRNAs. An 80% and 60% decrease of miR-23a-3p expression was respectively observed in human islets and in human EndoC-βH1 cells (Figures 4A and 4C) using a miR-23a-3p inhibitor, followed or not by cytokines exposure. A >90% decrease of miR-23b-3p expression was observed in human EndoC-βH1 cells using a miR-23b-3p inhibitor in both untreated and IL-1β+IFN-γ-treated cells (Figure 4D). Due to the close similarity between miR-23a-3p and miR-23b-3p, we observed an important cross-reaction between the inhibitors, which decreased expression of both miRNAs in parallel in human EndoC-βH1 cells (Figures 4C and 4D). Thus, findings with the individual inhibitors should be interpreted as mediated by inhibition of both miR-23a-3p and miR-23b-3p.

The miR-23a-3p inhibitor exacerbated basal apoptosis, as evaluated by nuclear dyes, in both human islets and human EndoC-βH1 cells (Figures 4B, 4E and Supplementary Figures 4A, and 4B, respectively). A similar increase in apoptosis was observed using the miR-23b-3p inhibitor in human EndoC-βH1 cells (Figure 4E and Supplementary Figures 4A and 4B). Cytokine-induced apoptosis was also augmented in both human islets and in human EndoC-βH1 cells using miR-23a-3p inhibitor (Figures 4B, 4E and Supplementary Figures 4A and 4B) and in EndoC-βH1 cells after exposure to the miR-23b-3p inhibitor (Figure 4E and

Supplementary Figures 4A and 4B). In line with these observations the inhibitors for miR-23a-3p and miR-23b-3p increased expression of cleaved caspase-3 in human EndoC-βH1 cells (Figures 4F and 4G), confirming apoptosis activation. Similar findings were observed by double immunofluorescence for insulin and cleaved caspase-3 in human islets after use of the miR-23a-3p inhibitor (Figure 5), indicating that at least part of the observed cell death in human islet cells takes place at the beta cell level, which is in line with the observed increase in cell death in the human beta cell line EndoC-βH1 cells (Figure 4B and Supplementary Figure 4).

Exposure of cells to the miR-23a-3p inhibitor increased mRNA expression of the pro-apoptotic Bcl-2 family members DP5, PUMA, BAX and BIM (Figures 6A, 6B, 6D and 6E, respectively), whereas inhibition of miR-23b-3p increased expression of DP5 and PUMA (Figures 6A and 6B) in human EndoC-βH1 cells following or not exposure to IL-1β+IFN-γ. No increase in NOXA was observed after inhibition of miR-23a-3p or miR-23b-3p (Figure 6C). A mild increase of protein expression of DP5 and PUMA was observed after the use of miR-23a-3p and miR-23b-3p inhibitors in human EndoC-βH1 cells (Supplementary Figures 5A-B and 5C-D, respectively), whereas no modification in BAX and BIM protein expression after miR-23 family inhibition was detected (data not shown). On the other hand, there was no change in expression of the anti-apoptotic Bcl-2 family members Mcl-1 and BCL-XL or in APAf-1 after the use of miR-23a-3p or miR-23b-3p inhibitors in human EndoC-βH1 cells following or not exposure to IL-1β+IFN-γ (data not shown). Taken together, these results suggest that miR-23a-3p and miR-23b-3p regulate beta cells apoptosis by modulating expression of key pro-apoptotic Bcl-2 protein family members.

In order to evaluate if DP5 is a direct target of miR-23a-3p HeLa cells were transfected with a luciferase construct containing the 3'UTR sequence of DP5 (supplementary Figure 6A). MiR-23a-3p did not decrease the luciferase activity in cells transfected with the construct

containing 3' UTR of DP5, suggesting an indirect effect of miR-23a-3p on DP5 modulation. We next evaluated whether depletion of the miR-23 family up-regulated expression of c-Jun, a key regulator of DP5 expression in beta cells [45]. Inhibition of miR-23a-3p and miR-23b-3p increased by >16-fold c-JUN mRNA expression in human EndoC-βH1 cells under basal condition or following exposure to IL-1β+IFN-γ (Figure 6F). Interestingly, analysis on whether miR-23 family members target negative regulators of c-JUN mRNA expression, identified ARNT, HDAC4, MEF2C, NCOA2, TNFAIP3, ZNF382 and ZNF384 as predicted targets of miR-23 family.

Knock-down of PUMA or DP5 protects human EndoC-βH1 cells from cytokine-induced apoptosis in the context of miR-23a-3p inhibition. To determine whether the observed effects of miR-23a-3p on pro-apoptotic Bcl-2 proteins is causally related to beta cell death, we next knocked down PUMA or DP5 in combination with the miR-23a-3p inhibitor. This abrogated miR-23a-3p inhibitor-dependent induction of PUMA and DP5 (Figures 7A and 7B, respectively) in both untreated and cytokine-treated human EndoC-βH1 cells; the inhibition of miR-23a-3p was confirmed by real-time qPCR (Figures 7C and 7D). Importantly, blocking PUMA and DP5 induction in parallel to miR-23a-3p inhibition also prevented the increase in apoptosis secondary to depletion of this miRNA (Figures 7E and 7F), indicating that up-regulation of these two BH3-only proteins is part of the mechanisms by which a cytokine-induced miR-23a-3p decrease contributes to apoptosis of human pancreatic beta cells. This, and the above-described activation of caspase-3, indicates that miR-23a-3p regulates the intrinsic or mitochondrial apoptotic pathway in human beta cells.

To investigate whether additional miRNAs contribute to cytokine-induced apoptosis mediated by Bcl-2 family members, we studied miR-149-5p, another miRNA observed as downregulated by cytokines in our initial screening (Supplementary Figures 1B and Supplementary Table 5). MiRNA target prediction programs indicated miR-149-5p as a

possible modulator of the Bcl-2 family members PUMA and BIM. Using a miR-149-5p inhibitor, there was a 60-80% decrease of miR-149-5p expression in human EndoC-βH1 cells exposed or not to cytokines (Supplementary Figure 7A). MiR-149-5p depletion in both untreated and cytokine-treated human EndoC-βH1 cells increased apoptosis, as evaluated by nuclear dyes (Supplementary Figure 7B). Furthermore, miR-149-5p inhibition led to up-regulation of DP5, PUMA and BAX (Supplementary Figures 7C, 7D and 7F) and a mild modulation of BIM (Supplementary Figure 7G), whereas no modification in Mcl-1 and BCL-XL was observed (data not shown). This supports the concept that individual genes can be regulated by different miRNAs and that several miRNAs can participate in the regulation of key genes [13], such as the ones involved in beta cell death. We next knocked down PUMA or DP5 in combination with the miR-149-5p inhibitor. This abrogated miR-149-5p inhibitor-dependent induction of PUMA and DP5 (Supplementary Figures 8A and 8B, respectively) in both untreated and cytokine-treated human EndoC-βH1 cells; the inhibition of miR-149-5p was confirmed by real-time qPCR (Supplementary Figures 8C and 8D). Importantly, blocking PUMA and DP5 induction in parallel to miR-149-5p inhibition also prevented the increase in apoptosis secondary to depletion of this miRNA (supplementary Figures 8E and 8F), as we previously observed for miR-23a-3p (Figures 7E and 7F).

DISCUSSION

A significant reduction of functional beta cell mass as a consequence of increased beta cell death is the key feature of T1D. During the early stages of the disease, pancreatic islets are exposed to pro-inflammatory mediators released by the invading immune cells. Available information, mostly from animal models and from *in vitro* experiments, suggests that this results into changes in the expression of key beta cell gene networks involved in beta cell function and phenotype, induction of ER stress and inflammation [1, 46, 47]. Locally produced pro-inflammatory mediators recruit further immune cells that enhance release of chemokines and cytokines, leading to amplification of the inflammatory process. Prolonged cytokine exposure and the consequent persistent ER stress, possibly in combination with environmental factors such as viral infections, may activate downstream pathways such as JNK phosphorylation and the transcription factors NF-κB and STAT1, eventually triggering expression of pro-apoptotic Bcl-2 proteins, cleavage and activation of caspases and cell death [1, 7, 48]. The fine regulation of this process, particularly in human beta cells, remains to be clarified.

The Bcl-2 family members can be subdivided into three different groups: anti-apoptotic (i.e. Bcl-2, Bcl-XL, Mcl-1 and A1), pro-apoptotic (i.e. BAX, BAK and BOK) Bcl-2 proteins and pro-apoptotic BH3-only proteins [48]. Additionally, the BH3-only proteins can be divided into two subgroups: the sensitizers (i.e. DP5, BAD and NOXA) and the activators (i.e. BID, BIM and PUMA) of apoptosis [48, 49]. It has been previously shown that PUMA and DP5, two of the most important regulators of the intrinsic apoptotic pathway, are induced in beta cells by exposure to the cytokines IL-1β+IFNγ or to chemical ER stressors, leading to BAX translocation to the mitochondria, caspase-3 activation and beta cell death [45, 50].

MiRNAs are key regulators of gene expression and some have been shown to sensitize beta cells to cytokine-induced apoptosis [24, 25]. Thus, prolonged exposure of mouse islets to IL-

1β or to TNF-α results in up-regulation of miR-146a-5p, miR-34a-5p and miR-21-5p [24]. Furthermore, cytokine-induced up-regulation of miR-101a-3p and miR-30b-5p decreases expression of anti-apoptotic Bcl-2 proteins in the mouse insulin producing cell line MIN6 [26], whereas suppression of Bcl-2 upon miR-34a-5p induction contribute to palmitate-induced apoptosis in MIN6 cells [51]. In line with these observations, cytokines up-regulate miR-29-5p which exacerbates cytokine-induced apoptosis via inhibition of the anti-apoptotic Bcl-2 protein Mcl-1 [25]. On the other hand, the possible contribution of miRNAs to the cytokine-induced up-regulation of pro-apoptotic BH3-only proteins in beta cells remains to be clarified. Against this background, we presently performed a global miRNA expression profile on three independent human islets preparations treated or not with the cytokines IL-1β+IFN-γ. Twenty-two and thirty-five miRNAs were found respectively up- and down-regulated in human islets after IL-1β+IFN-γ treatment. Of particular interest, these cytokines induced a significant down-regulation of miR-23a-3p, miR-23b-3p and miR-27a-3p, all belonging to the same miRNA family. MiR-23b-3p is also downregulated in islets from 4- and 8-weeks NOD mice [25]. Downregulation of miR-23a-3p and miR-27a-3p in post-traumatic brain injury contributes to neuronal cell death by directly up-regulating the pro-apoptotic Bcl-2 family members BAX, NOXA and PUMA [42], suggesting that this may be an important mechanism by which cytokines up-regulate these key pro-apoptotic Bcl-2 family members. To test this hypothesis, miR-23a-3p and miR-23b-3p were inhibited in human beta cells. Since miR-23a-3p and miR-23b-3p are very similar and differ by only 1 nucleotide in their 3' ends, which is less important for microRNA action than the 5' end, the inhibitors of miR-23a-3p and miR-23b-3p utilized led to inhibition of both miRNAs. Thus, all data obtained based on these inhibitors should be interpreted as indicating broad effects of the miR-23 family. MiR-23 inhibition increased apoptosis under both basal condition and following cytokine treatment. This was paralleled by up-regulation of several BH3-only

proteins, with the most consistent findings observed for DP5 and PUMA. Importantly, knocking down PUMA or DP5 reverted the pro-apoptotic effects of miR-23a-3p inhibition, suggesting that these two BH3-only proteins are regulated in an indirect way by pro-inflammatory cytokines, i.e. via the inhibition of miRNAs of miR-23 family. Using HeLa cells we did not observe a direct interaction between the miR-23 family and DP5 (for technical reasons these experiments could not be performed in human EndoC-βH1 cells). This suggests the possibility of an indirect effect of these miRNAs on DP5 via up-regulation of c-Jun (present data), a transcription factor previously shown by us to regulate DP5 expression [45]. It remains to be determined how miR-23 cross-talk with PUMA. Of note, the results described above show additive effects of PUMA/DP5 and miR-23 in that they both affect apoptosis, and the BH3-only proteins seem to be regulated downstream of the miRNAs. It remains to be proven, however, that these signals interact and the ultimate mechanisms involved. The present data should be thus interpreted with caution.

The fact that these novel observations were obtained in human islets and in a human beta cell line increases their potential relevance to human type 1 diabetes. Additional miRNAs targeting Bcl-2 family members, such as miR-211-3p and miR-149-5p, were also found down-regulated in our screening, suggesting a more complex crosstalk between miRNAs and pro-apoptotic Bcl-2 family members in beta cells. In line with this, inhibition of miR-149-5p also led to up-regulation of DP5, PUMA and BAX. An overview of this complex cross talk is provided in Figure 8.

In conclusion, the present results identify a potential novel cross-talk between a family of miRNAs and key pro-apoptotic Bcl-2 proteins in human pancreatic beta cells, providing a new mechanistic understanding of inflammation-induced beta cell apoptosis.

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AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: FAG, GS, RR, PM, FD and DLE. Acquired data: FAG, GS, JJM, OV, LM, LL, KT and MB. Supervised the study and contribute to reagent: PM, FD and DLE. Wrote the paper: FAG and DLE. All authors revised the manuscript. DLE is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Duality of interest

The authors declare that there is no duality of interest associated with this study.

LEGENDS TO THE FIGURES

Figure 1. MiRNA profile of human islets exposed or not to pro-inflammatory cytokines. Human islets (A-C) were left untreated or treated with IL-1β+IFN-γ for 48h. MiRNAs were then isolated and miRNA expression profiling was performed using TaqMan Array Human MicroRNA Cards Panel A v2.1 as described in Material and Methods. (A) Dendogram of the global miRNA expression profile. Columns correspond to individual samples exposed or not to cytokines, whereas lines correspond to the individual miRNAs analysed. The scale colour from blue to red corresponds to the abundance of miRNA expression reported as normalized dCT (blue: dCT value = -2.18; Red: dCT value = 18). All raw data were normalized using three small nucleolar RNAs included in the card (RNU6, RNU44 and RNU48). Expression of miR-23a (B) and miR-23b (C) was assayed in the same samples by RT-PCR and normalized by two different small nucleolar RNAs (RNU6 and RND61). The results are from 3 independent human islets preparations (A-C).

Figure 2. Cytokines co-regulate the expression of miR-23a-3p, miR-23b-3p, NOXA and PUMA in human pancreatic islets. Human islets were left untreated or treated with IL-1β+IFN-γ for 48h. Expression of miR-23a-3p (A) and miR-23b-3p (B) was assayed by RT-PCR and normalized by two different small nucleolar RNAs (RNU6 and RND61). Expression of DP5 (C), PUMA (D), NOXA (E), BAX (F), BIM-total (G) and BIM-small (BIMs) (H) were assayed by RT-PCR and normalized by the housekeeping gene β-actin. Results are represented as box plot, indicating lower quartile, median, and higher quartile, with whiskers representing the range of the remaining data points (A-H). * $p<0.05$ and ** $p<0.01$ *versus* untreated cells; paired Student's *t*-test. Data are shown as mean ± SEM of 4-7 independent experiments.

Figure 3. Cytokines co-regulate miR-23a-3p, miR-23b-3p and pro-apoptotic Bcl-2 family members in human EndoC-βH1 cells. Human EndoC-βH1 cells were left untreated or treated with IL-1β+IFN-γ for 48h. Expression of miR-23a-3p (A) and miR-23b-3p (B) was assayed by RT-PCR and normalized by two different small nucleolar RNAs (RNU6 and RND61). Expression of DP5 (C), PUMA (D), NOXA (E), BAX (F), BIM-total (G) and BIM-small (BIMs) (H) were assayed by RT-PCR and normalized for the housekeeping gene β-actin. Rresults are represented as box plot, indicating lower quartile, median, and higher quartile, with whiskers representing the range of the remaining data points (A-H). * $p<0.05$,

** p<0.01 and *** p<0.001 *versus* untreated cells; paired Student's *t*-test. Data are shown as mean ± SEM of 7 independent experiments.

Figure 4. Inhibition of miR-23a-3p and miR-23b-3p exacerbates apoptosis in human islets and in human EndoC-βH1 cells. Dispersed human islets (A and B) and human EndoC-βH1 cells (C-G) were transfected with siControl (CTRL) or with anti-miRNAs targeting miR-23a-3p (human islets and EndoC-βH1 cells) or miR-23b-3p (EndoC-βH1 cells) for 8h. After 48h recovery, cells were left untreated or treated with IL-1β+IFN-γ for 48h as indicated. Expression of miR-23a-3p (A and C) and miR-23b-3p (D) were assayed by RT-PCR and normalized by two different small nucleolar RNAs (RNU6 and RND61) (A) or by miR-375-3p expression (C and D). Apoptosis was evaluated by Hoechst / propidium iodide staining after 48h of cytokine treatment (B and E). Cleaved caspase-3 and β-actin protein expression were evaluated by western blot after 48h of cytokine treatment; one representative blot of five independent experiments is shown (F). The optical density quantification of cleaved caspase-3 (as shown in F) was quantified and corrected by β-actin expression (G). Results are represented as box plot, indicating lower quartile, median, and higher quartile, with whiskers representing the range of the remaining data points (A-E). In (G) results were normalized against the highest value in each independent experiment, considered as 1. * p<0.05, ** p<0.01 and *** p<0.001 *versus* siCTRL untreated cells; § p<0.05, §§§ p<0.001, # p<0.05 and ## p<0.01 as indicated by bars; ANOVA followed by paired Student's *t*-test with Bonferroni's correction. Data are shown as mean ± SEM of 5-7 independent experiments.

Figure 5. Inhibition of miR-23a-3p augments cleaved caspase-3 expression in human beta cells. Human dispersed islets were transfected with siControl (CTRL) (A-D and I-L) or a anti-miRNA targeting miR-23a-3p (E-H and M-P) for 8h. After 48h recovery, cells were left untreated (A-H) or treated with IL-1β+IFN-γ (I-P) for 48h. After cytokine treatment, cells were fixed and used for histological studies. Fluorescent microscopy analysis of insulin (A, E, I, and M in green) and cleaved caspase-3 (F, J and N in red) shows the presence of double-positive cells for insulin and cleaved caspase-3 (D, H, L and P merged panels in yellow). Hoechst staining (C, G, K and O in blue) shows the presence of nuclear condensation in the apoptotic cleaved-caspase-3 positive cells (G, K and O). Double-positive cells for insulin and cleaved caspase-3 are indicated by the arrows (E, F, H, I, J, L, M, N, P).

Figure 6. Inhibition of miR-23a-3p and miR-23b-3p increases expression of pro-apoptotic Bcl-2 family members in human EndoC-βH1 cells. EndoC-βH1 cells were

transfected with siControl (CTRL) or with anti-miRNAs targeting miR-23a-3p and miR-23b-3p for 8h. After 48h recovery, cells were left untreated or treated with IL-1β+IFN-γ for 48h as indicated. Expression of DP5 (A), PUMA (B), NOXA (C), BAX (E), BIM (E) and c-JUN (F) were assayed by RT-PCR and normalized by the housekeeping gene β-actin. Results are represented as box plot, indicating lower quartile, median, and higher quartile, with whiskers representing the range of the remaining data points (A-F). * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ *versus* siCTRL untreated cells; § $p<0.05$, §§ $p<0.01$, §§§ $p<0.001$, # $p<0.05$, ## $p<0.01$ and ### $p<0.001$ as indicated by bars; ANOVA followed by paired Student's *t*-test with Bonferroni's correction. Data are shown as mean ± SEM of 6-7 independent experiments.

Figure 7. KD of PUMA or DP5 protects human EndoC-βH1 cells against cytokine-induced apoptosis in the context of miR-23a-3p inhibition. EndoC-βH1 cells were transfected with siControl (CTRL), siPUMA, anti-miR-23a-3p or co-transfected with siPUMA+anti-miR-23a-3p (A, C and E) for 8h. After 48h recovery, cells were left untreated or treated with IL-1β+IFN-γ for 48h as indicated. The KD of PUMA (A) was confirmed by RT-PCR and normalized by the housekeeping gene β-actin. Expression of miR-23a-3p (C) was assayed by RT-PCR and normalized by miR-375-3p expression. Apoptosis was evaluated by propidium iodide / Hoechst staining after 48h of cytokine treatment (E). EndoC-βH1 cells were transfected with siControl (CTRL), siDP5, anti-miR-23a-3p or co-transfected with siDP5+anti-miR-23a-3p (B, D and F) for 8h. After 48h recovery, cells were left untreated or treated with IL-1β+IFN-γ for 48h as indicated. The KD of DP5 (B) was confirmed by RT-PCR and normalized by the housekeeping gene β-actin. Expression of miR-23a-3p (D) was assayed by RT-PCR and normalized by miR-375-3p expression. Apoptosis was evaluated by propidium iodide / Hoechst staining after 48h of cytokine treatment (F). Results (A and B) were normalized against the highest value in each independent experiment, considered as 1. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ *versus* siCTRL untreated cells; § $p<0.05$, §§ $p<0.01$ and §§§ $p<0.001$ versus siCTRL cytokine-treated cells; # $p<0.05$, ## $p<0.01$, ### $p<0.001$ and @@@ $p<0.001$ as indicated by bars; ANOVA followed by paired Student's *t*-test with Bonferroni's correction. Data are shown as mean ± SEM of 5 independent experiments.

Figure 8. Proposed model for the cytokine-induced cross-talk between miRNAs and pro-apoptotic Bcl-2 proteins in beta cells. The pro-inflammatory cytokines IL-1β+IFN-γ down-regulate expression of miR-23a-3p, miR-23b-3p and miR-149-5p in beta cells.

Decreased expression of these inhibitory miRNAs leads to increased mRNA expression of key pro-apoptotic Bcl-2 proteins. Specifically, miR-23a-3p and miR-23b-3p modulate the BH3-only sensitizer DP5 (at least in part via up-regulation of c-JUN) and the BH3-only activator PUMA, whereas miR-23a-3p regulates both the BH3-only sensitizer BIM and the pro-apoptotic Bcl-2 protein BAX. Additionally, inhibition of miR-149-5p expressions results in increased mRNA expression of both DP5 and PUMA. This complex interaction between miRNAs, transcription factors and downstream pro-apoptotic proteins leads to cleavage and activation of caspase-3 and, subsequently, to beta cell apoptosis.

Figure 1. MiRNA profile of human islets exposed or not to pro-inflammatory cytokines.

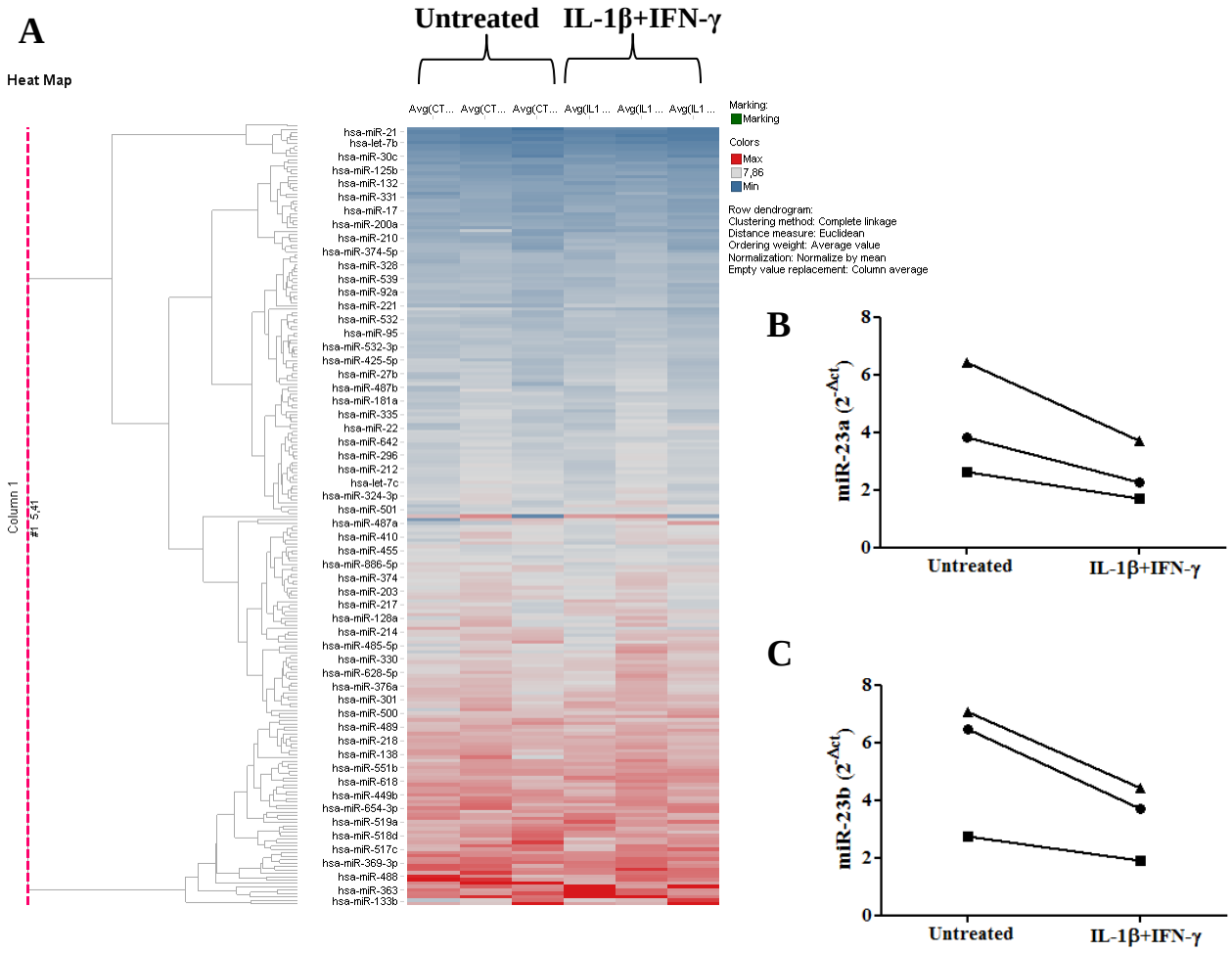


Figure 2. Cytokines co-regulate the expression of miR-23a-3p, miR-23b-3p, NOXA and PUMA in human pancreatic islets.

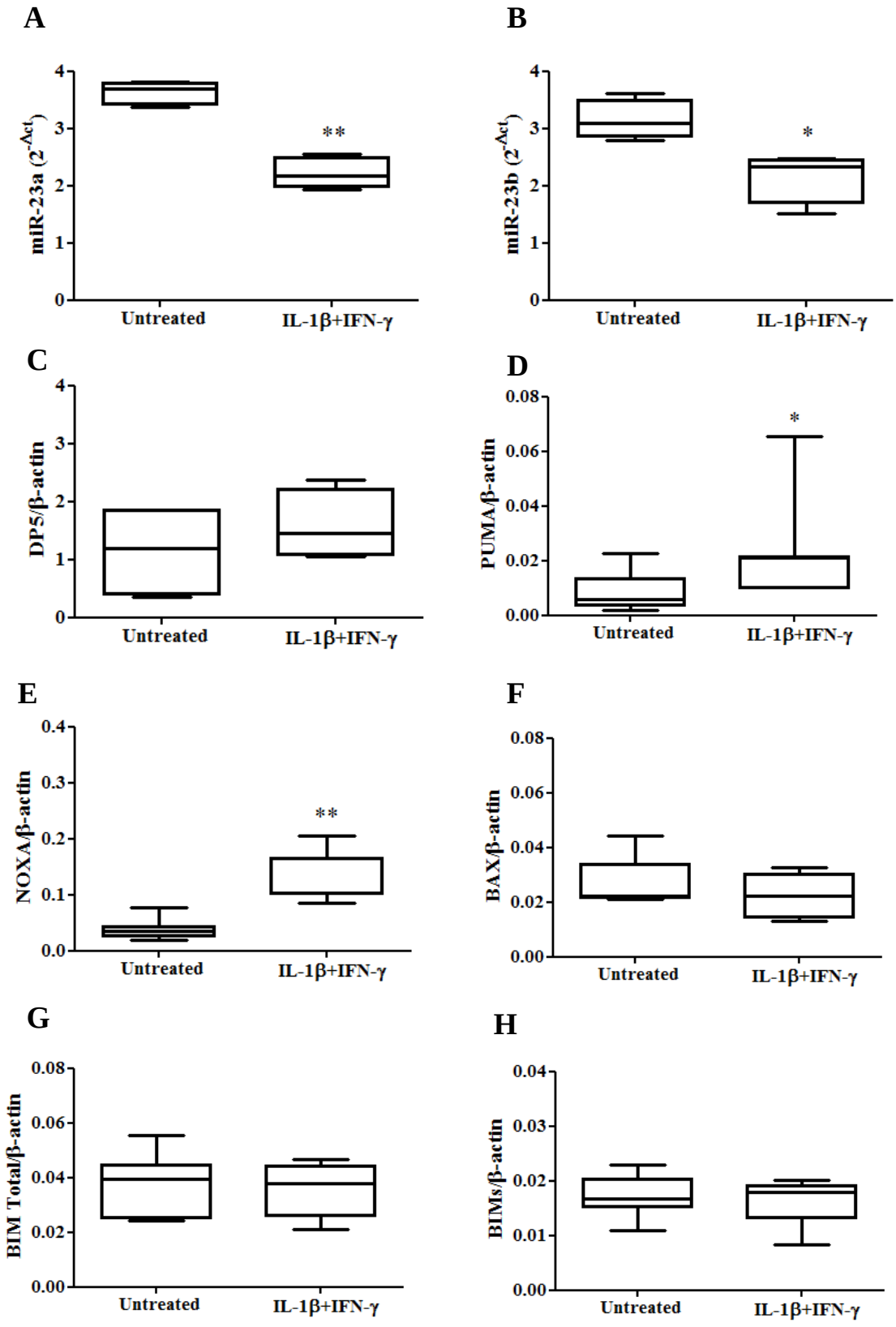


Figure 3. Cytokines co-regulate miR-23a-3p, miR-23b-3p and pro-apoptotic Bcl-2 family members in human EndoC-βH1 cells.

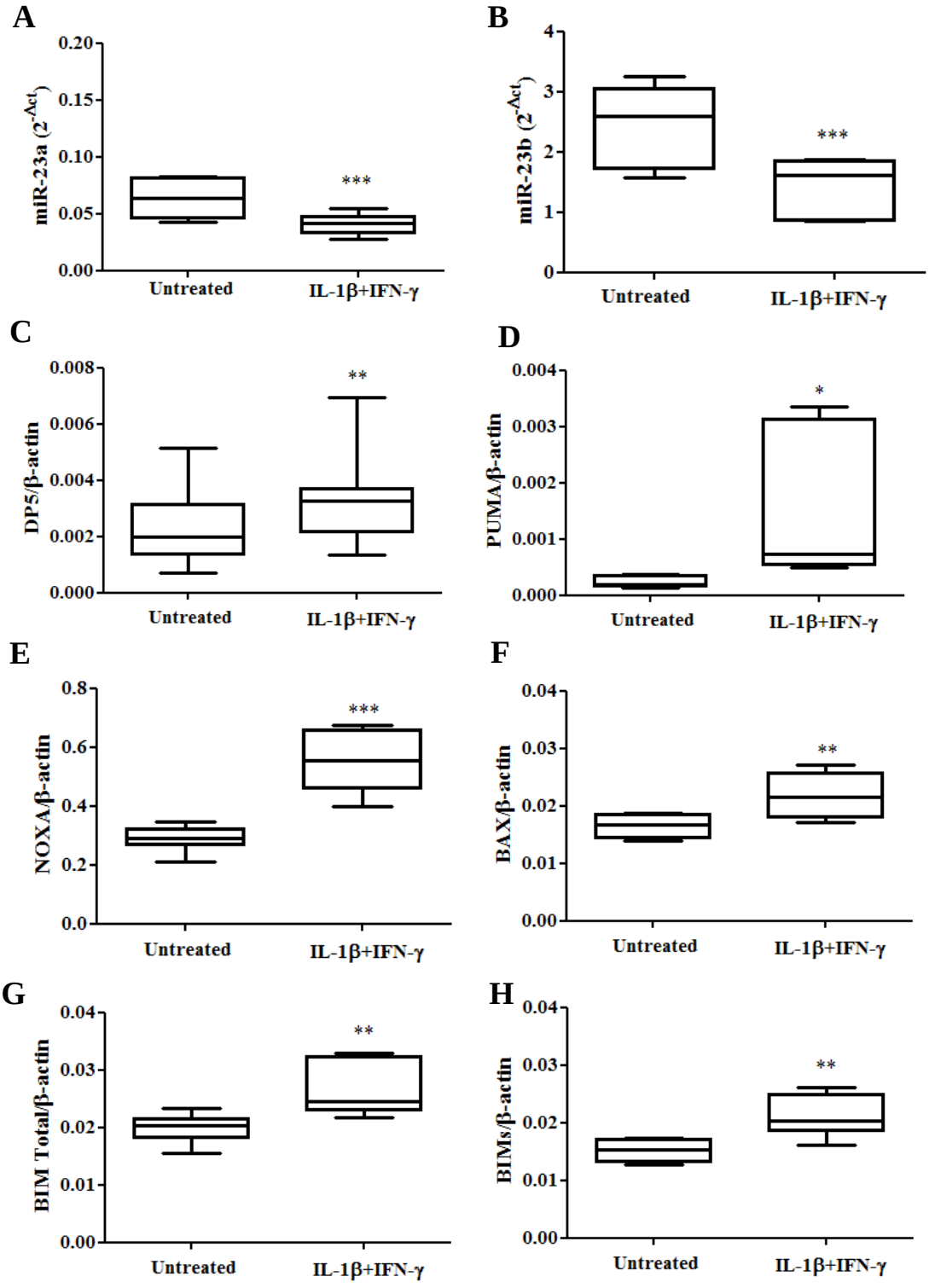


Figure 4. Inhibition of miR-23a-3p and miR-23b-3p exacerbates apoptosis in human islets and in human EndoC-βH1 cells.

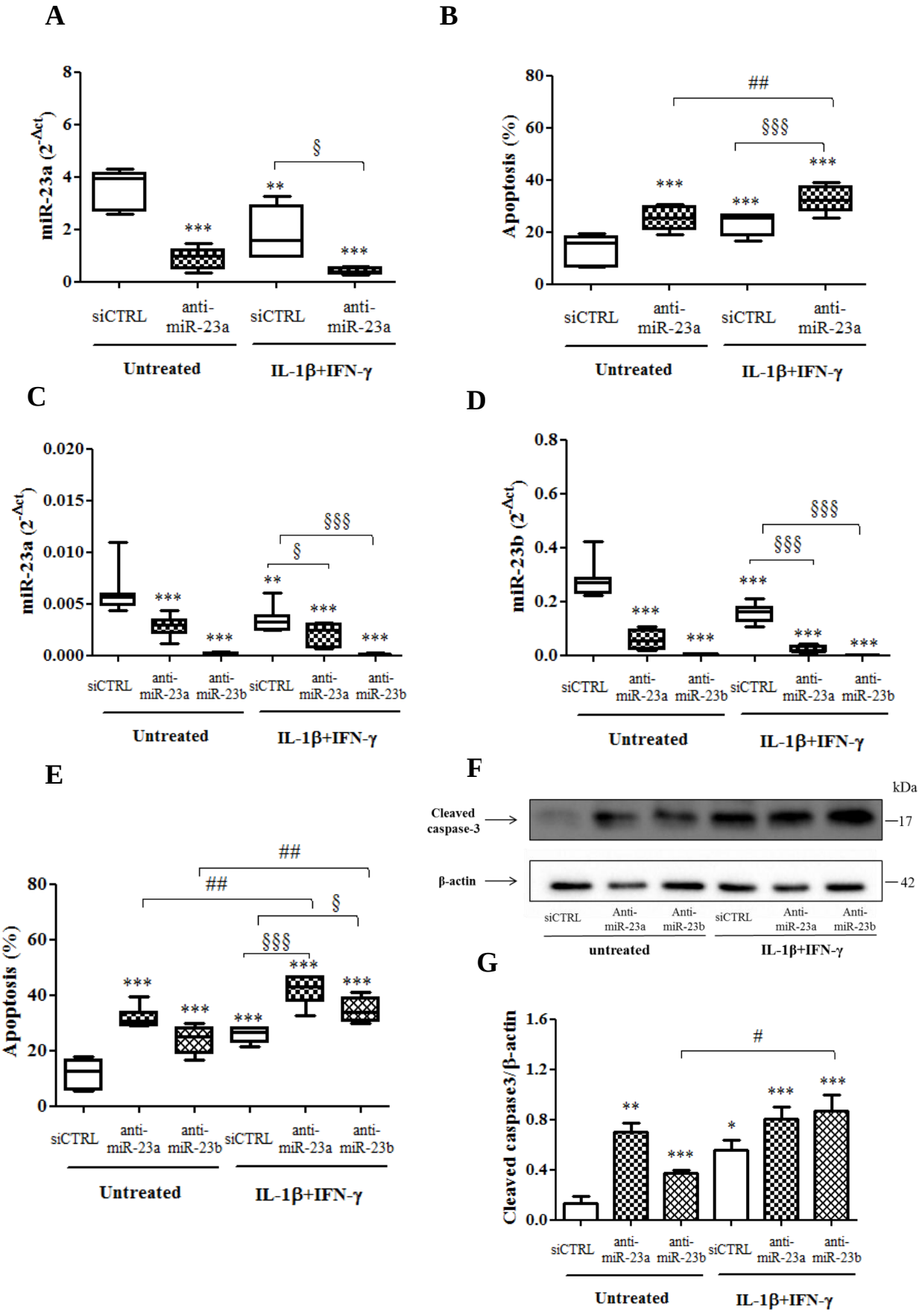
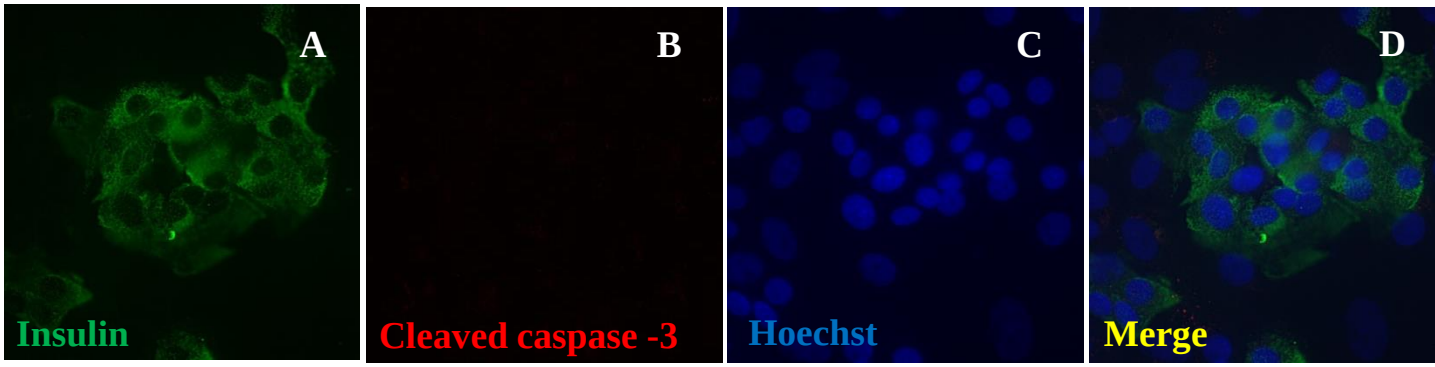
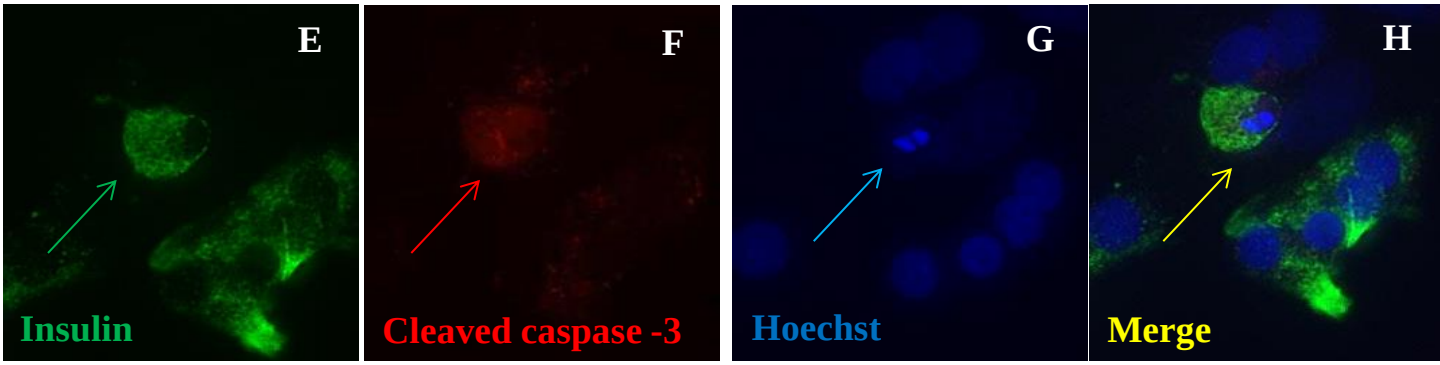


Figure 5. Inhibition of miR-23a-3p augments cleaved caspase-3 expression in human beta cells.



siCTRL untreated



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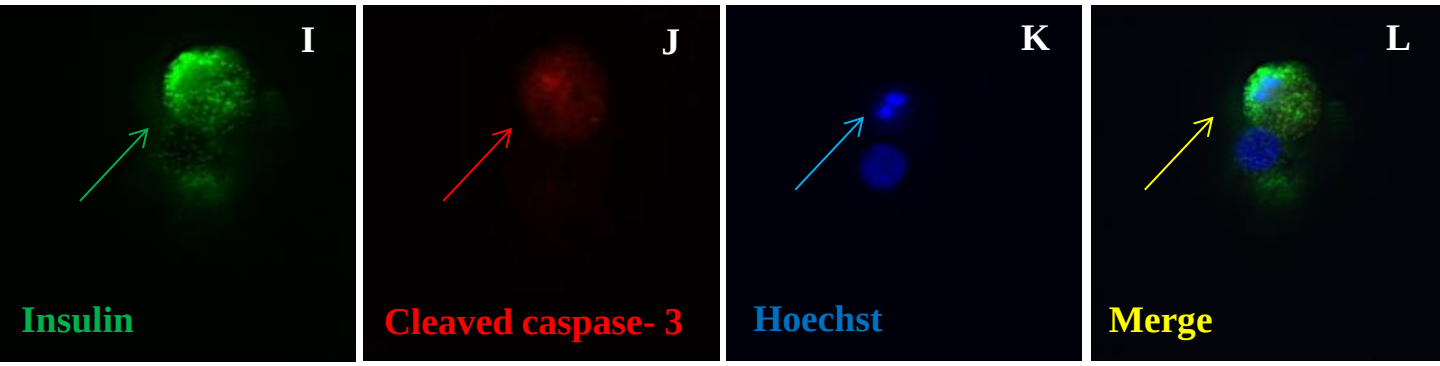
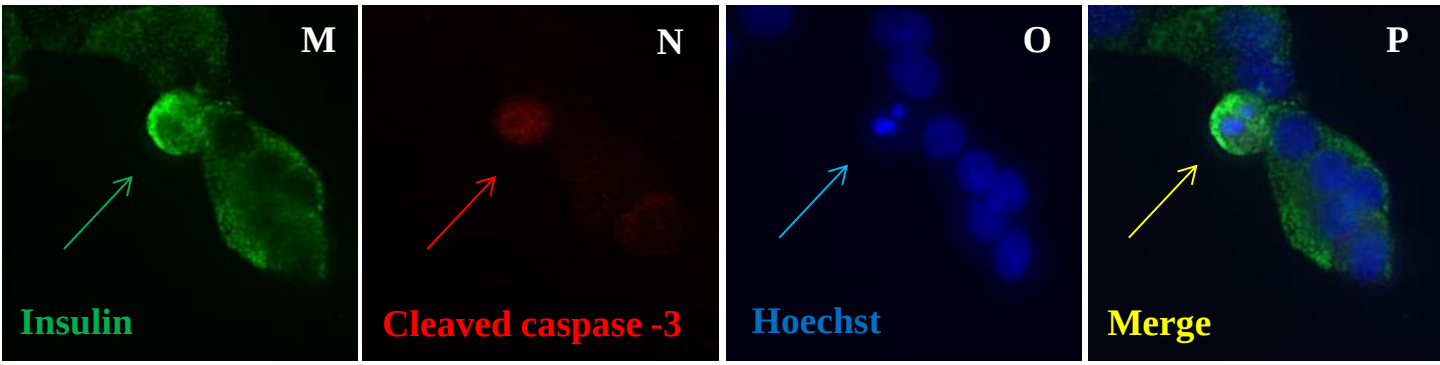
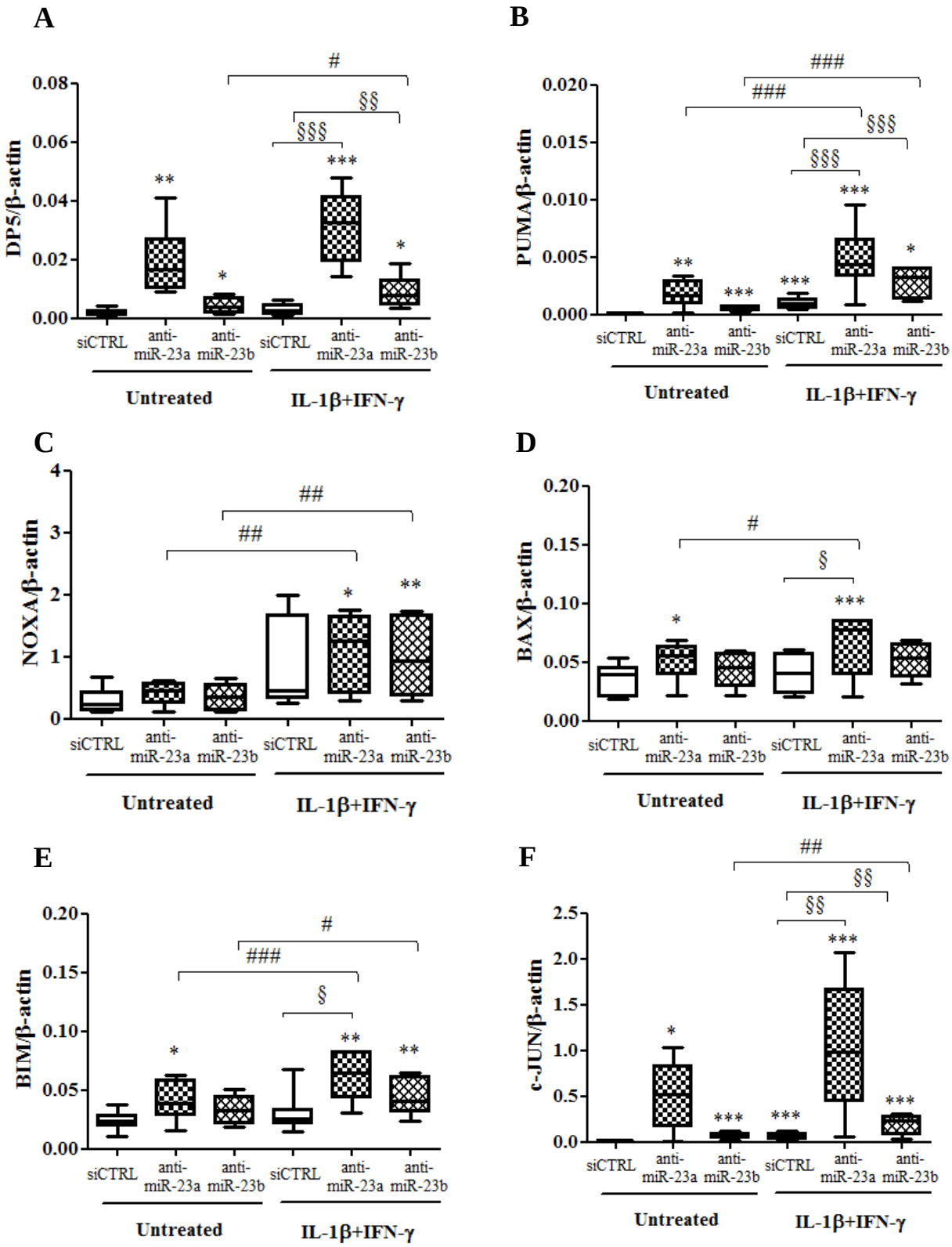


siCTRL IL-1β+IFN-γ



anti-miR-23a IL-1β+IFN-γ

Figure 6: Inhibition of miR-23a-3p and miR-23b-3p increases expression of pro-apoptotic Bcl-2 family members in human EndoC-βH1 cells.



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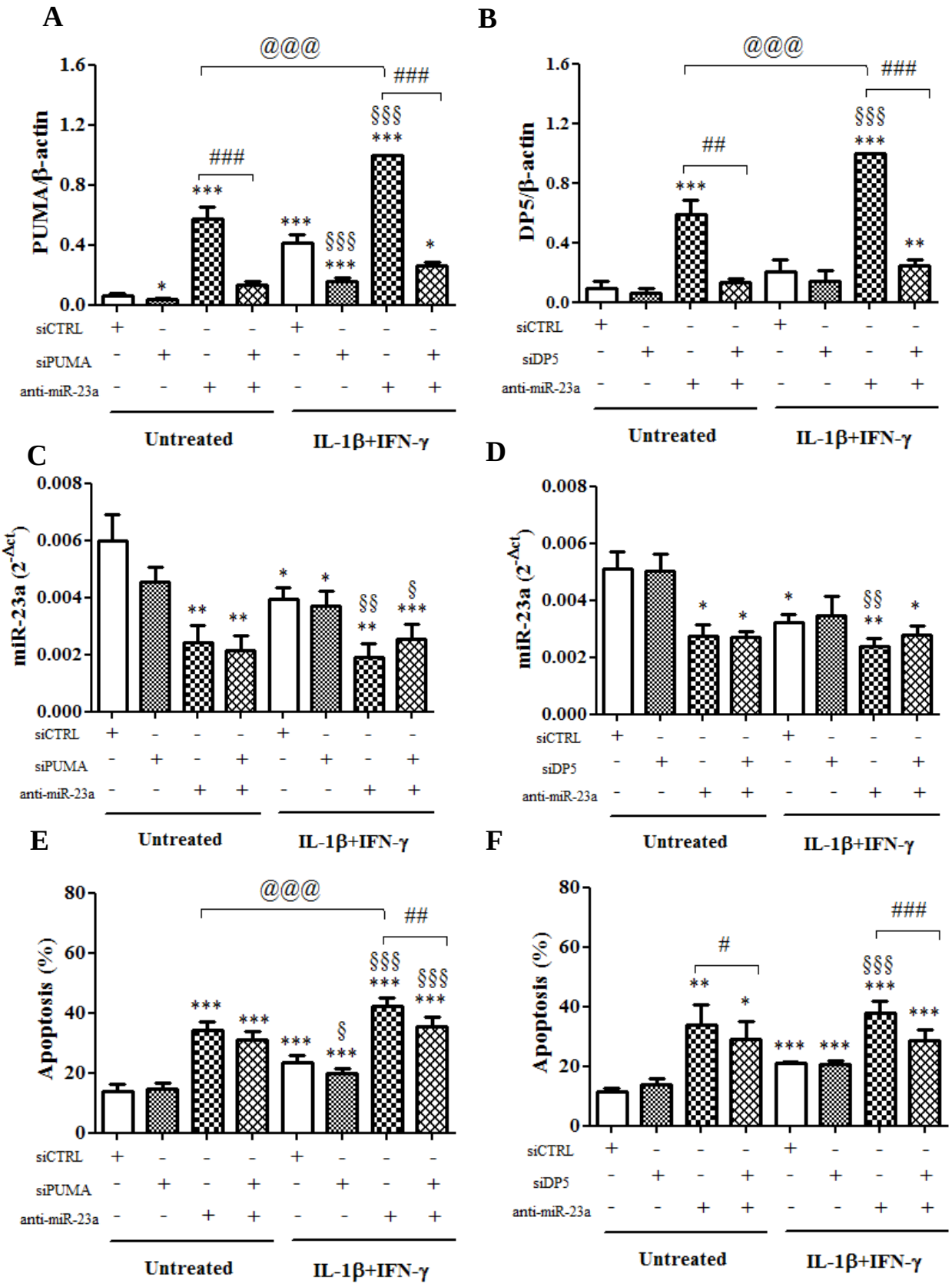
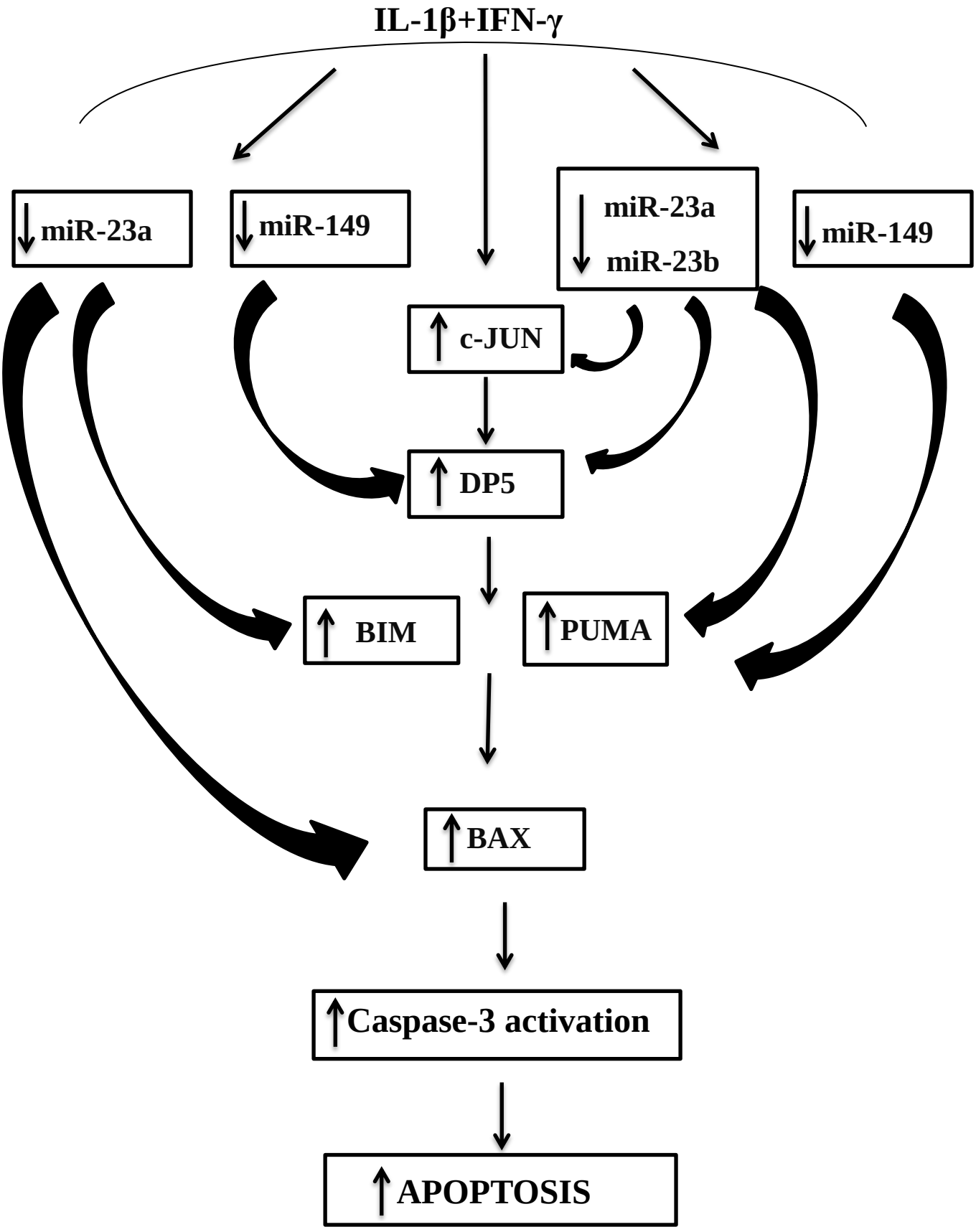


Figure 8. Proposed model for the cytokine-induced cross-talk between miRNAs and pro-apoptotic Bcl-2 proteins in beta cells.



LEGENDS TO THE SUPPLEMENTARY FIGURES

Supplementary Figure 1. Confirmation by RT-PCR of miRNAs detected as cytokine-modulated by micro-array. Human islets were left untreated or treated with IL-1β+IFN-γ for 48h (similar preparations and experimental conditions as in Figure 1). Expression of miR-375-3p (A), miR-149-5p (B), miR-221-3p (C), miR-27a-3p (D), miR-146a-5p (E) and miR-155-5p (F) were assayed by RT-PCR and normalized by two different small nucleolar RNAs (RNU6 and RND61). Results shown are from 3 independent human islets preparations (A-F).

Supplementary Figure 2. Confirmation by RT-PCR in independent samples of miRNAs detected as cytokine-modulated by micro-array. Human islets were left untreated or treated with IL-1β+IFN-γ for 48h. Expression of miR-375-3p (A), miR-149-5p (B), miR-221-3p (C), miR-27a-3p (D), miR-146a-5p (E) and miR-155-5p (F) were assayed by RT-PCR and normalized by two different small nucleolar RNAs (RNU6 and RND61). Results are represented as box plot, indicating lower quartile, median, and higher quartile, with whiskers representing the range of the remaining data points (A-F). * $p<0.05$ *versus* untreated cells; paired Student's *t*-test. Data are shown as mean ± SEM of 4 independent experiments respect to Figure 1 and Supplementary Figure 1.

Supplementary Figure 3. Cytokine-modulated miRNAs in the human beta cell line EndoC-βH1. Human EndoC-βH1 cells were left untreated or treated with IL-1β+IFN-γ for 48h. Expression of miR-375-3p (A), miR-149-5p (B), miR-221-3p (C), miR-27a-3p (D) and miR-146a-5p (E) were assayed by RT-PCR and normalized by two different small nucleolar RNAs (RNU6 and RND61). Results are represented as box plot, indicating lower quartile, median, and higher quartile, with whiskers representing the range of the remaining data points (A-E). ** $p<0.01$ and *** $p<0.001$ *versus* untreated cells; paired Student's *t*-test. Data are shown as mean ± SEM of 7 independent experiments.

Supplementary Figure 4. Inhibition of miR-23a-3p and miR-23b-3p exacerbates apoptosis in human EndoC-βH1 cells. Human EndoC-βH1 cells were transfected with siControl (CTRL) or with anti-miRNAs targeting miR-23a-3p or miR-23b-3p for 8h. After 48h recovery, cells were left untreated or treated with IL-1β+IFN-γ for 48h as indicated. Apoptosis was evaluated by Hoechst / propidium iodide staining after 48h of cytokine treatment (A). A representative image of stained cells, for each individual experimental condition, is shown in (B). Hoechst staining (a, c, e, g, i and k in blue) shows living cells; propidium iodide staining (b, d, f, h, j and l in red) shows dying cells. Results (A) are

represented as box plot, indicating lower quartile, median, and higher quartile, with whiskers representing the range of the remaining data points. *** $p<0.001$ *versus* siCTRL untreated cells; §§ $p<0.01$, §§§ $p<0.001$, # $p<0.05$ and ### $p<0.001$ as indicated by bars; ANOVA followed by paired Student's *t*-test with Bonferroni's correction. Data in (A) are mean ± SEM of 4 independent experiments.

Supplementary Figure 5. Inhibition of miR-23a-3p and miR-23b-3p modify expression of pro-apoptotic Bcl-2 family members in human EndoC-βH1 cells. EndoC-βH1 cells were transfected with siControl (CTRL) or with anti-miRNAs targeting miR-23a-3p and miR-23b-3p for 8h. After 48h recovery, cells were left untreated or treated with IL-1β+IFN-γ for 48h as indicated. DP5 (A), PUMA (C), and α-tubulin (A and C) protein expression were evaluated by western blot after 48h of cytokine treatment; one representative blot of six-eight independent experiments is shown (A and C). The optical density quantification of DP5 (as shown in A) and PUMA (as shown in C) was quantified and corrected by α-tubulin expression (B and D respectively). Results (B and D) were normalized against the highest value in each independent experiment, considered as 1. * $p<0.05$ and ** $p<0.01$ *versus* siCTRL untreated cells; § $p<0.05$ *versus* siCTRL cytokine-treated cells; paired Student's *t*-test. Data are shown as mean ± SEM of 6-8 independent experiments.

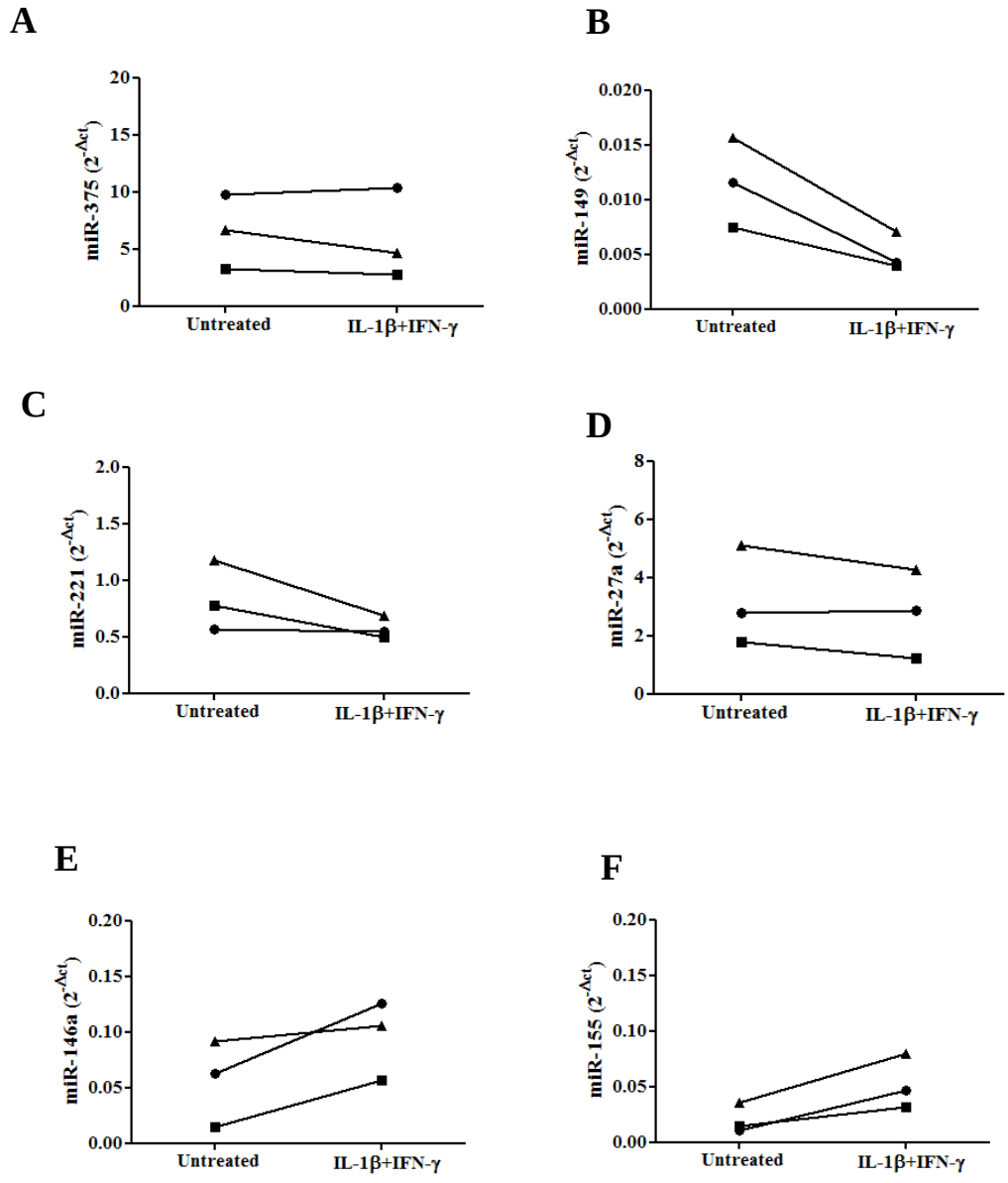
Supplementary Figure 6. DP5 is not a direct target of miR-23a-3p. HeLa cells were transfected with 3'UTR Firefly dual Luciferase reporter plasmids pEZXMT04-DP5-3'UTR together with precursor expressing vectors pEZX-miR-23a as indicated. pEZX-miR scramble vector was used as control. Firefly Luciferase activity was measured using GLOMAX 20/20 luminometer and divided by the Renilla luciferase activity to correct eventual differences in transfection efficiency. Data are shown as mean ± SEM of 9 independent experiments.

Supplementary Figure 7. Inhibition of miR-149-5p exacerbates apoptosis and up-regulates pro-apoptotic Bcl-2 family member expression in human EndoC-βH1 cells. EndoC-βH1 cells were transfected with siControl (CTRL) or with an anti-miRNA targeting miR-149-5p for 8h. After 48h recovery, cells were left untreated or treated with IL-1β+IFN-γ for 48h as indicated. Expression of miR-149-5p (A) was assayed by RT-PCR and normalized by miR-375-3p expression. Apoptosis was evaluated by propidium iodide / Hoechst staining after 48h of cytokine treatment (B). Expression of DP5 (C), PUMA (D), NOXA (E), BAX (F) and BIM (G) were assayed by RT-PCR and normalized by the housekeeping gene β-actin. Results were normalized against the highest value in each independent experiment,

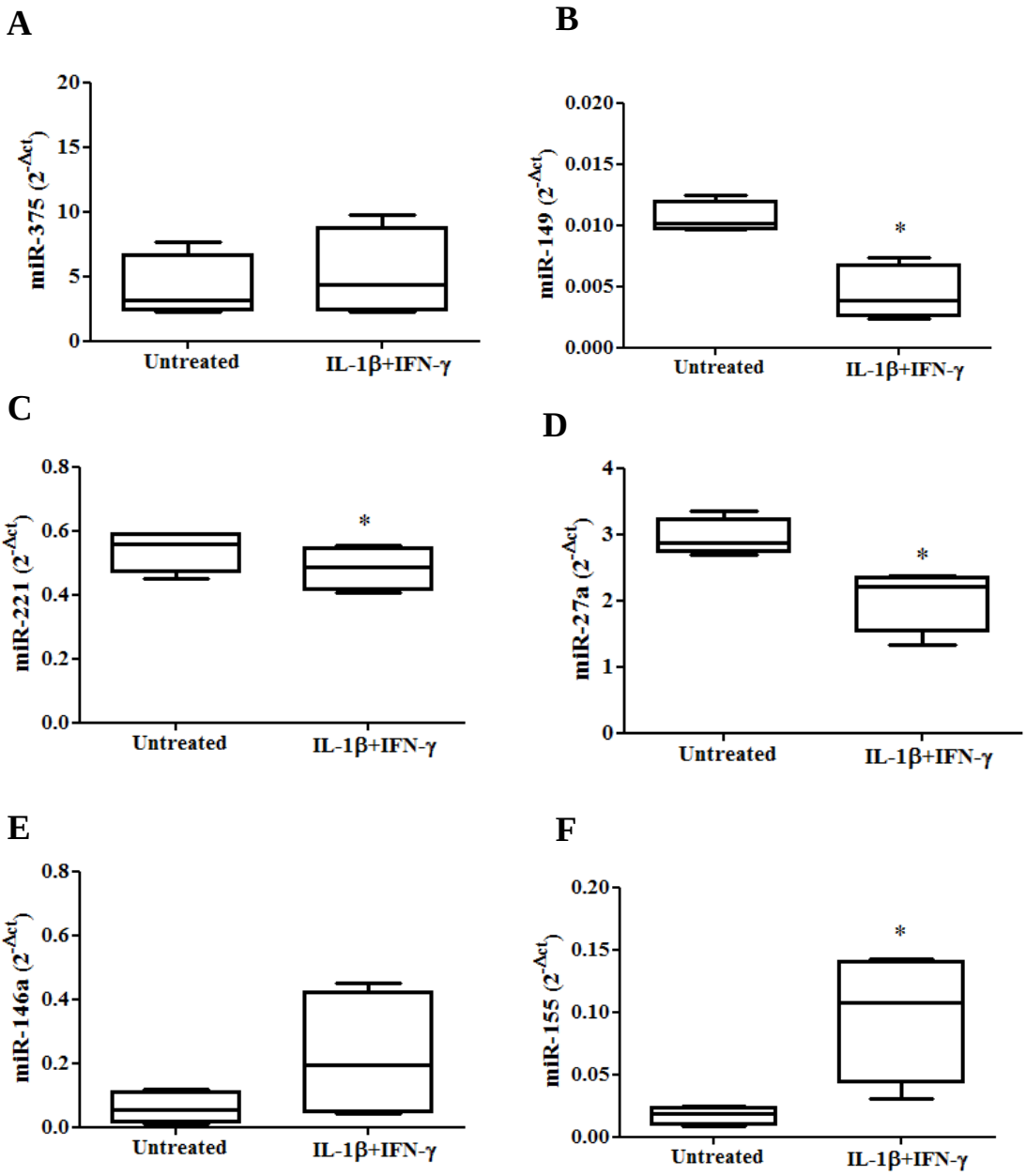
considered as 1 (C-G). * p<0.05, ** p<0.01 and *** p<0.001 *versus* siCTRL untreated cells; § p<0.05, §§ p<0.01 and §§§ p<0.001, # p<0.05, ## p<0.01 and ### p<0.001 as indicated by bars; ANOVA followed by paired Student's *t*-test with Bonferroni's correction. Data are shown as mean ± SEM of 7 independent experiments.

Supplementary Figure 8. KD of PUMA or DP5 protects human EndoC-βH1 cells against cytokine-induced apoptosis in the context of miR-149-5p inhibition. EndoC-βH1 cells were transfected with siControl (CTRL), siPUMA, anti-miR-149-5p or co-transfected with siPUMA+anti-miR-149-5p (A, C and E) for 8h. After 48h recovery, cells were left untreated or treated with IL-1β+IFN-γ for 48h as indicated. The KD of PUMA (A) was confirmed by RT-PCR and normalized by the housekeeping gene β-actin. Expression of miR-149-5p (C) was assayed by RT-PCR and normalized by miR-375-3p expression. Apoptosis was evaluated by propidium iodide / Hoechst staining after 48h of cytokine treatment (E). EndoC-βH1 cells were transfected with siControl (CTRL), siDP5, anti-miR-149-5p or co-transfected with siDP5+anti-miR-149-5p (B, D and F) for 8h. After 48h recovery, cells were left untreated or treated with IL-1β+IFN-γ for 48h as indicated. The KD of DP5 (B) was confirmed by RT-PCR and normalized by the housekeeping gene β-actin. Expression of miR-149-5p (D) was assayed by RT-PCR and normalized by miR-375-3p expression. Apoptosis was evaluated by propidium iodide / Hoechst staining after 48h of cytokine treatment (F). Results (A and B) were normalized against the highest value in each independent experiment, considered as 1. * p<0.05, ** p<0.01 and *** p<0.001 *versus* siCTRL untreated cells; § p<0.05, §§ p<0.01 and §§§ p<0.001 versus siCTRL cytokine-treated cells; # p<0.05, ## p<0.01, ### p<0.001, @ p<0.05 and @@@ p<0.001 as indicated by bars; ANOVA followed by paired Student's *t*-test with Bonferroni's correction. Data are shown as mean ± SEM of 6 independent experiments.

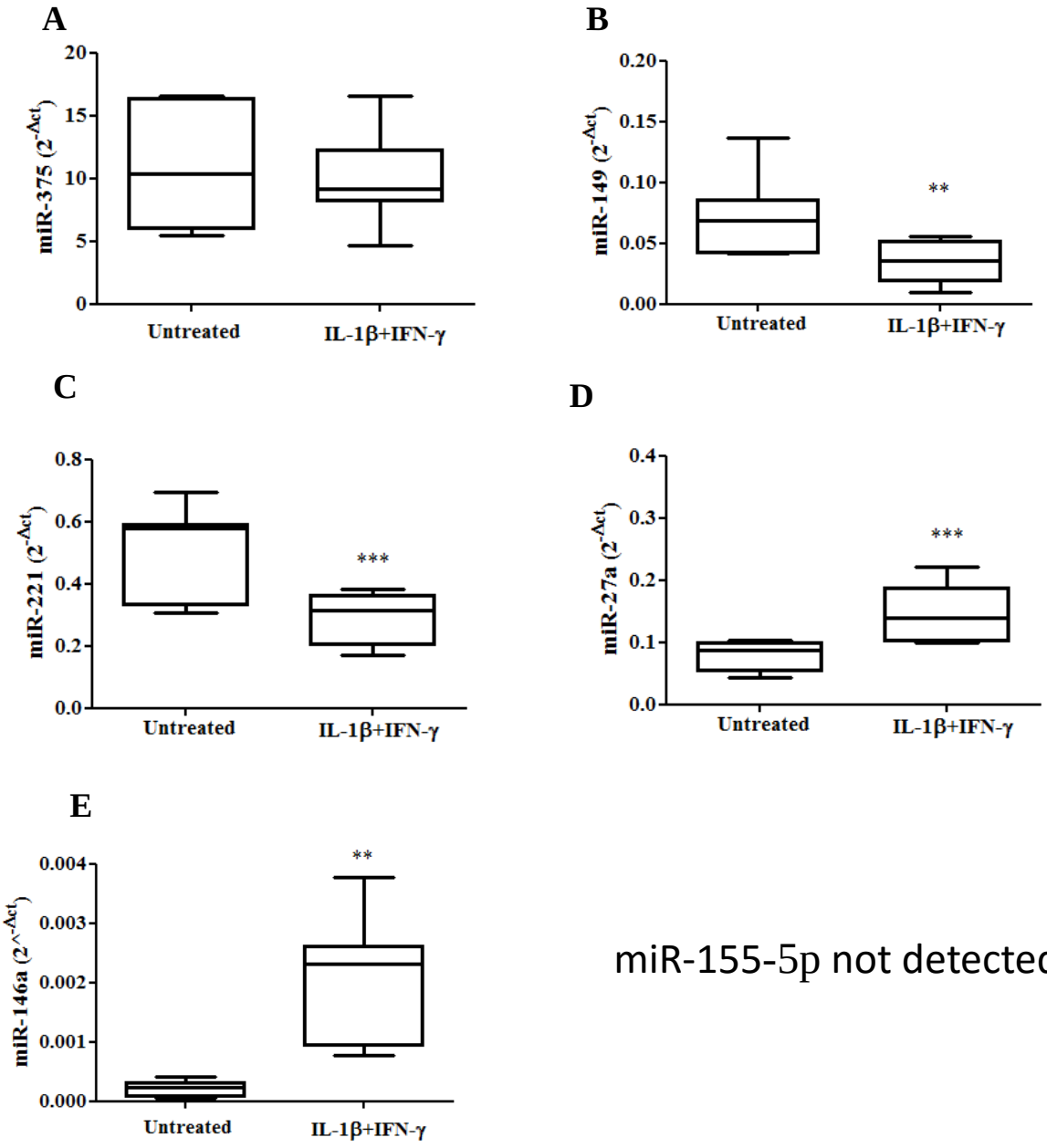
Supplementary Figure 1. Confirmation by RT-PCR of miRNAs detected as cytokine-modulated by micro-array.



Supplementary Figure 2. Confirmation by RT-PCR in independent samples of miRNAs detected as cytokine-modulated by micro-array.

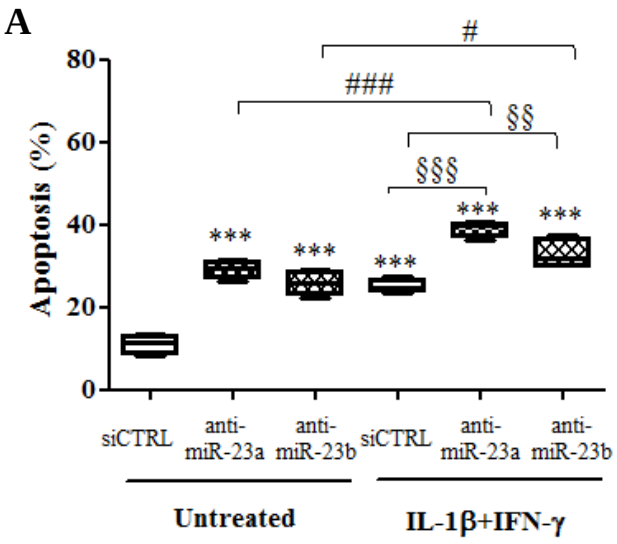


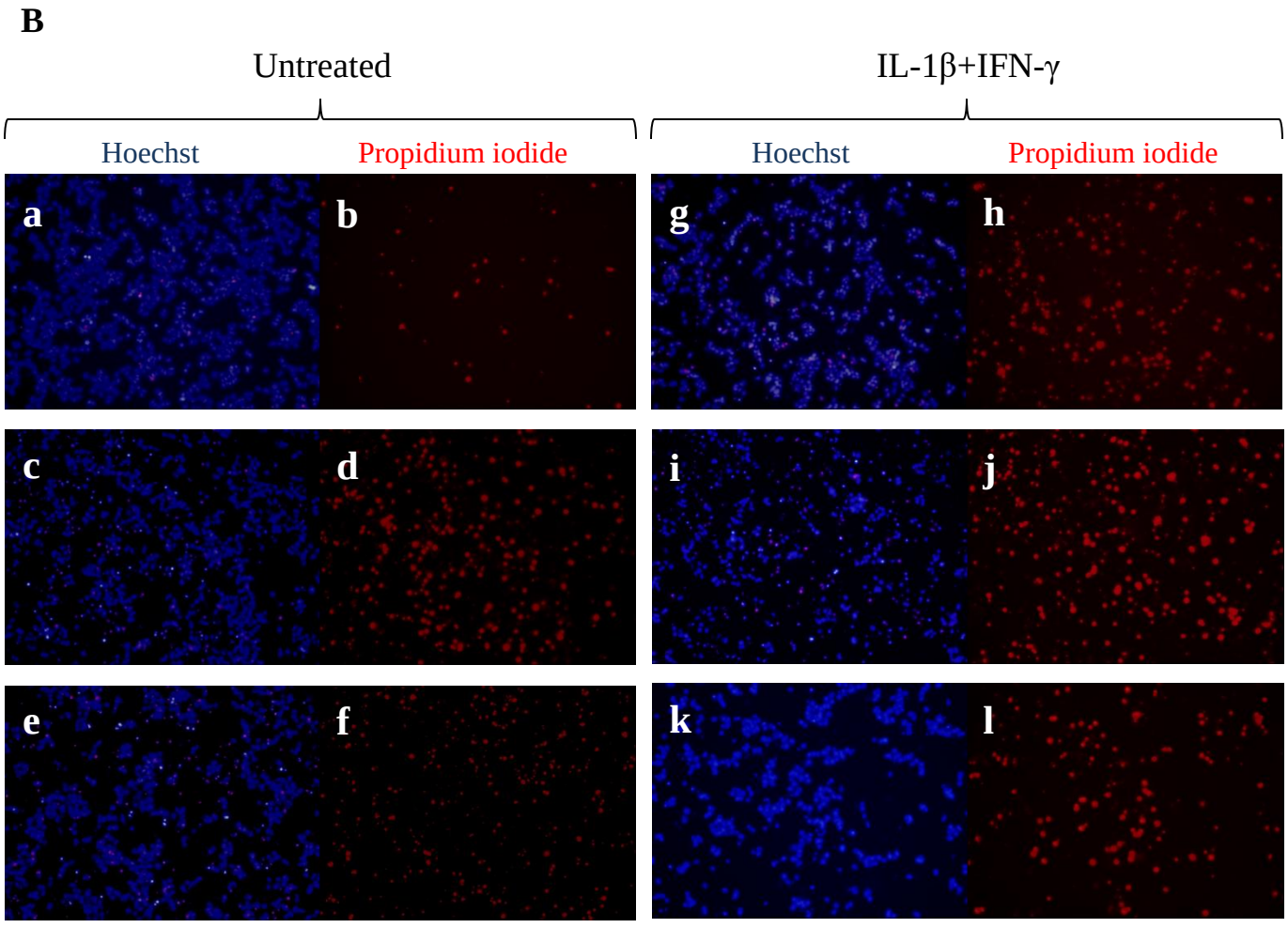
Supplementary Figure 3. Cytokine-modulated miRNAs in the human beta cell line EndoC-βH1.



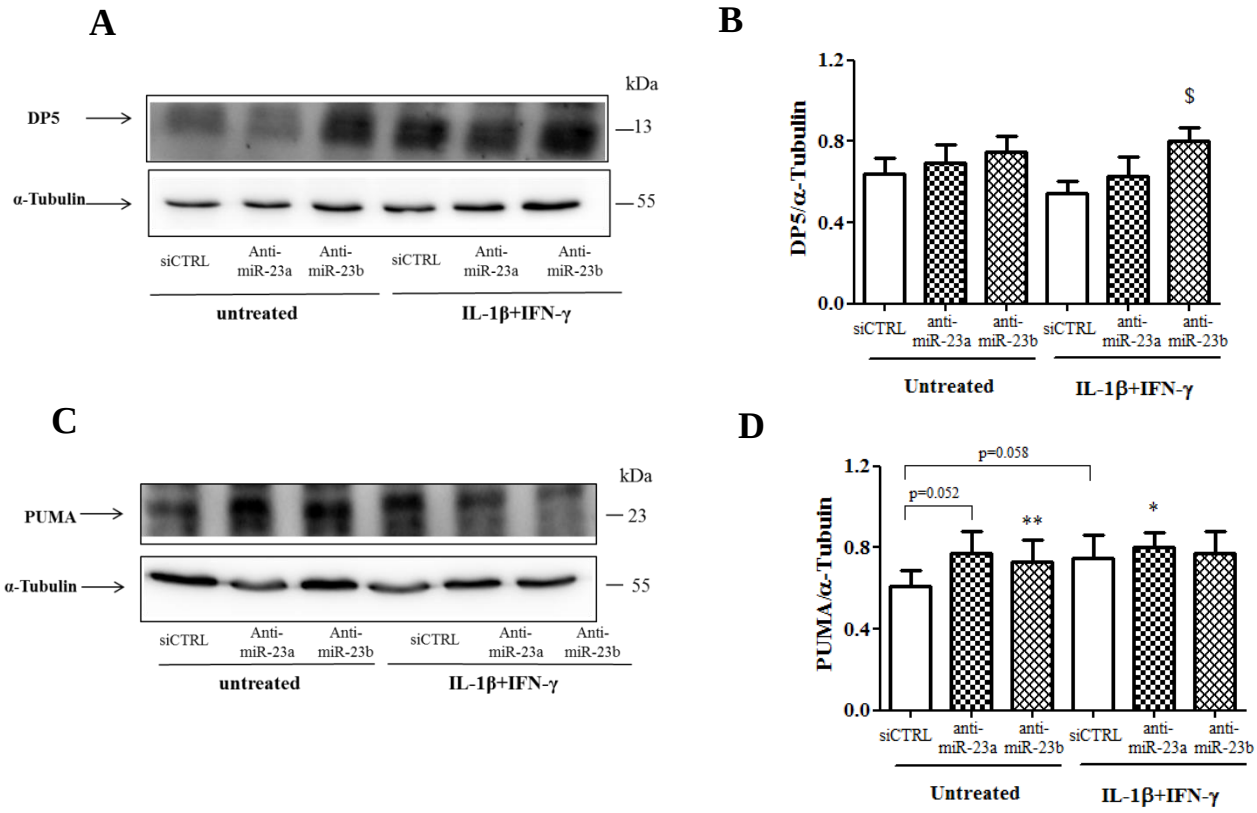
miR-155-5p not detected

Supplementary Figure 4. Inhibition of miR-23a-3p and miR-23b-3p exacerbates apoptosis in human EndoC-βH1 cells.



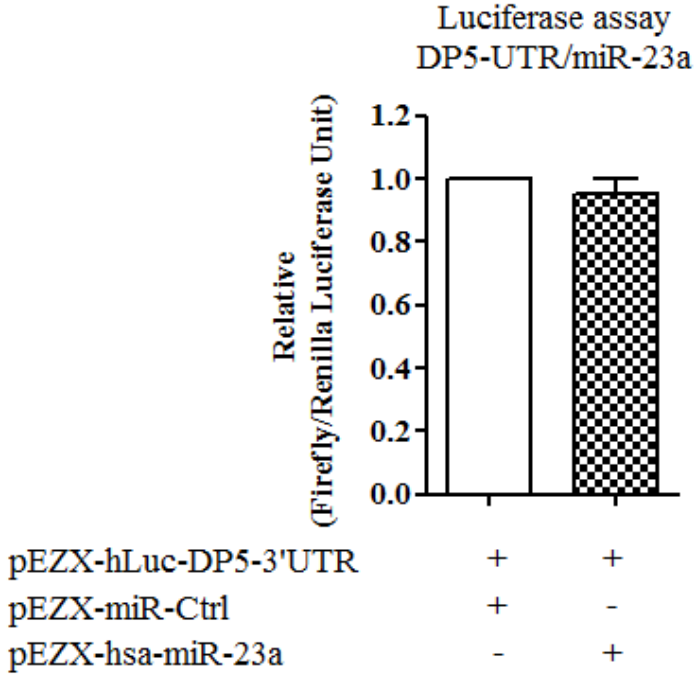


Supplementary Figure 5. Inhibition of miR-23a-3p and miR-23b-3p modify expression of pro-apoptotic Bcl-2 family members in human EndoC-βH1 cells.

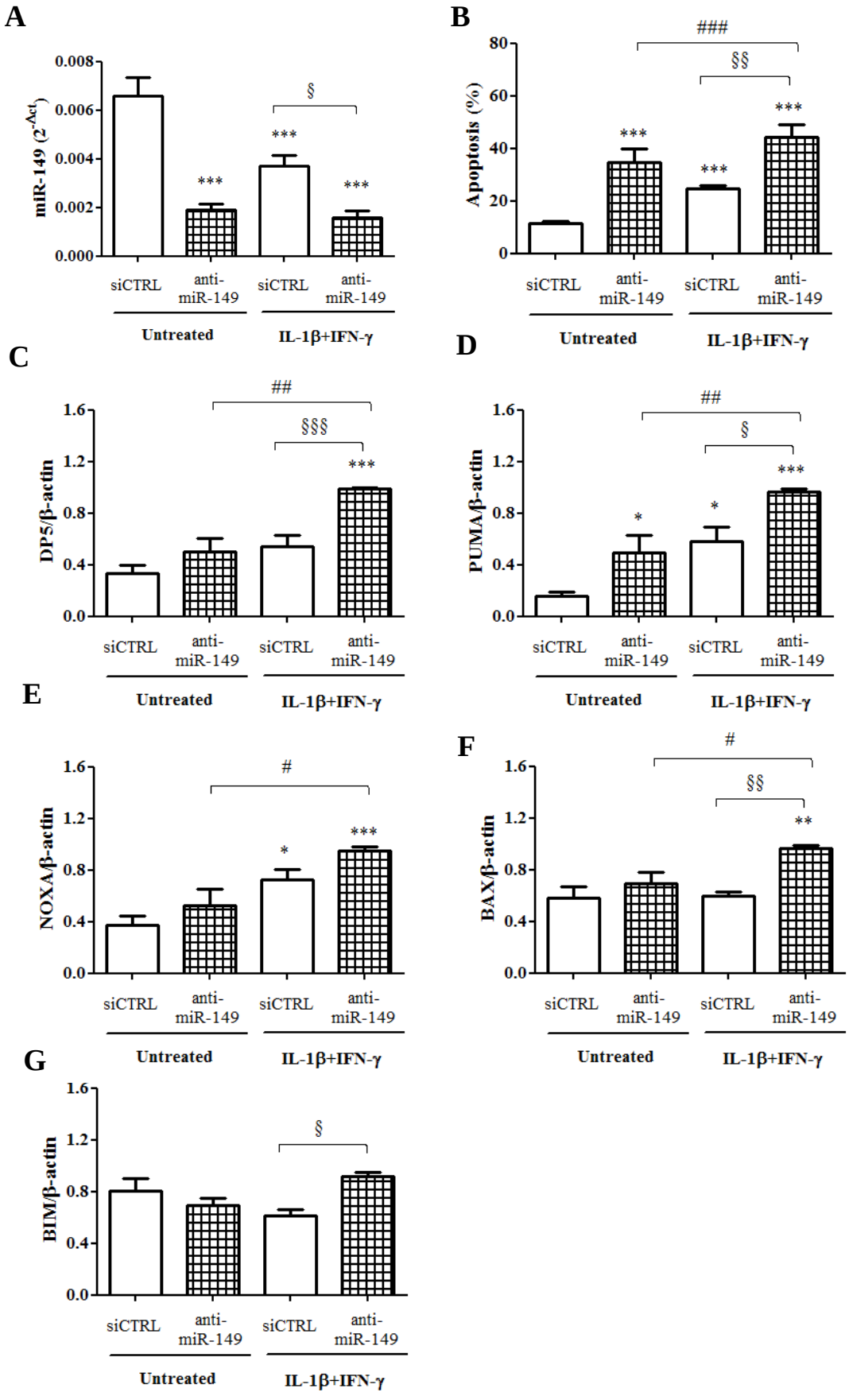


Supplementary Figure 6. DP5 is not a direct target of miR-23a-3p

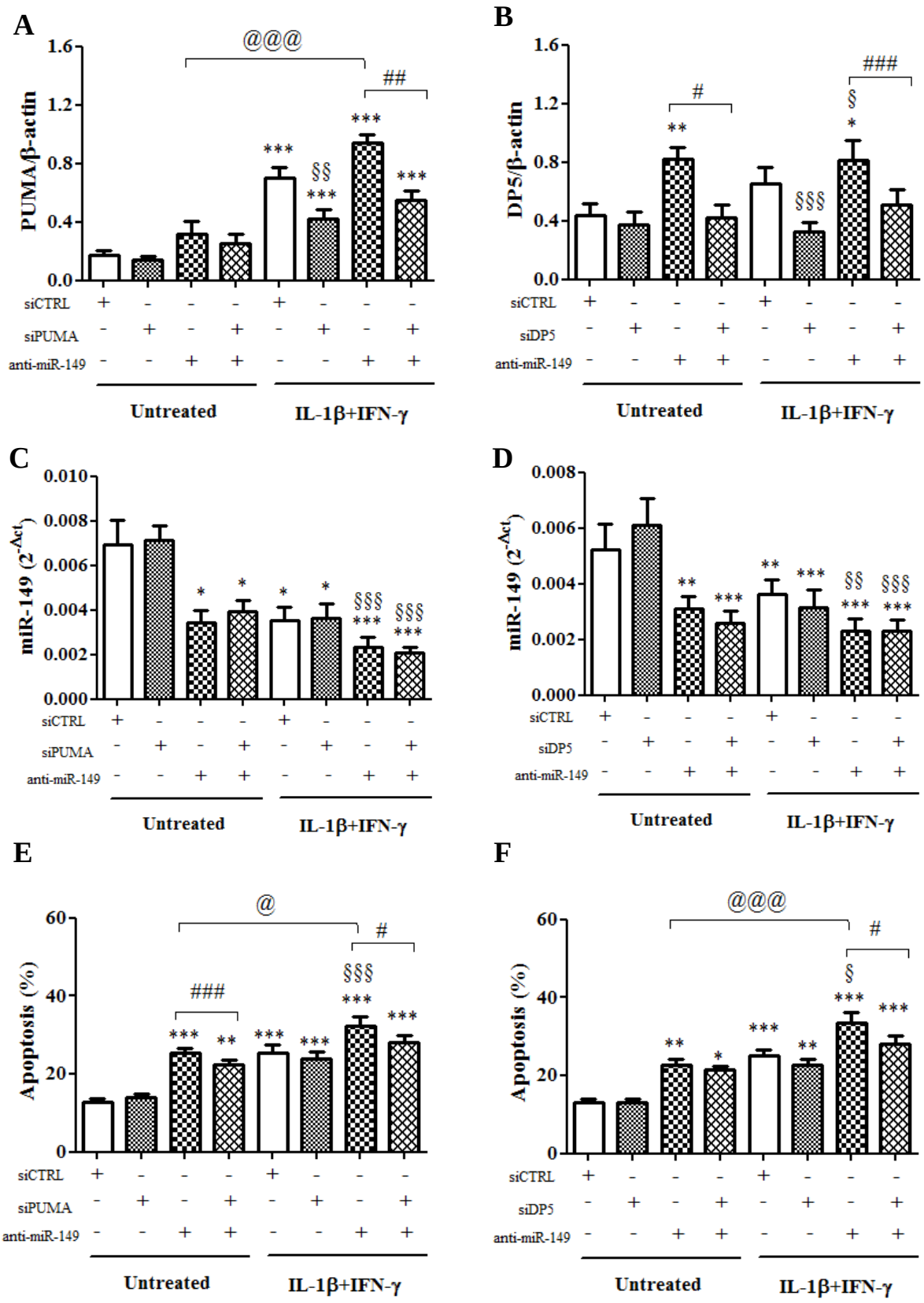
A



Supplementary Figure 7. Inhibition of miR-149-5p exacerbates apoptosis and up-regulates pro-apoptotic Bcl-2 family member expression in human EndoC-βH1 cells.



Supplementary Figure 8. KD of PUMA or DP5 protects human EndoC-βH1 cells against cytokine-induced apoptosis in the context of miR-149-5p inhibition.



Supplementary Table 1 – Characteristics of the human islet donors

Subject	Age (years)	Gender	BMI (kg/m²)	Islet beta cell content
C1	74	M	26	82%
C2	78	F	25	65%
C3	59	M	27	58%
C4	51	M	26	54%
C5	68	M	28	42%
C6	76	F	25	39%
C7	79	F	21	41%
C8	85	M	25	46%
C9	85	F	22	51%
C10	70	M	22	60%
C11	82	F	28	54%
C12	64	F	22	55%
C13	51	M	25	60%
X ± SEM	**71 ± 3**	**-**	**25 ± 1**	**54 ± 3%**

Supplementary Table 2 – Antibodies used in the study

Antibody	Company	Reference	Dilution
Insulin	Sigma-Aldrich, Bornem, Belgium	I2018	IHC: 1/1000
Donkey anti-mouse IgG rhodamine	Lucron Bioproducts, De Pinte, Belgium	715-026-156	IHC: 1/200
Cleaved caspase-3 (D175) (rabbit)	Cell Signaling, Danvers, MA, USA	9661S	WB: 1/1000 IHC: 1/200
HrK (DP5) (rabbit)	ABCAM, Cambridge, United Kingdom	ab45419	WB: 1/500
PUMA (rabbit)	ABCAM, Cambridge, United Kingdom	ab9645	WB: 1/1000
α-tubulin (mouse)	Sigma-Aldrich, Bornem, Belgium	T9026	WB: 1/5000
β-actin (rabbit)	Cell Signaling, Danvers, MA, USA	4967	WB: 1/5000
HRP-conjugated anti-rabbit IgG	Lucron Bioproducts, De Pinte, Belgium	711-036-152	WB: 1/5000
HRP-conjugated anti-mouse IgG	Lucron Bioproducts, De Pinte, Belgium	715-036-150	WB: 1/5000
Alexa Fluor 488 goat anti-mouse	Molecular Probes Life Technologies-Invitrogen, Paysley, UK	A-11008	IHC: 1/500
Alexa Fluor 546 goat anti-rabbit	Molecular Probes Life Technologies-Invitrogen, Paysley, UK	A-11030	IHC: 1/500

Supplementary Table 3 – siRNAs and single-stranded miRNA inhibitors used in the study

Name	Type	Distributors	Sequence
siCTRL	Allstar Negative Control siRNA	Qiagen, Venlo, Netherlands	Seq not provided
Human siDP5	Silencer Selected Pre-designed (Inventoired) siRNA	Ambion, Life Technologies, Paysley, UK	1: 5'-GAGCGAUCGUAGAAACACAtt-3' 2: 3'-UGUGUUUCUACGAUCGCUCca-5'
Human siPUMA (BBC3)	ON-TARGETplus siRNA	Dharmacon, Lafayette, Colorado, USA	1: CGGACGACCUCAACGCACA 2: CCGAGAUGGAGCCCAAUUA 3: CCUGGAGGGUCCUGUACAA 4: GUAGAUCCGGAAUGAAUU
Hs-miR-23a-3p	miRCURY LNA Power Inhibitor	Exiqon, Vedbaek, Denmark	Sequence 5'-3': GGAAATCCCTGGCAATGT
Hs-miR-23b-3p	miRCURY LNA Power Inhibitor	Exiqon, Vedbaek, Denmark	Sequence 5'-3' : TAATCCCTGGCAATGTGA
Hs-miR-149-5p	miRCURY LNA Power Inhibitor	Exiqon, Vedbaek, Denmark	Sequence 5'-3' : AGTGAAAGACACGGAGCCA

Supplementary Table 4 - Sequence of the primers

miScript Primer Assay	Accession	Sequence
Hs_RNU6	RNU6 small nuclear 6, pseudogene	Not provided
Hs_SNORD61	SNORD61 Small nucleolar RNA, C/D box 61	Not provided
Hs_miR-375	MIMAT0000728	5' - UUUGUUCGUUCGGCUCGCGUGA
Hs_miR-23a	MIMAT0000078	5' – AUCACAUUGCCAGGGAUUUCC
Hs_miR-23b	MIMAT0000418	5' - AUCACAUUGCCAGGGAUUACC
Hs_miR-149	MIMAT0000450	5' - UCUGGCUCCGUGUCUUCACUCCC
Hs_miR-221	MIMAT0000278	5' - AGCUACAUUGUCUGCUGGGUUUC
Hs_miR-27a	MIMAT0000084	5' - UUCACAGUGGCUAAGUUCCGC
Hs_miR-146a	MIMAT0000449	5' - UGAGAACUGAAUUCCAUGGGUU
Hs_miR-155	MIMAT0000646	5' - UUAAUGCUAAUCGUGAUAGGGGU
Primer	**Forward sequence**	**Reverse sequence**
β-actin (human)	5'-CTGTACGCCAACACAGTGCT-3'	5'-GCTCAGGAGGAGCAATGATC-3'

DP5 (human)	5’- GAGCCCAGAGCTTGAAAGG -3’	5’- CCCAGTCCCATTCTGTGTTT -3’
PUMA (human)	5'-TTGTGCTGGTGCCCGTTCCA-3'	5'-AGGCTAGTGGTCACGTTTGGCT-3'
BAX (human)	5’- TCTGACGGCAACTTCAACTG- 3’	5’- TTGAGGAGTCTCACCCAACC-3’
NOXA (human)	5’- GCAAGAATGGAAGACCCTTG-3’	5’- CGCCCAGTCTAATCACAGGT-3’
BIM TOTAL (human)	5’-TTCTTGCAGCCACCCTGC-3’	5’-CTTGCGTTTCTCAGTCCGA-3’
BIM SMALL (human)	5’-GAGCCACAAGCTTCCATGAG-3’	5’-TAACCATTCGTGGGTGGTCT-3’
c-JUN (human)	5’-ACCTGATGTACCTGATGCTATGG-3’	5’-CATCGCACTATCCTTTGGTAAGC-3’

Supplementary Table 5. **MiRNA profile of human islets exposed or not to pro-inflammatory cytokines**. Human islets were left untreated (CTRL) or treated with IL-1β+IFN-γ for 48h as described in Material and Methods. Total RNAs was extracted and miRNA expression profiling was performed using TaqMan Array Human MicroRNA Cards Panel A v2.1 The table shows all individual miRNAs differentially modified after cytokines treatment. Results are expressed as fold change versus control (non-cytokine treated) human islets cells. Up-regulated miRNAs are shown in green, whereas down-regulated miRNAs are shown in red. MiRNAs consistently modulated (i.e. changed in the same direction in each individual experiment) are indicated in bold.

microRNA	IL-1β+ IFN-γ vs CTRL	microRNA	IL-1β+ IFN-γ vs CTRL	microRNA	IL-1β+ IFN-γ vs CTRL	microRNA	IL-1β+ IFN-γ vs CTRL
miR-101	2.648	**miR-216a**	**0.676**	miR-431	2.258	miR-523	4.357
miR-10a	6.546	**miR-22**	**0.672**	miR-449	2.378	miR-576-3p	0.429
miR-125a	**0.685**	**miR-221**	**0.317**	miR-449b	2.841	miR-597	7.747
miR-129	**0.492**	miR-23a	0.711	**miR-455-3p**	**0.553**	**miR-618**	**0.478**
miR-142-3p	**2.185**	**miR-23b**	**0.717**	**miR-485-5p**	**0.495**	miR-654-3p	2.930
miR-145	**0.701**	**miR-27a**	**0.606**	miR-486-3p	0.705	**miR-671-3p**	**0.518**
miR-146a	**4.117**	**miR-27b**	**0.684**	**miR-487a**	**0.277**	miR-708	2.173
miR-146b	**2.158**	**miR-299-5p**	**0.233**	miR-488	45.069	**miR-758**	**0.323**
miR-149	**0.277**	**miR-330**	**0.630**	**miR-494**	**0.725**	**miR-886-3p**	**6.677**
miR-154	**0.558**	miR-338-3p	3.477	**miR-501**	**0.738**	**miR-886-5p**	**2.837**
miR-155	**4.599**	**miR-342-5p**	**2.803**	**miR-509-5p**	**0.257**	miR-888	0.708
miR-193b	**0.728**	miR-346	0.746	miR-518d	2.822	miR-891a	2.775
miR-19b	**0.682**	**miR-361**	**0.541**	**miR-518f**	**0.308**		
miR-202	2.461	**miR-362-3p**	**2.889**	**miR-519a**	**0.219**		
miR-211	**0.260**	miR-363	0.563	miR-522	0.555		