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Contribution of intronic miR-338-3p and its Hosting Gene AATK to Compensatory β-cell Mass Expansion

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Abstract

The elucidation of the mechanisms directing β-cell mass regeneration and maintenance is of interest since the deficit of β-cell mass contributes to diabetes onset and progression. We previously found that the level of the microRNA miR-338-3p is decreased in pancreatic islets from rodent models displaying insulin resistance and compensatory β-cell mass expansion, including pregnant rats, diet-induced obese mice and *db/db* mice. Transfection of rat islet cells with oligonucleotides that specifically block miR-338-3p activity increased the fraction of proliferating β-cells *in vitro* and promoted survival under pro-apoptotic conditions without affecting the capacity of β-cells to release insulin in response to glucose. Here, we evaluated the role of miR-338-3p *in vivo* by injecting mice with an Adeno-Associated viral (AAV) vector permitting specific sequestration of this microRNA in β-cells. We found that the AAV construct increased the fraction of proliferating β-cells confirming the data obtained *in vitro*. miR-338-3p is generated from an intron of the gene coding for Apoptosis-Associated Tyrosine Kinase (AATK). Similarly to miR-338-3p, we found that AATK is down-regulated in rat and human islets and INS832/13 β-cells in the presence of the cAMP-raising agents exendin-4, estradiol and a GPR30 agonist. Moreover, AATK expression is reduced in islets of insulin resistant animal models and selective silencing of AATK in INS832/13 cells by RNA interference promoted β-cell proliferation. The results point to a coordinated reduction of miR-338-3p and AATK under insulin resistance conditions and provide evidence for a cooperative action of the microRNA and its hosting gene in compensatory β-cell mass expansion.

Introduction

MicroRNAs (miRNAs) form a large family of short (≈22nt) single-stranded non-coding RNAs which are critical posttranscriptional regulators of gene expression (1,2). These small RNA molecules function by partially pairing to the 3' untranslated region (UTR) of target mRNAs thereby inhibiting their translation and/or stability. Numerous miRNAs have been shown to play major roles in the regulation of β-cell functions, including insulin secretion, proliferation and survival (3,4). Mammalian miRNAs are generated from diverse genomic locations and can be either intergenic and transcribed from their own independent unit or intronic and transcribed from introns of protein-coding genes or from introns of non-coding genes (5,6). Some intronic miRNAs are co-regulated with their hosting genes and have analogous functions, whereas others display opposite expression profiles and have antagonistic roles (7). Bioinformatic studies revealed that about one third of the intronic miRNAs possess their own promoters, while the others are co-transcribed with their hosting genes and are processed from the same primary transcript (8,9).

Recently, we showed that miR-338-3p is down-regulated in islets under conditions of insulin resistance, such as pregnancy and obesity, and upon exposure of β-cells to 17-β estradiol and to the GLP1-analogue exendin-4. We also observed that blockade of miR-338-3p *in vitro* and in transplanted islets leads to increased β-cell proliferation and improved survival under pro-apoptotic conditions (10). However, the beneficial impact of reduced miR-338-3p activity on β-cell function *in vivo* remained to be proven. This particular miRNA is generated from the seventh intron of the Apoptosis-Associated Tyrosine Kinase (AATK) gene, also called Lemur Kinase 1 (LMTK1) (5). Recent studies in other cell systems have evidenced a co-regulation of miR-338-3p and its hosting gene, and this miRNA was proposed to serve the interest of AATK by silencing several genes that act antagonistically to AATK (11). Although, AATK has been shown to play a role in cell differentiation, growth and apoptosis (12-17) its biological relevance in insulin-producing cells is unknown.

The objective of our study was first to verify whether the proliferative effect of miR-338-3p observed *in vitro* is conserved *in vivo* and second, to determine whether the hosting gene AATK synergizes or antagonizes the action of the miRNA.

Materials and methods

Chemicals

TNFα and IFNγ were from R&D Systems. IL-1β, Tnfrsf1b, exendin-4, 17-β estradiol, 3-Isobutyl-1-methylxanthine (IBMX) and G1 were obtained from Sigma-Aldrich.

Animals

Male and pregnant Wistar rats were obtained from Charles River laboratories and were housed on a 12:12 h light-dark cycle in climate-controlled and pathogen-free facilities. 8-week-old male ICR mice were used to inject AAV8 intraductally. All procedures were performed in accordance with the National Institutes of Health guidelines, and were approved by the Swiss Research Councils and Veterinary Office and by the Ethics Committee in Animal and Human Experimentation of the Universitat Autònoma de Barcelona. Four week-old male C57BL/6 mice were purchased from Charles River Laboratories (Saint-Constant, QC, Canada) and fed a normal diet or HFD (Bio-Ser Diet number F3282, Frenchtown, NJ, USA; 60% [wt/wt] energy from fat) for 8 weeks. The detailed sources of *db/db* and diet-induced obese mice have been described previously (18,19).

Isolation and culture of islet cells

Pancreatic islets were isolated as described (20) by collagenase digestion followed by purification on a Histopaque (Sigma-Aldrich) density gradient. The islets were first cultured overnight in RPMI 1640 Glutamax medium (Invitrogen) supplemented with 10% fetal calf serum (FCS; Amimed), 50 U/ml penicillin, 50ug/ml streptomycin, 1 mmol/l Na Pyruvate and 250 μmol/l Hepes. Human islets were obtained from the Cell Isolation and Transplantation Center from the University of Geneva, through the ECIT "Islets for Research" distribution program supported by the Juvenile Diabetes Research Foundation. The use of human islets was approved by the Geneva institutional ethical committee. After isolation, the islets were kept in culture for one or two days before treatment. Whole human islets were cultured in CMRL medium (Invitrogen) supplemented with 10% FCS, 100 U/ml penicillin,

100μg/ml streptomycin, 2mmol/l glutamine and 250 μmol/l Hepes. Detailed information about the human islet preparations used in this study is presented in the Supplemental Table 3.

Transfection and modulation of miR-338-3p and AATK levels

INS832/13 cells or MIN6B1 cells were transfected using Lipofectamine 2000™ (Invitrogen) with single-stranded miScript miRNA inhibitors (Qiagen) that specifically block endogenous miR-338-3p or with the miScript miRNA reference inhibitor (Qiagen) as a negative control. To modulate the level of the hosting gene, the cells were transfected with a small interfering RNA (siRNA) duplex against AATK or with a custom-designed siRNA duplex against green fluorescent protein that was used as negative control. To experimentally sequester and inhibit the activity of miR-338-3p, MIN6B1 cells were transfected for three days with plasmids expressing enhanced green fluorescent protein (EGFP)-labelled constructs driven by the Rat Insulin Promoter II (RIP-II) containing either a control sponge or the miR-338-3p sponge.

Measurement of miRNA and mRNA expression

Mature miRNA expression was assessed by qRT-PCR using the miRCURY LNA™ Universal RT microRNA PCR kit (Exiqon). miRNA primers were purchased from Exiqon. Messenger RNA expression was measured by conventional reverse transcription (Promega) followed by qRT-PCR (Biorad) with custom-designed primers (Microsynth). Primer sequences are available on request. miRNA expression was normalized to the level of U6 small nuclear ribonucleoprotein. mRNA expression was normalized to the amount of 18S present in the same samples.

Insulin secretion

Three days after transfection, INS832/13 cells were pre-incubated during 30 minutes at 37°C in Krebs-Ringer buffer (25 mM HEPES, pH 7.4, 127 mM NaCl, 4.7 mM KCl, 1 mM CaCl2, 1.2 mM KH2PO4, 1.2 mM MgSO4 and 5 mM NaHCO3) containing 2 mmol/l glucose. The pre-incubation medium was discarded and the cells incubated for 45 minutes in the same buffer (basal condition) or with Krebs-Ringer buffer containing 20 mmol/l glucose, 10 μM Forskolin and 100 μM IBMX (stimulatory

condition). At the end of the incubation period, the medium was collected and total cellular insulin contents recovered with ethanol acid (75% ethanol, 0.55% HCl). The amount of insulin in the samples was determined using an insulin enzyme immunoassay kit (ELISA, Mercodia).

Cell death assessment

Three days after transfection, transfected INS832/13 or MIN6B1 cells were incubated with 1 μg/ml Hoechst 33342 (Invitrogen) during 1 minute. The fraction of cells displaying picnotic nuclei was scored under fluorescence microscopy (AxioCam MRc5, Zeiss). Apoptosis was triggered by exposing the cells during 24h to cytokines (10 ng/ml TNFα, 0.1 ng/ml IL-1β and 30 mg/ml IFNγ) or during 48h to culture medium containing 5% FCS and supplemented with 0.5 mmol/l palmitate bound to 0.5% BSA.

Proliferation assay

Three days after transfection, transfected INS832/13 or MIN6B1 cells and rat islet cells cultured on Poly-L-lysine coated glass coverslips, were fixed with ice-cold methanol and permeabilized with 0.5% saponin (Sigma-Aldrich). The coverslips were incubated with a rabbit anti-Ki67 antibody (ab66155 Abcam) at 1:1500 and then with goat anti-rabbit Alexa Fluor 488 (A11008 Invitrogen). Pictures were collected using a fluorescence microscope (AxioCam MRc5, Zeiss).

Protein extraction and western Blotting

Proteins lysates (30-50 μg) from INS832/13 cells were separated on polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were incubated overnight at 4°C with primary antibody against AATK (Ab100587 Abcam; 1:500), AKT (Cell Signaling #2920; 1:500), phospho-AKT (Cell Signaling #9275; 1:500) or against α-Tubulin (T9026 Sigma Aldrich, 1:10000). After one hour exposure to IRDye (Li-Cor® Biosciences), the bands were visualized via the Odyssey imaging system (Li-Cor® Biosciences). Band intensity was quantified by using ImageJ software.

Recombinant AAV vectors

Single-stranded AAV vectors of serotype 8 were generated by triple transfection of human embryonic kidney 293 cells and purified by the CsCl-based gradient method (21). Transgenes used expressed a construct coding for EGFP driven by the rat insulin promoter II (RIP-II) and carried either a sponge against miR-338-3p or against a scrambled sequence. Purified AAV vectors were dialyzed against PBS, filtered and stored at -80°C. Titers of viral genomes (vg) were determined by qRT-PCR as described (22).

Administration of AAV vectors

Retrograde pancreatic intraductal injections were performed as described previously (23). Mice were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg). A dose of 10^{12} vg/mouse (100 µL AAV solution/mouse) was intraductally administered.

Luciferase assays

A miR-338-3p sensor plasmid was generated by cloning a sequence complementary to the miRNA between the EcoR1 and XhoI restriction sites of psiCHECK-1 (Promega). An analogous approach was used to generate the plasmid including the 3'UTR of rat Tnfrsf1b containing the putative binding site of miR-338-3p. Luciferase activity was measured in INS832/13 or MIN6B1 cells with a dual-luciferase reporter assay (Promega, Madison, WI) three days after transfection. Renilla luciferase activity was normalized for transfection efficiency to the SV40-driven Firefly activity generated by the PGL3 promoter vector (Promega).

Immunohistochemistry

Tissues were fixed for three days in 10% formalin, embedded in paraffin and sectioned. The pancreatic slices were then incubated overnight at 4°C with the following antibodies: 1:300 goat anti-GFP (ab6673 Abcam), 1:200 guinea pig anti-insulin (ab7842 Abcam) or 1:300 rabbit anti-Ki67 (ab66155 Abcam). As secondary antibodies, biotinylated donkey anti-goat (sc-2042 Santa Cruz), streptavidin

Alexa Fluor 488 (S-11223 Molecular Probes), goat anti-guinea pig Alexa Fluor 555 (A21435 Invitrogen), goat anti-rabbit Alexa Fluor 555 (A21428 Invitrogen) at 1:300 were used. Sections were counterstained with Hoechst 33342 (Invitrogen) for nuclear labeling. A Zeiss Axiovision fluorescence microscope was used. For measurement of β-cell proliferation, three pancreatic slices (200 μm apart) were stained with anti-Ki67 and anti-GFP antibodies, and nuclei were counterstained with Hoechst. Replicative cells were identified by double Ki67 and GFP immunostaining. The β-cell mass was calculated by multiplying pancreas weight by percentage of β-cell area. The percentage of β-cell area per pancreas was analyzed in three insulin-stained sections 200 μm apart, by dividing the area of all insulin positive cells in one section by the total area of that section. An islet was considered as infected by the viruses if at least one insulin-positive cell was also positive for EGFP.

Statistical analysis

Statistical differences were tested using a Student's t-test or, for multiple comparisons, with ANOVA followed by a post-doc Dunnett test, with a discriminating p value of 0.05 (SAS statistical package).

Results

β-cell specific inhibition of miR-338-3p promotes cell proliferation *in vivo*

To inhibit the activity of miR-338-3p *in vivo*, we engineered a "microRNA sponge" (24,25) capable of specifically inhibiting the activity of this miRNA in β-cells. Our construct is driven by the rat insulin promoter and contains the coding region of EGFP with 7 binding sites for miR-338-3p in the 3'UTR. By capturing miR-338-3p, the sponge prevents the interaction with the endogenous targets of the miRNA (Figure 1A). Indeed, the transfection of the miR-338-3p sponge in the insulin-secreting cell line MIN6B1 led to an increase in the expression of a luciferase reporter construct containing the binding site of the miRNA (Supplemental Fig. 1A) and reproduced the functional effects of anti-miR-338-3p (10) resulting in an increased proliferation rate and enhanced survival under pro-apoptotic conditions (Supplemental Fig. 1B, C). To examine whether inhibition of miR-338-3p activity *in vivo* promotes the replication of β-cells, a dose of 1×10^{12} viral genomes/mouse of AAV8 vectors carrying the RIP-II-GFP-miR-338-3p sponge cassette or RIP-II-GFP-control sponge were injected into the pancreatic duct of adult ICR mice. One month after AAV administration, immunostaining revealed that more than 80% of the islets were transduced by these AAV8 vectors and expressed the construct (Figure 1B). Double immunostaining for insulin and GFP confirmed that the construct was exclusively expressed in β-cells. Quantification of the number of GFP^+ β-cells demonstrated an average of 20% transduced β-cells (Figure 1C, D). The sponge was not expressed by glucagon-positive cells (Supplemental Fig. 2). The fraction of proliferating GFP^+ β-cells receiving the control sponge was identical to that of non-transduced islet cells (Figure 1E). In contrast, the GFP^+ β-cells receiving the AAV8-miR-338-3p sponge displayed a significant increase in the proliferation rate (Figure 1E, F). Consistent with these observations, the fraction of proliferating insulin-positive cells was increased in islets transduced with the miR-338-3p sponge compared to islets receiving the control sponge (Figure 1G, H). One month after viral injection, no significant change in β-cell mass was observed between the two groups of mice (Figure 1I). Analysis of individual glycemic profiles revealed no differences between the two groups (data not shown). Overall the data demonstrate the conserved beneficial impact of reduced activity of miR-338-3p in promoting β-cell proliferation capacity *in vivo*.

miR-338-3p and its hosting gene AATK are co-regulated

We previously reported reduced expression of miR-338-3p in islets of insulin resistant animals displaying compensatory β-cell mass expansion (10). Since this miRNA is encoded within one of the introns of AATK (Supplemental Fig. 3), we analyzed in the same samples the expression of the hosting gene. Similarly to miR-338-3p, we found that AATK mRNA decreases in islets of rats during pregnancy reaching the minimal level at day 14 of gestation (corresponding to the peak of β-cell mass expansion) and returned close to pre-pregnancy levels 3-days post-partum (Figure 2A). AATK expression is also reduced to a similar extent than miR-338-3p in islets of two animal models characterized by insulin resistance and compensatory β-cell mass expansion (Supplemental Tables 1 and 2): 6 week-old pre-diabetic *db/db* mice (Figure 2B) and diet-induced obese mice fed a high-fat diet for 8 weeks (Figure 2C). These results suggest a common regulation of the expression profile of the gene coding for AATK and its intronic miRNA in pancreatic islets under conditions of insulin resistance.

In view of these findings, to determine if miR-338-3p and AATK expression is coordinated we measured AATK mRNA levels in INS832/13 cells exposed to the cAMP-raising agent IBMX. We found that the changes in the level of miR-338-3p and AATK follow the same kinetics and are maximal after about 4 hours of treatment (Figure 3). We then exposed rat and human islets to estradiol, a hormone capable of eliciting a decrease of miR-338-3p expression by binding to its non-canonical GPR30 receptor that activates the cAMP-dependent pathway (10). We found that exposure of rat and human islets to 17-β estradiol for 48 hours decreases the expression of AATK (Figure 4A, B). Treatment with G1, a specific GPR30 agonist, produced the same effect in rat islets (Figure 4C). GLP1 is also able to reduce the level of miR-338-3p by activating the cAMP-dependent pathway (10). In line with the effect of other hormones triggering cAMP-dependent signaling, the stable GLP1 analog exendin-4 represses AATK levels both in rat and human islets (Figure 4D, E). A similar effect was also observed in INS832/13 cells both at the mRNA and at the protein level (Supplemental Fig. 4).

Functional roles of AATK in insulin-secreting cells

In order to investigate the functional role of AATK in β-cells and compare it to that of miR-338-3p, we used RNA interference to silence AATK mRNA and protein levels in INS832/13 cells. The siRNA treatment, faithfully reproduced the decrease of approximately 50% of AATK mRNA observed in islets of insulin resistant animal models, without affecting the level of miR-338-3p (Figure 5A). AATK protein levels were also reduced by about half (Figure 5B, C). A specific antisense LNA-oligonucleotide (anti-miR) was also transiently transfected in INS832/13 cells to target and inhibit the endogenous miRNA without altering the mRNA level of AATK (Figure 5D). We first assessed the impact of the silencing of miR-338-3p and of its hosting gene on β-cell proliferation. Interestingly, inhibition of either miR-338-3p or AATK increased the proliferation of INS832/13 cells (Figure 6A) without significantly affecting insulin release (Figure 6B) and insulin content (Figure 6C). Moreover, in agreement with our previous results, the inhibition of miR-338-3p protected the cells against the toxic effects of pro-inflammatory cytokines and palmitate (Figure 6D, E). In contrast, the reduction of AATK did not promote cell survival under these pro-apoptotic conditions (Figure 6D, E).

The precise mechanism through which miR-338-3p affects β-cell proliferation and survival remains to be elucidated. Functional analysis of the potential targets revealed enrichment in genes involved in cancer development, MAP kinase pathway and cytokine signaling (Supplemental Table 4). We indeed obtained evidence indicating that miR-338-3p directly targets Tnfrsf1b (26), potentially explaining part of the anti-apoptotic but not of the proliferative effect of the miRNA (Supplemental Fig. 5). We previously identified several key gene expression changes elicited by miR-338-3p blockade in rat and human islets (10) that mimic the mRNA expression profile observed in islets of insulin resistant animals (10,27-30). As expected, similar modifications of genes involved in proliferation and survival (Birc5, Foxm1, Cyclin D2, Igf1r, Irs2, Bcl2, Bcl-xl, Bad) were observed upon repression of miR-338-3p in tumoral INS832/13 β-cells (Figure 6F) and led to the activation of the AKT signaling (Supplemental Fig. 6). Consistent with its functional impact on cell proliferation, silencing of AATK in INS832/13 cells led to an increase in the expression of Igf1r and Irs2, two genes that are part of an autocrine loop involved in regulating β-cell proliferation (31,32) (Figure 6F). In contrast, reduced

279 levels of AATK did not trigger any modification in the expression of the anti- or pro-apoptotic genes
280 Bcl2, Bcl-xl and Bad (Figure 6F) as miR-338-3p did. Altogether these results strongly support the
281 hypothesis of a cooperative action of miR-338-3p and its hosting gene AATK on β-cell proliferation
282 and in promoting the expansion of the functional β-cell mass under insulin resistant conditions.

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Discussion

In the present study, we have identified β-cell specific down-regulation of miR-338-3p as a powerful strategy to promote β-cell proliferation *in vivo*. Furthermore, we extend our understanding of the regulation and functional role of the miR-338-3p hosting gene AATK. The results reveal a parallel down-regulation of the miRNA and its hosting gene in islets of insulin resistant animal models and in response to activation of the cAMP-dependent pathway. In addition, we were able to show that AATK contributes to the regulation of β-cell proliferation by activating signaling pathways that are common to those of the miRNA.

Conventional knock-out (KO) mouse models are often used to study the role of miRNAs *in vivo*. However, about 30-40% of known mammalian miRNAs are located within introns of protein coding genes (5,33). Thus, the phenotype of miRNA KO animals can be influenced by the simultaneous invalidation of their hosting gene (34). To circumvent this problem, strategies avoiding to manipulate the genome and permitting direct interference with the function of the mature miRNA have been developed. *In vivo* delivery of miRNA sponges constitutes an interesting approach to study miRNA loss-of-function phenotypes and is emerging as a very attractive alternative to the use of KO mouse models. miRNA sponges have been demonstrated to be powerful inhibitors of miRNA activity due to the presence of multiple binding sites that sequester their target miRNA, thus preventing the interaction with their endogenous targets (24). A major advantage of this strategy is that it permits repression of miRNA activity in adult animals, avoiding problems associated with the invalidation of the miRNA during the fetal period and possible developmental defects. An efficient strategy of miRNA sponge delivery in mouse tissues is the use of viral vectors (25). In the past decade, new AAV vectors have been engineered, providing efficient gene transfer vehicles driven by tissue-specific promoters that have the tremendous advantage of lacking pathogenicity and displaying low immunogenicity (35). Studies showed that intraductal delivery of single stranded AAV vectors permits high gene transfer efficiency in endocrine islet cells (23).

Here we have combined a miRNA sponge approach with an AAV delivery strategy to study miR-338-3p action *in vivo*. To our knowledge, this is the first study with AAV-mediated delivery of a miRNA sponge specifically in pancreatic β-cells. Our results now confirm *in vivo* the involvement of miR-338-3p in the control of β-cell proliferation and suggest that AAV-based delivery of miR-338-3p inhibitors in diabetic animal models may represent an attractive therapeutic approach to promote the β-cell expansion and restore an appropriate mass of insulin-secreting cells. Further investigations will be required to improve the efficiency of the delivery of the sponge since under our experimental condition only a fraction of the β-cells were transduced and this was insufficient to significantly increase the total β-cell mass. An additional aspect that will need to be re-evaluated is the AAV delivery approach. Intraductal injection is a rather invasive method to deliver the sponge. In the future, it will be possible that capsid modifications in the AAV particles will allow them to target the β-cells *in vivo* by means of less invasive strategies such as intraperitoneal or intravenous injections.

Intronic miRNAs are usually thought to be coordinately expressed with their hosting gene, suggesting that they are transcribed from a common precursor under the control of a single promoter (5,8). Consistent with the data of a previous study carried out in a neuroblastoma cell line, we found a parallel regulation of the expression of miR-338-3p and of its hosting gene AATK (11). Our findings suggest that the concomitant decrease of miR-338-3p and AATK in β-cells of animals with insulin resistance showing β-cell mass expansion involves the cAMP-dependent pathway. This observation is consistent with the activation of GPR30 or GLP1R signalling cascades that increase cAMP levels (36,37). The reduction of miR-338-3p and AATK levels elicited by these two hormones reproduced the changes observed in islets of pregnant and obese animals. Our results point to a common regulation of the expression of miR-338-3p and its hosting gene in pancreatic islets under insulin resistance conditions that involves at least in part the cAMP signaling. The possibility of a differential regulation of the level of the miRNA and its hosting gene under other specific conditions cannot be ruled out. This could potentially be achieved by the use of an alternative promoter permitting an independent transcription of miR-338-3p (38) or via post-transcriptional control mechanisms allowing a fine tuning of the level of the AATK mRNA and of miR-338-3p (39).

Thanks to its tyrosine kinase activity, AATK has been proposed to play a role in the terminal differentiation and growth of neurons as well as cell proliferation and to be involved in the apoptosis of mature neuronal and cancer cells (12-17). So far, this gene has not yet been investigated in pancreatic β-cells. In contrast to neuronal cells, the reduction of AATK in insulin-secreting cells did not impact on cell survival. This may be explained by the fact that, in contrast to miR-338-3p blockade, AATK knock-down does not lead to the up-regulation of the anti-apoptotic genes Bcl2 and Bcl-xl. However, silencing of AATK resulted in a significant increase of replicating β-cells, suggesting that its down-regulation under conditions of insulin resistance may contribute to compensatory β-cell mass expansion. This view is supported by the fact that both miR-338-3p and AATK down-regulation elicit β-cell proliferation in association with increased expression of *Igf1r* and *Igf2* genes. In fact, these two proteins are the central components of an autocrine signaling loop that controls β-cell mass expansion under insulin resistance conditions (31,32). None of the genes differentially expressed in the presence of the anti-miRNA are predicted targets of miR-338-3p. Thus, the activation of the Igf1r/Igf2 autocrine loop observed after the reduction of the level of miR-338-3p is probably indirect. Interestingly, the inhibition of miR-338-3p has been proposed to play a prominent role in regulating the proliferation of liver and gastric cancer cells by directly targeting the 3'UTR of mRNAs encoding oncogenes and cell cycle activators (40,41). Analogous mechanisms may also contribute to the proliferative effect observed in insulin-secreting cells following the reduction of AATK or its intronic miRNA.

In conclusion, we have identified miR-338-3p and it host gene AATK as candidates for gene-based therapies or drug targets for approaches aiming at promoting β-cell proliferation and β-cell mass expansion. Moreover, we have shown that the expression of miR-338-3p and AATK is regulated by common mechanisms, activates overlapping downstream signaling pathways and has similar functional impacts on insulin-secreting cells. Whether miR-338-3p and AATK have, redundant, complementary or synergetic actions in promoting β-cell mass expansion under conditions of increased insulin needs remains to be determined.

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Figure legends

FIGURE 1. Intraductal delivery of AAV8-miR-338-3p sponge promotes β-cell proliferation. A, Schematic representation of the construct used to specifically block the activity of miR-338-3p in pancreatic beta-cells. The multimerized binding sites present on the "sponge" selectively sequester miR-338-3p and prevent its interaction with its endogenous targets. The expression of the sponge is driven by the rat insulin promoter insuring beta-cell-specific expression of large amounts of the construct. **B-I,** Mice were injected intraductally with 1×10^{12} viral genomes of AAV8-control sponge or AAV8-miR-338-3p sponge. Both expression cassettes also encoded the GFP reporter gene and were driven by the rat insulin promoter. Animals were analyzed 1 month after injection. **B,** Quantification of transduced islets per pancreas (%). **C,** Quantification of transduced β-cells per islets (%). **D,** Immunohistochemical analysis of GFP (green) and insulin (red) abundance in islets. Transduced cells were exclusively β-cells. **E,** β-cell and non β-cell replication was assessed by Ki67 and GFP co-immunostaining (%). **F,** Image showing examples of double-positive Ki67+/GFP+ islet cells (Ki67 staining in red; GFP+ cells in green; nuclear staining in blue). **G,** β-cell replication assessed by co-immunostaining with Ki67 and insulin. **H,** Ki67+ β-cells, red; β-cells, green; nuclei, blue; arrows indicate Ki67+ β-cells. **I,** β-cell mass was measured after injection of AAV8-control sponge or AAV8-sponge miR-338-3p in ICR mice by using insulin staining and weighting pancreas (mg). Results are expressed as mean ± SD; n=4-5 animals per group. *$p<0.05$ was considered significant versus control.

FIGURE 2. AATK expression is reduced in islets of insulin resistant animals. AATK mRNA levels were measured by qRT-PCR in islets from pregnant rats at different stages of gestation **A,** 6-week-old pre-diabetic db/db mice **B,** and diet-induced obese (DIO) mice fed a high-fat-diet for 8 weeks **C**. Data are the mean ± SD from 4 animals, normalized by 18S. *$p<0.05$ versus control.

FIGURE 3. miR-338-3p and AATK expression is reduced in INS832/13 cells treated with a cAMP-raising agent. miR-338-3p and AATK mRNA levels were measured by qRT-PCR in

INS832/13 cells exposed to 1mM IBMX for the indicated periods. Data were normalized by 18S or U6 levels for mRNAs and miRNAs, respectively. They represent the mean ± SD from 3 independent experiments. *p<0.05 versus control.

FIGURE 4. 17-β estradiol and the GLP1-analogue exendin-4 elicit AATK decrease.

AATK expression was measured by qRT-PCR in rat **A, C, D** and human **B, E** islets. **A, B,** Islets were treated for 48h with 100 nM of 17-β estradiol, **C,** 100 nM of the GPR30 agonist G1 or **D, E,** 100 nM of the GLP1 analogue exendin-4. Data are the mean ± SD from 3 independent experiments normalized by 18S. *p<0.05 versus control.

FIGURE 5. Silencing of AATK and inhibition of miR-338-3p in INS832/13 cells reproduce their decreased levels observed in islets of insulin resistant animal models. A, miR-338-3p expression and AATK mRNA levels were measured by qRT-PCR three days following transfection with siGFP or siAATK, normalized by U6 or 18S. **B,** AATK protein levels were assessed by western blot 72h after transfection of siGFP or siAATK and normalized by tubulin. **C,** Western blot quantification. **D,** INS832/13 cells were transfected with anti-miR-338-3p or with a scrambled sequence single stranded used as an anti-miR-control. miR-338-3p expression and AATK mRNA levels were measured by qRT-PCR and normalized to U6 or 18S levels. Results represent the mean ± SD from 3-6 independent experiments. * p<0.05 versus control.

FIGURE 6. Functional role of AATK and miR-338-3p in INS832/13 cells. INS832/13 cells were transfected for 72 hours either with anti-miR-338-3p (anti-338-3p), anti-miR-control (anti-ctrl), siAATK or siGFP as control. **A,** Cell replication was assessed by scoring the Ki67-stained cells. Insulin secretion. **B,** and insulin content. **C,** in response to 2 mM glucose as basal condition (Black bars) or 20 mM glucose, 10 μM forskolin, 100μM IBMX as a cocktail for stimulatory condition (grey bars). Insulin release is expressed as percentage of insulin content (IC). **D,** Cells were incubated for 24 hours with (+) or without (-) a mix of proinflammatory cytokines (10 ng/ml TNF-α, 0.1 ng/ml IL-1β, 30 ng/ml IFN-γ) or **E,** for 48 hours either with 0.5% BSA (-) or with 0.5% BSA coupled to 0.5 mM

palmitate (+). **D, E,** Apoptosis was assessed by staining the cells with Hoechst and counting the picnotic nuclei. **F,** Birc5, Foxm1, Cyclin D2, Igf1r, Irs2, Bcl2, Bcl-xl, and Bad expression were measured by qRT-PCR and compared with the level in cells transfected with a scrambled antimiR or siGFP as controls. Data are expressed as fold changes. Data are expressed as mean ± SD from 3-5 independent experiments. *, # $p<0.05$.

Figure 1

Figure 1. Jacovetti et al.

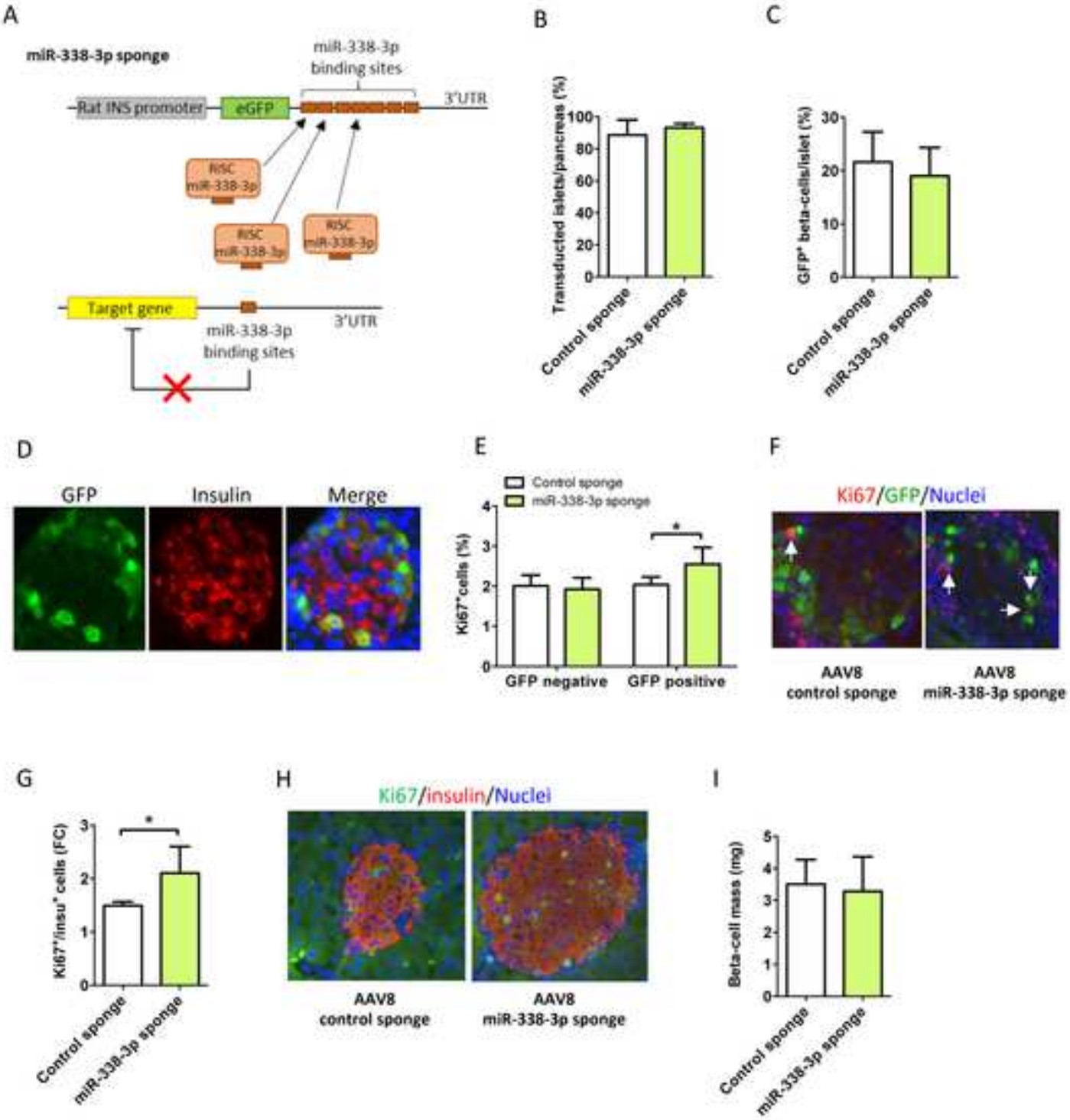

Figure 2

Figure 2. Jacovetti et al.

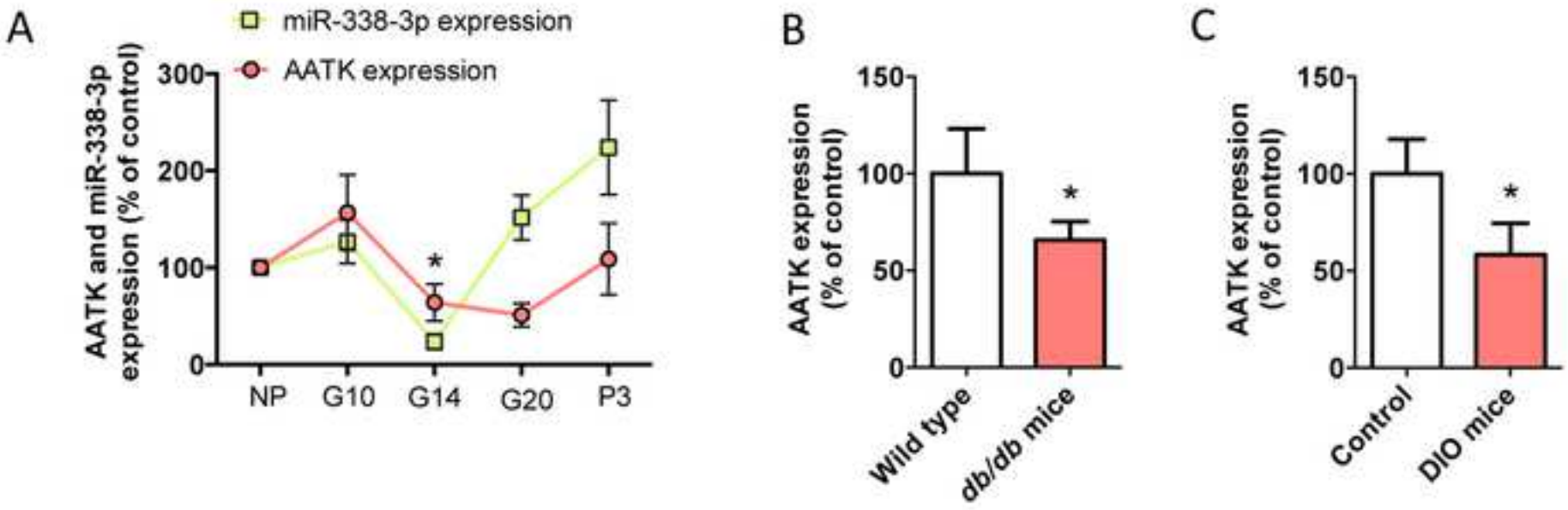

Figure 3

Figure 3. Jacovetti et al.

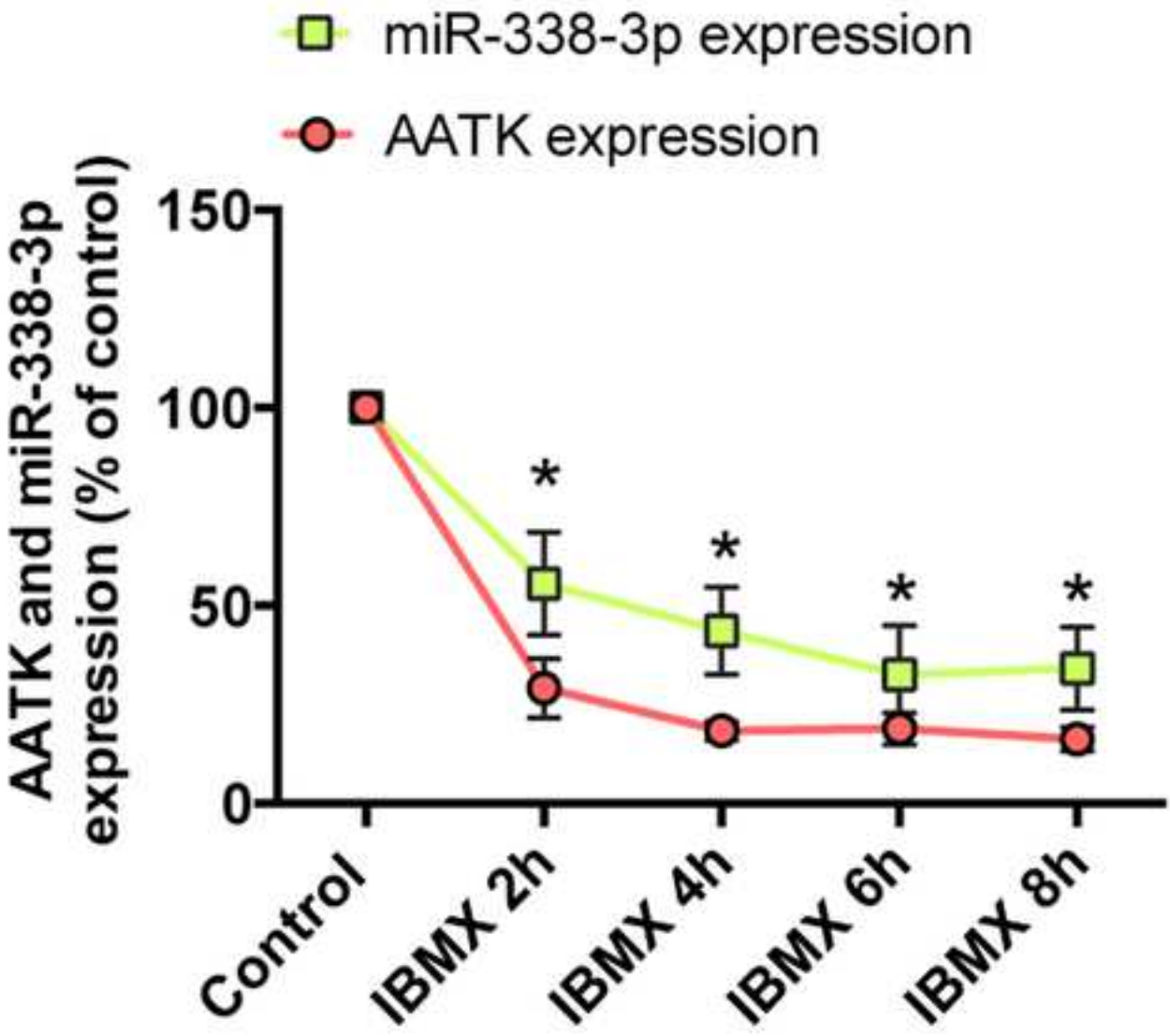

Figure 4

Figure 4. Jacovetti et al.

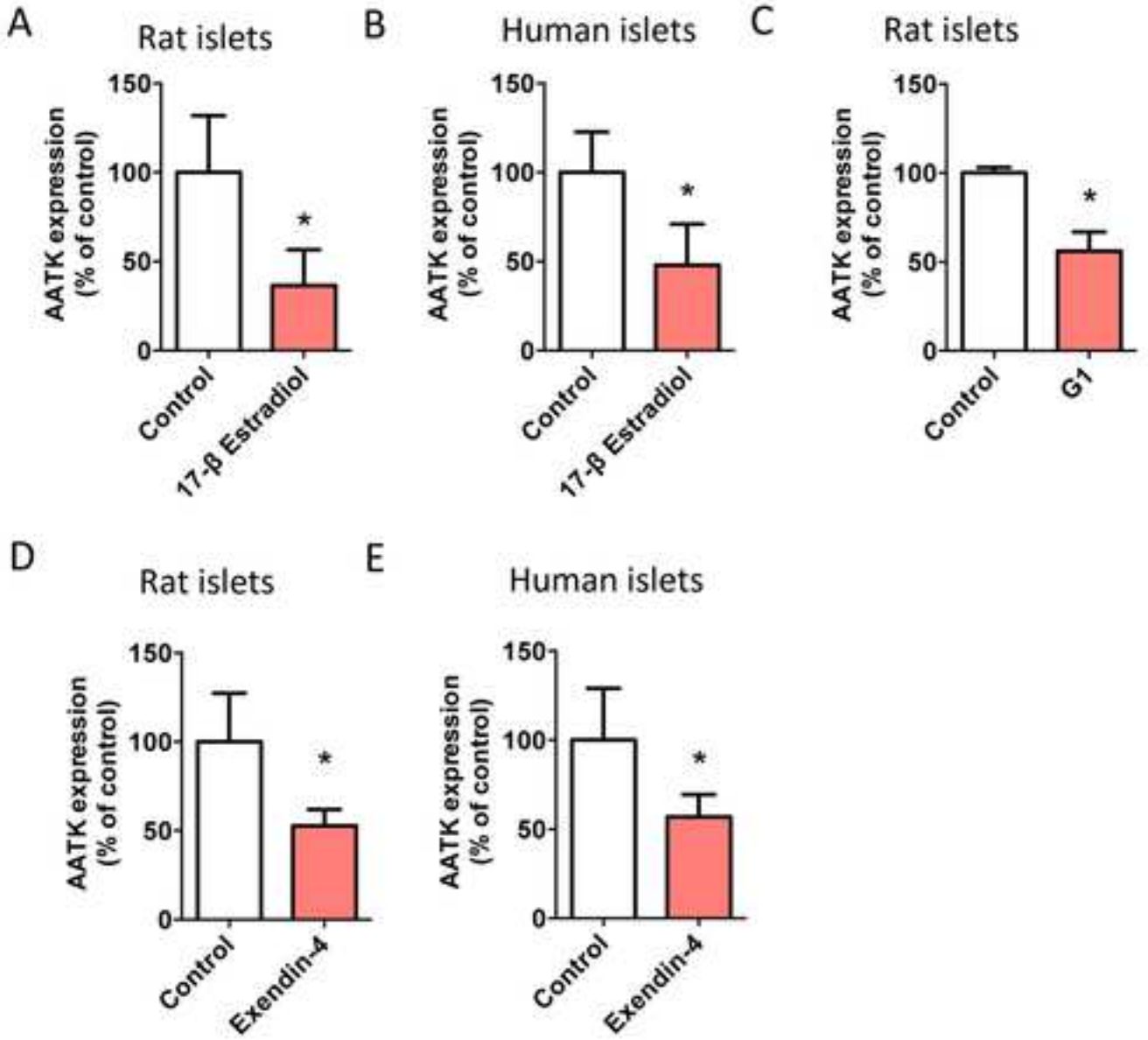

Figure 5

Figure 5. Jacovetti et al.

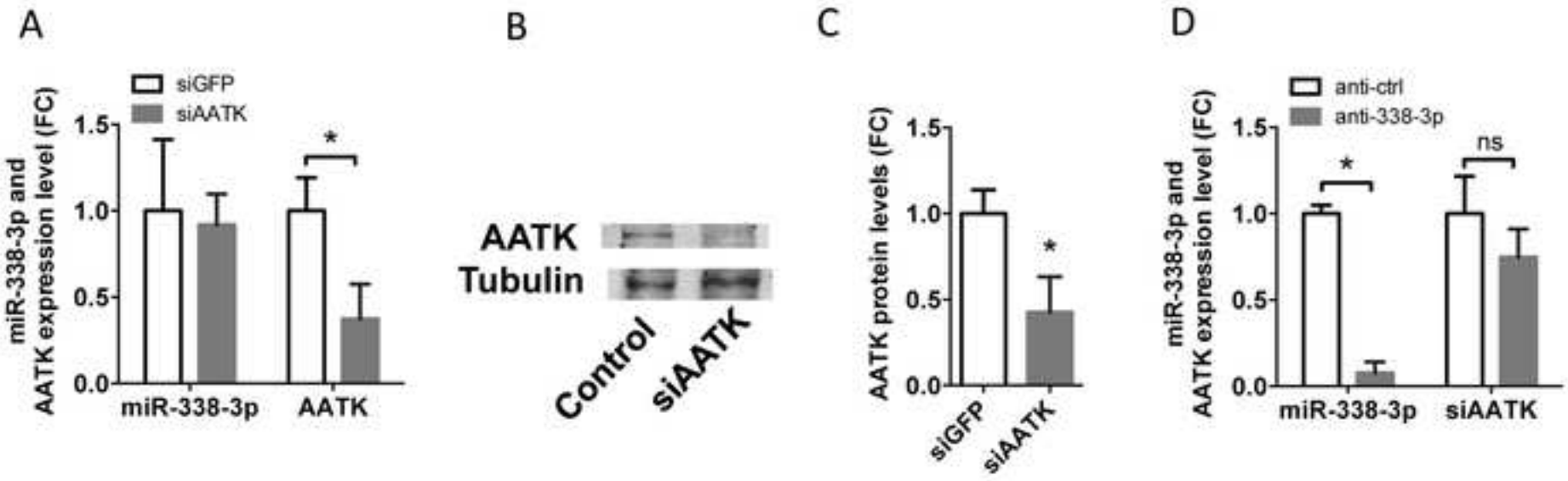

Figure 6

Figure 6. Jacovetti et al.