

Metformin and Rapamycin Reduce Pancreatic Cancer Growth in Obese Prediabetic Mice by Distinct MicroRNA-Regulated Mechanisms

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Metformin and Rapamycin Reduce Pancreatic Cancer Growth

in Obese Prediabetic Mice by Distinct MicroRNA-Regulated Mechanisms

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Abstract

Metformin treatment is associated with a decreased risk and better prognosis of pancreatic cancer (PC) in patients with type 2 diabetes, but the mechanism of metformin's PC growth inhibition in the context of a prediabetic state is unknown. We used a Panc02 pancreatic tumor cell transplant model in diet-induced obese (DIO) C57BL/6 mice to compare the effects of metformin and the direct mTOR inhibitor rapamycin on PC growth, glucose regulation, mTOR pathway signaling, and candidate microRNA (miR) expression. In DIO/prediabetic mice, metformin and rapamycin significantly reduced pancreatic tumor growth and mTOR-related signaling. The rapamycin effects centered on decreased mTOR-regulated growth and survival signaling, including increased expression of let-7b and cell cycle-regulating miRs. Metformin (but not rapamycin) reduced glucose and insulin levels, expression of miR-34a and its direct targets Notch, Slug, and Snail. Metformin also reduced the number and size of Panc02 tumor spheres *in vitro* and inhibited the expression of Notch in spheroids. Our results suggest that metformin and rapamycin can both inhibit pancreatic tumor growth in obese, prediabetic mice through shared and distinct mechanisms. Metformin and direct mTOR inhibitors, alone or possibly in combination, represent promising intervention strategies for breaking the diabetes-pancreatic cancer link.

Introduction

Type 2 diabetes (T2D) is a progressive metabolic disorder affecting nearly 30 million Americans (~9.3% of the population) and associated with obesity and increased risk of developing and dying from cancer (1). More specifically, the risk of developing pancreatic cancer (PC) is twice that of the non-diabetic population (2; 3). Prediabetes,

defined as having elevated fasting glucose and impaired glucose tolerance, is rising in prevalence even faster than T2D, with nearly 90 million prediabetic Americans (including 51% of Americans over 65 years of age) (1). This is of particular concern regarding PC since >80% of PC cases develop after age 60 (2). Putative mechanistic explanations for the link between prediabetes, T2D and pancreatic cancer include chronic hyperinsulinemia, oxidative stress, and inflammation (4).

Epidemiologic studies suggest that metformin, a widely used drug for the treatment of T2D (5), is associated with reduced risk among diabetics of being either diagnosed with, or dying from, PC (6-8). However, whether these associations reflect a genuine protective action of metformin, as compared to other anti-diabetic drugs, or whether they are the result of confounding (since T2D patients prescribed metformin may have very different clinical characteristics than T2D patients taking sulfonylurea or other glucose lowering medications), remains unclear (9; 10). Moreover, the potential PC chemoprotective effects of metformin are not well understood

Metformin can diminish hepatic glucose output, resulting in improved insulin sensitivity; it can also exert direct effects on tumor cell signaling (11; 12). Direct cellular effects of metformin involve inhibition of ATP production, increased AMP kinase (AMPK) activity (13), and inhibition of the mammalian target of rapamycin (mTOR), a complex that couples protein synthesis to external growth factors and intracellular energy stores (14). Recent evidence indicates that this affect on mTOR is accomplished through transcription factor, Sp1-mediated downregulation of IGF-1 signaling (12). Inhibition of mTOR decreases cell proliferation in several cancer cell lines, including pancreatic cancer cells (12; 15).

Rapamycin, a specific mTOR inhibitor, inhibits several processes involved in tumor cell proliferation and survival (16; 17). Despite their shared mTOR inhibitory effects, rapamycin and metformin differentially influence other targets, including several microRNAs (miRs), noncoding RNA molecules that post-transcriptionally regulate gene expression (18). MiRs regulate cellular processes involved in cancer initiation, recurrence, and metastasis (19).

To our knowledge, a direct comparison between the effects of metformin and rapamycin on PC outcomes in the context of diet-induced obesity (DIO) and prediabetes has not yet been reported. We chose to use a model of prediabetes characterized by hyperglycemia and insulin resistance since prediabetes is an established risk factor for PC (1), and prediabetics are becoming more prevalent in the US and represent an important population for cancer prevention and treatment. The aim of this study was to compare metformin and rapamycin with respect to PC growth, systemic glucose metabolism, mTOR pathway signaling, candidate miR expression, and CSC and EMT characteristics using a syngeneic murine pancreatic cancer transplant model in DIO/prediabetic mice.

Materials and Methods

Mice, diet, and experimental design

Mice were singly housed in a semibarrier facility in the Animal Resource Center at the University of Texas; the Institutional Animal Care and Use Committee approved all experimentation.

Seventy-five male 6-week old C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME) were administered a DIO diet (Research Diets; #D12492) throughout the study.

After 15 weeks of DIO, mice were randomized (25/group) to receive either a) metformin (250 mg/kg continuously in drinking water), b) rapamycin (2.5 mg/kg, administered i.p. every other day; LC Laboratories, Woburn, MA), or c) vehicle control (0.1% DMSO, in 0.9% saline, i.p. every other day) for 5 weeks. The metformin group received vehicle injections every other day, while the rapamycin and vehicle control groups were provided *ad libitum* access to drinking water without metformin. The rapamycin dose of 2.5 mg/kg was chosen to achieve inhibition of phosphorylation of mTOR based on our previous studies (20). At week 19 on study (4 weeks of drug treatment), 10 mice/group underwent GTT and ITT (as previously described (20; 21)) and then were killed and their tissues collected and stored. At 20 weeks on study, the remaining mice ($n=15$ /group) were injected (s.c.) in the right flank with 5×10^5 Panc02 murine pancreatic cancer cells (maintained in McCoy's media as previously described (22)), and continued on their diet regimen for 4 additional weeks while tumor growth was monitored. Food intake and body weights were recorded weekly. At study termination (week 24), all mice were fasted for 6 hours, anesthetized by CO₂ inhalation and then blood was collected via cardiac puncture. Blood samples were centrifuged at $9,300 \times g$ for 5 minutes and serum collected and stored at -80°C . Pancreatic tumors were either snap-frozen in liquid nitrogen and stored at -80°C , or fixed with 10% neutral-buffered formalin overnight, paraffin embedded, and used for histologic and immunohistochemical analyses as described below.

Serum hormones and adipokines

Serum insulin, IGF-1, resistin, and adiponectin concentrations were measured in mice (vehicle $n=11$; metformin $n=14$; rapamycin $n=14$) using a LINCOplex® bead-based

array assay (Millipore Corporation, Billerica, MA) on a BioRad BioPlex® multianalyte detection system (BioRad, Inc., Hercules, CA) according to manufacturer's directions.

Histopathology and Immunohistochemistry

Formalin-fixed pancreatic tumors were embedded in paraffin, cut into 4- μ m sections, and processed for either hematoxylin and eosin (H&E) or immunohistochemical staining at the Histology Core Laboratory at The University of Texas M.D. Anderson Cancer Center (Smithville, TX). Antibodies used for immunohistochemical analyses were optimized using both positive and negative controls. Slides were deparaffinized and sequentially hydrated in ethanol and water. Antigen retrieval was achieved by microwaving slides for 10 min in 10mM citrate buffer. Endogenous peroxidase was quenched with 3% hydrogen peroxide for 10 min. Nonspecific binding was inhibited with Biocare blocking reagent (Biocare Medical, Concord, CA) for 30 min at RT followed by incubation with primary antibodies (previously described (20)) diluted in blocking buffer. Slides were washed five times with PBS and developed with diaminobenzidine followed by hematoxylin counterstain.

For each IHC marker, randomly selected slides (8 mice/group) were digitized using the Aperio Scanscope System (Scanscope XT; Aperio Technologies, Vista, CA). Analysis of immunohistochemically-stained tumor sections was performed on 4 fields/slide using the standard ImageScope membrane/cytoplasmic-specific algorithms to quantify the percentage of cells stained positive for pAkt, pmTOR, pACC, cyclin D1, and cdk4 (cdk4, control and metformin groups had n=7).

Gene expression analysis

RNA was extracted from untreated and treated pancreatic tumor spheres (n=3/group) and tumor tissue (n=6/group) using TRI Reagent® (Sigma-Aldrich, St. Louis, MO) according to manufacturer's instructions. Complimentary DNA was synthesized from extracted RNA using the high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA) according to manufacturer's directions. For miR analysis, cDNA was synthesized using TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems). Gene expression for EMT and miRs were measured by quantitative real-time polymerase chain reaction (qRT-PCR) using Taqman® gene expression assays with TaqMan® Universal PCR Master Mix (Applied Biosystems) on an Applied Biosystems ViiA 7™ Real-Time PCR System. Expression data for mRNA were normalized to the housekeeping gene, β -actin, whereas miR expression levels were normalized to miR-16 expression. Relative quantitation (RQ) was calculated using the $\Delta\Delta C_t$ method.

Notch1 Luciferase assay

Luciferase assays were performed to assess whether miR-34a targets the Notch 3'UTR in Panc02 cells using LightSwitch 3'UTR Reporter GoClones, LightSwitch microRNA mimics, LightSwitch Assay reagents, and associated protocols (SwitchGear Genomics, Menlo Park, CA). The experiment was repeated three independent times and data are presented as mean \pm SEM. The mutant Notch1 3'UTR vector was generated from LightSwitch Notch1 3'UTR GoClone vector DNA (SwitchGear Genomics) using the QuikChange Lightning Multi Site-Directed Mutagenesis Kit (Agilent Technologies,

Santa Clara, CA) following manufacturer's instructions. Briefly, QuikChange reagents, Notch1 vector (100ng), and mutagenic primers (Notch 201, 380ng; Notch 930, 200ng) were combined and cycled through the thermal cycling program indicated in the manufacturer's protocol. Mutagenic primers were designed to alter the sequence of the Notch1 3'UTR at the two sites complimentary to the miR-34a seed sequence (target sites) as specified by miRDB, TargetScan, and PicTar. Mutant Notch1 3'UTR GoClone vector was amplified and selected per manufacturer's instructions. Plasmids were sequenced at the University of Texas at Austin DNA Core Facility using the following primers:

Forward- GGAAGTACATCAAGAGCTTCGT, Reverse-
CCCCCTGAACCTGAAACATAAA.

Tumor sphere assay

Panc02 cells were seeded in 96-well low-adherence plates (Corning Incorporated, Corning, NY) in a serial dilution (from 2000 to 0 cells/well; 6 replicates per dilution), as previously described (23). Cells were cultured under serum-free conditions using McCoy's media supplemented with insulin, B27, N2, EGF and FGF (all from BD Biosciences, San Jose, CA) with/without metformin (0.5 mM) or rapamycin (0.1 mM). Cell media was changed weekly and tumor spheres were quantified after 14 days. Pancreatic tumor spheres (only those with >5 cells/sphere) were quantified by visual counting at 20X magnification. The diameter of each tumor sphere was measured using Spot software (Diagnostic Instruments, Sterling Heights, MI). Three independent experiments were performed.

Identification of the CD24⁺CD44⁺ population in Panc02 Cells

Panc02 cells (~50,000 cells/well) were seeded into 6-well plates (Corning Incorporated) and left overnight in a 37°C incubator at 5% CO₂. The following day, cells were washed with PBS and then treated with fresh complete McCoy's media in presence or absence of 0.5 mM metformin. After 24-72 hours of treatment, cells were washed with PBS, harvested and stained with anti-mouse antibodies against phycoerythrin (PE)-CD24, and allophycocyanin-(APC)-CD44 (BD Pharmigen) as previously described (23). Each antibody was used at saturating concentrations optimized by initial titrations for flow cytometry staining. The negative control panels were mixtures of isotype controls (E-Bioscience) diluted to an identical immunoglobulin concentration. Cells were analyzed using Guava Flow Cytometry (Guava Technologies, Hayward, CA) and at least 15,000 events were collected for each sample.

Cell cycle analysis

Panc02 cells were treated with complete McCoy's media in presence or absence of either metformin (0.5mM) or rapamycin (0.1mM). Panc02 cells were stained with propidium iodide (PI) following the manufacturer's instruction (Guava[®] Cell Cycle Reagent, Millipore). Cells serum starved for 24 hours were used as a control for G0/G1 phase (quiescent phase). After a 30-minute incubation with PI, cells were analyzed using Guava EasyCyte Flow Cytometry. Histograms exhibiting G0/G1, S and G2/M phases were generated using Modofit LT Software (Verity Software House, Topsham, ME). A minimum of three independent experiments was run in which each condition was tested in six replicates.

Statistical analysis

All statistics were performed using GraphPad Prism 4 (GraphPad Software, San Diego, CA, USA). One-way ANOVA was used to assess differences between vehicle and treatment groups. Two-tailed Student's t-test was used to assess differences between vehicle control and an individual treatment group. Values of $P < 0.05$ were considered statistically significant.

Results

Body weight, caloric intake, glucose tolerance and serum factors

An obese phenotype was attained in all mice prior to drug treatment initiation (15 weeks) as indicated by the increased mean body weight (40.8 ± 4.7 g) relative to the mean baseline measurement (20.3 ± 29.0 g, $p=0.0005$; Fig. 1a). After just four weeks of treatment, metformin significantly reduced mean body weight relative to vehicle group (41.1 ± 2.7 g vs. 44.8 ± 3.2 g, respectively; $p=0.0062$), whereas rapamycin treatment did not (46.2 ± 3.5 g; Fig. 1a). Despite these varying effects on body weight, the treatments had no effect on caloric intake (Figure S1). As expected, the metformin group exhibited improved glucose clearance ($p=0.027$) as well as enhanced insulin sensitivity ($p=0.038$) relative to vehicle (Fig. 1b-c). The rapamycin group displayed worsened glucose sensitivity ($p=0.023$), but it did not affect insulin sensitivity relative to the vehicle group ($p=0.71$; Fig. 1b-c). Fasting levels of circulating insulin was reduced in response to metformin treatment (1.9 ± 0.8 ng/mL vs. 2.7 ± 0.6 ng/mL, $p=0.03$; Fig. 1d). Mean

circulating levels of the mitogen IGF-1 were non-significantly reduced by metformin treatment relative to vehicle (400 ± 35 ng/mL vs. 420 ± 38 ng/mL; $p=0.08$; Fig. 1e). No statistical differences were detected between the rapamycin group and the vehicle group regarding insulin (2.6 ± 1.1 ng/mL) or IGF-1 (367 ± 50 ng/mL) levels. Resistin was decreased in the metformin group (2.8 ± 0.4 ng/mL vs. 3.8 ± 1.5 ng/mL; $p=0.02$) as well as in the rapamycin group (2.05 ± 0.4 ng/mL) relative to vehicle ($p<0.001$; Fig. 1f). Serum adiponectin, relative to control, was significantly increased in the metformin group (13.2 ± 1.6 μ g/mL vs. 10.5 ± 2.3 μ g/mL; $p=0.05$), and decreased in the rapamycin group (6.6 ± 0.7 μ g/mL; $p<0.001$; Fig. 1g).

Effect of metformin on tumor growth and intracellular signaling intermediates

At 20 weeks on study, Panc02 cells were injected in the right flank of mice ($n=15$ /group) that continued on their diet regimen for 4 additional weeks while tumor growth was monitored. At study termination, tumors were collected from mice. Five mice (control, $n=4$; metformin, $n=1$; rapamycin, $n=1$) were censored from the study due to injection errors. Both metformin (0.62 ± 0.27 g; $p=0.04$) and rapamycin (0.25 ± 0.33 g; $p<0.01$) significantly reduced tumor burden compared to vehicle (0.90 ± 0.22 g), although the effect of rapamycin was more dramatic (Fig. 2a). Metformin ($10.6 \pm 3.5\%$, $p=0.006$) and rapamycin ($19.1 \pm 5.7\%$, $p=0.05$) both significantly decreased tumoral mTOR activity, assessed by measuring phosphorylation of mTOR, relative to untreated tumors ($43.7 \pm 8.7\%$; Fig. 2b-c). Despite inhibitory effects on phosphorylation of mTOR, rapamycin treatment ($86.3 \pm 1.9\%$; $p=0.024$), but not metformin treatment ($77.2 \pm 3.3\%$), resulted in significantly increased pAKT (Fig. 2b-c). Metformin ($53.7 \pm 3.6\%$, $p=0.04$) and rapamycin ($52.6 \pm 3.2\%$, $p=0.06$) resulted in a mean increase in phosphorylation of

ACC, an inhibitory phosphorylation indicative of AMPK activity as compared to vehicle ($37.9 \pm 5.6\%$; Fig. 2b-c).

Metformin increases expression of miR-34a

Since miRNAs are aberrantly expressed in many types of cancers, we screened the expression of miRNAs highly regulated in pancreatic cancer during obesity and diabetes (11) using q-RT-PCR (Supplementary Table 1). Validation analysis was performed for those miRNAs that had p -values ≤ 0.15 . Validation analysis showed that metformin significantly increased miR-34a expression (RQ = 2.15 ± 1.07 vs 1.07 ± 0.43 , $p=0.046$; Fig. 3a) and downregulated Notch (RQ = 0.56 ± 0.42 vs 1.10 ± 0.49 , $p=0.041$; Fig. 3b), an EMT-associated target of miR-34a (24-27), relative to untreated/vehicle controls in both tumors (RQ = 0.61 ± 0.07 vs 1.50 ± 0.15 , $p=0.041$) and *in vitro* Panc02 cells (RQ = 0.65 ± 0.20 vs. 1.13 ± 0.09 in untreated cells, $p=0.034$). When Panc02 cells were transfected with Notch reporter plasmid and a miR-34a mimic, luciferase signal was significantly decreased relative to control ($p=0.007$, Fig. 3c), confirming that Notch is regulated by miR34-a in Panc02 cells. Moreover, after mutating the two miR-34a target sites within the Notch 3' UTR in the reporter plasmid, no effect of miR-34a on luciferase signal was observed (Fig. 3d), indicating that the inhibition of luciferase activity seen in the Notch + miR-34a condition is due to direct binding of miR-34a to Notch in Panc02 cells.

Metformin decreases expression of Snail, Vimentin, and Slug in tumor samples

Besides Notch, additional EMT markers were found regulated by metformin in

tumors. As shown in Figure 4, mRNA expression levels of Snail ($p=0.049$) and Vimentin ($p=0.049$) were significantly decreased in the metformin group relative to vehicle. Expression of Slug was also reduced in response to metformin; however, this finding only approached significance ($p=0.059$).

Rapamycin increases expression of let-7b in tumors and inhibits cell cycle progression

Let-7b expression in Panc02 tumors was significantly increased in the rapamycin group relative to vehicle controls (RQ= 1.49 ± 0.22 vs. 1.05 ± 0.37 ; $p=0.044$; Fig. 5a). Metformin had no effect on let-7b expression (0.99 ± 0.05 ; $p=0.88$; Fig. 5a); however induction of let-7b expression by metformin has been reported in pancreatic cancer cells (28). Immunohistochemical staining of downstream cell cycle regulatory proteins corroborates this finding by demonstrating that rapamycin significantly reduced expression of cdk4 and cyclin D1 ($28.2 \pm 3.8\%$, $p<0.001$ and $32.0 \pm 4.7\%$, respectively, $p=0.008$; Fig. 5b-c) relative to vehicle ($43.5 \pm 4.0\%$ and $48.4 \pm 3.6\%$, respectively). Metformin had no significant effect on cyclin D1 ($39.4 \pm 0.9\%$) relative to vehicle although it reduced cdk4 ($30.8 \pm 4.8\%$, $p<0.001$; Fig. 5b-c).

Treating Panc02 cells with rapamycin (0.1mM) significantly increased the percentage of cells arrested in the G0/G1 phase of the cell cycle ($50.7 \pm 1.7\%$ of positive cells) compared to untreated cells ($36.4 \pm 1.0\%$; $p=0.011$; Fig. 5d-e). Rapamycin also reduced the percentage of cells in S phase ($10.5 \pm 0.45\%$) compared to untreated cells ($21.3 \pm 1.4\%$; $p=0.048$; Fig. 5d-e). No major differences were observed in the G2/M phase between treatments. Metformin did not impact cell cycle kinetics relative to untreated cells. Serum starved Panc02 cells were used as a positive control, causing 70%

of total cells to arrest in G0/G1 phase. The decrease in cell cycle progression was not driven by increased apoptosis, since we did not observe any difference in the generation of DNA fragments in rapamycin-treated Panc02 cells, compared to untreated cells (Figure S2).

Metformin reduced pancreatic tumor sphere formation

Since metformin drastically inhibited pancreatic tumor growth *in vivo*, we investigated whether this effect was achieved by acting on CSCs, a population with enhanced proliferation and self-renewal properties and characterized by the expression of the cell surface markers CD24 and CD44 (29). We observed that the CD24⁺/CD44⁺ population of Panc02 cells was significantly reduced by metformin compared to untreated controls ($63.5 \pm 8.5\%$ vs. $76.7 \pm 9.4\%$; $p=0.024$; Fig. 6a-b). Since tumor sphere-forming capacity has been used to define a more tumorigenic cell population (29), we grew Panc02 cells into tumor spheres in the presence or absence of metformin and assessed spheroid size and number. After 14 days, metformin (relative to untreated) significantly reduced mean size of Panc02 tumor spheres grown under non-adherent culture conditions by approximately 50% regardless of initial cell seeding number (from 500-2000 cells; Fig. 6c-e). Metformin significantly decreased expression of Notch in tumor spheres (RQ= 0.23 ± 0.006 vs 1.17 ± 0.64 ; $p = 0.037$) compared to untreated tumor spheres (Fig.6f).

Discussion

Our findings establish that both metformin and rapamycin inhibit pancreatic

tumor growth in a mouse model of DIO and prediabetes through common and divergent mechanisms. We found that both pharmacologic agents blunted the DIO-associated tumoral activation of mTOR, a crucial complex involved in the regulation of protein translation. Additional data, however, indicate several distinct systemic and intracellular effects of metformin relative to rapamycin, including effects on glucose clearance, circulating levels of energy balance-responsive hormones and growth factors, miR expression, cell cycle regulatory protein expression, and stem cell properties. These findings support epidemiologic data suggesting that metformin, but not other glucose-lowering therapies, may decrease PC risk in the prediabetic and diabetic population as well as minimize PC-related death in diabetics (6-8). They also provide novel insights regarding the shared and distinct mechanisms associated with the anticancer effects of metformin versus the direct mTOR inhibitor, rapamycin.

We have previously shown that rapamycin exerts significant tumor growth-inhibitory effects in association with diminished mTOR signaling in normoweight mice injected with the same Panc02 tumors used in the present studies (20). In the current report, we show this growth-prohibitive effect of rapamycin in the context of obesity and insulin resistance. Additionally, these findings suggest that a potential mechanism for the inhibitory effects of rapamycin is the increase in let-7b, a miR involved in the regulation of cell proliferation and differentiation, at least in part through regulation of cell cycle-regulatory proteins (30-32). In fact, rapamycin treatment resulted in reduced expression of cdk4 and cyclin D1. The composite effect of these outcomes in response to rapamycin treatment was cell cycle inhibition, as evidenced by an accumulation of cells in G0/G1, a finding corroborated by others (33; 34).

Specific mTOR inhibition with rapamycin resulted in tumor growth suppression but unfortunately, was also accompanied by decreased insulin responsiveness and hyperglycemia, consistent with previous reports (35). In contrast, metformin significantly blunted pancreatic tumor growth (albeit to a lesser extent than rapamycin) without apparent adverse metabolic effects, similar to previous observations (36). In our study, we found that metformin resulted in improved glucose and insulin metabolism and a serum profile (decreased IGF-1, insulin, resistin; increased adiponectin) associated with normalization of metabolic state and tumor growth inhibition in DIO mice (20; 37; 38). Similar to previous reports (39), we found that metformin induced tumoral expression of miR-34a, a miR typically lost during cancer progression, including pancreatic (24; 40) and has been shown to contribute to reduced survivability in those diagnosed with pancreatic cancer (41). MicroRNA-34a suppresses EMT by acting as a negative regulator of the signaling pathway activated by transforming growth factor- β (TGF- β), a master regulator of EMT (42). Interestingly, metformin has been shown to inhibit TGF- β -induced EMT in breast cancer cells, particularly stem cell-enriched populations, as well as alter expression of EMT mediators (43). Expression of these mediators, such as Snail is intimately linked to TGF- β signaling and miR-34a expression. Specifically, as TGF- β increases, expression of miR-34a is diminished and Snail is enhanced (42), while re-expression of miR-34a reduces expression of Snail and other EMT mediators such as Notch and Slug (44). Our *in vitro* results corroborated these findings by demonstrating that targeted binding of miR-34a regulated expression of Notch in Panc02 cells, further suggesting that metformin may modulate EMT-related targets, such as Notch, in a miR-34a-dependent manner in Panc02 tumors. Panc02 tumor spheres also had reduced Notch

expression in response to metformin. Dysregulation of Notch signaling, a targeted perturbation in many cancer types (24; 45), has been shown to enhance hypoxia-induced tumor cell migration and invasion and influence expression of Snail and Slug (46; 47). Additionally, metformin reduced the CD24⁺/CD44⁺ cell population and decreased the tumor sphere-forming capacity of Panc02 cells, corroborating previous findings that metformin significantly reduced CSC enrichment in pancreatic tumors (28). The importance of this TGF- β /miR34a pathway cannot be understated in the context of Panc02 cells, which have a mutation in Smad4 (a downstream regulator of TGF- β signaling), a genetic anomaly that is found in approximately 55% of all pancreatic ductal adenocarcinomas and is predictive of poor prognosis (48). Loss of Smad functioning can alter miR expression profiles (49) but metformin, in our study, overcame the loss of miR-34a. This suggests that metformin may be able to blunt the protumorigenic nature of genetic alterations such as Smad4 mutation, possibly in part through negatively regulating the EMT.

In summary, these preclinical findings suggest metformin and rapamycin can both inhibit diabetes-associated pancreatic tumor growth through common and distinct microRNA-mediated mechanisms. We conclude that metformin and direct mTOR inhibitors, alone or possibly in combination, represent promising interventions that should be tested in future translational studies evaluating new strategies for breaking the diabetes-pancreatic cancer link.

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Authors' contributions included the following: V.C. performed the majority of in vitro and animal studies, analyzed and interpreted the data, wrote and critically revised the article. L.M.L. helped perform animal study, analyzed and interpreted the data and

critically revised the article. K.L.D. performed miR experiments and critically revised the article. S.M.D. conceptualized and assisted with the in vitro experiments. J.H. analyzed and interpreted immunohistochemical images and critically revised the article. R.K. and M.N.P. conceptualized the study and critically revised the article. S.D.H. (the guarantor) conceptualized the study, coordinated the research team, interpreted the data and revised the article.

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Figure Legends

Figure 1. Effect of metformin versus rapamycin on body weight, insulin sensitivity and serum hormones in obese/prediabetic mice. Treatment conditions (n=15/group): vehicle (0.1% DMSO in saline); metformin (250 mg/kg); rapamycin (2.5 mg/kg). Due to Panc02 cells injection error, 5 mice were censored from the study (vehicle=11 mice; metformin=14 mice ; rapamycin=14 mice). (a) Mean body weight, recorded weekly until study termination (b) GTT and (c) ITT performed prior to Panc02 cell injection at week 19 and 20, respectively (n=10/group). Fasting sera harvested at study termination were analyzed for levels of (d) insulin, (e) IGF-1, (f) resistin, and (g) adiponectin. The bar graph represents mean \pm SD. * p <0.05 by two tailed Student *t* test.

Figure 2. Metformin and rapamycin decreased tumor weights in obese prediabetic mice. (a) Scatter plot depicting tumor weights measured at study termination in mice receiving either metformin (250 mg/kg) (n=14), rapamycin (2.5 mg/kg) (n=14) or vehicle (0.1% DMSO in saline) (n=11). (b-c) Representative images of pancreatic tumors analyzed by immunohistochemical staining of pAkt, pmTOR, and pACC. (c) Quantification of pAkt, pmTOR and pACC positively-stained cells in tumor tissues. Images captured at 40X magnification. Scale bar represents 100nm. Data are expressed as mean \pm SEM number of positive cells/field in tumors (n=8/group). * p <0.05 by one-way ANOVA.

Figure 3. Metformin induced miR-34a expression in pancreatic tumors through direct binding to Notch. (a) Bar graph of miR-34a expression in pancreatic tumor

tissues (n=6/group). Data were normalized based on U6 expression. The bar graph represents mean \pm SEM. * p <0.05 by two tailed Student t test. (b) Quantification of Notch mRNA expression, relative to β -actin, in tumor samples and Panc02 cells. The bar graph represents mean \pm SD. * p <0.05 by two tailed Student t test. (c) Luciferase assay performed on Notch-transfected Pan02 cells in 3 independent experiments. (d) Luciferase assay performed on mutated Notch-transfected Pan02 cells in 3 independent experiments (mutations at two sites in Notch 3' UTR that are complimentary to the miR-34a seed sequence). The bar graph in c) and d) represents mean \pm SEM. * p <0.05 by one-way ANOVA.

Figure 4. Metformin decreased expression of EMT markers in pancreatic tumors from obese mice. mRNA expression of (a) Snail ($p=0.049$), (b) Vimentin ($p=0.049$), and (c) Slug ($p=0.059$) in metformin (250 mg/kg)- and vehicle (0.1% DMSO in saline)-treated tumors (n=6/group). The bar graph represents mean \pm SEM. * p <0.05 by Student t test.

Figure 5. Rapamycin increased let7b level in pancreatic tumors and inhibited cell cycle progression. (a) Bar graph representing mean let-7b mRNA expression in tumor tissues (n=3-6/group). The bar graph represents mean \pm SEM. * p <0.05 by Student t test. (b) Representative images of tumors immunohistochemically stained for the cell cycle-regulator proteins cdk4 and cyclin D1 and (c) respective quantification of positively-stained cells (n=8/group). Scale bar represents 100nm. (d) Cell cycle analysis (propidium iodide staining) of Panc02 cells treated with metformin (0.5 mM) or rapamycin (0.1 mM)

for 24 hours (n=3/treatment in six replicates). (e) Histogram representation of the cell cycle phase distribution. The bar graph represents mean \pm SEM. * p <0.05 by one-way ANOVA. The white sphere in Figure 5b (CND1 expression for the rapamycin-treated group) is the cellular outline of a large adipocyte, since histological processing with organic solvents extracts the lipid content of cells.

Figure 6. Metformin decreased tumor-sphere formation and EMT-associated genes expression in vitro. (a) Distribution and (b) quantification of the CD24⁺CD44⁺ population in Panc02 cells treated with metformin (0.5 mM) for 24 hours (p =0.024). (c) Representative image of Panc02 cells treated with metformin (0.5 mM) for 14 days, (d) quantification of the mean diameter and (e) number of tumor spheres of Panc02 cells treated with metformin (0.5 mM) for 14 days, relative to untreated control cells. (f) Bar graph representing Notch mRNA expression in pancreatic tumor spheres treated with metformin (0.5mM) or control after 14 days of treatment (n=3/treatment in six replicates). The bar graph represents mean \pm SD. * p <0.05 by Student *t* test.

Supplemental Figure 1 (S1). Effect of metformin versus rapamycin on caloric intake in obese/diabetic mice. Caloric intake was measured throughout the study in C57BL/6 male mice fed the DIO diet and treated with either metformin (250mg/kg), rapamycin (2.5 mg/kg) or vehicle (0.1% DMSO in saline). The bar graph represents mean \pm SD. No differences were observed between vehicle and treatment conditions.

Supplementary Table 1. Screening of miRNAs expression in tumor samples treated with metformin or rapamycin in pre-diabetic obese mice. Expression profile of highly regulated miRNAs in pancreatic cancer during obesity and diabetes performed in pancreatic tumors (n=3/group) by q-RT-PCR. Statistical analysis was performed by two-tailed Student *t* test.

Supplemental Figure 2 (S2). Rapamycin treatment did not induce apoptosis in Panc02 cells. (a-b) Level of DNA fragmentation was measured in Panc02 cells exposed to rapamycin (0.1 mM) or regular growth media for 24 h using a Bromodeoxyuridine (BrdU) immunofluorescence staining kit. BrdU positive cells were detected by flow cytometry (n=3/treatment). The bar graph represents percentage \pm SD. * p <0.05 by Student *t* test.

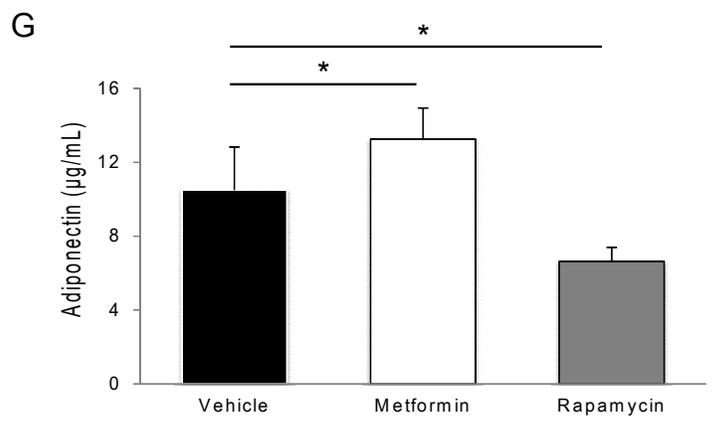
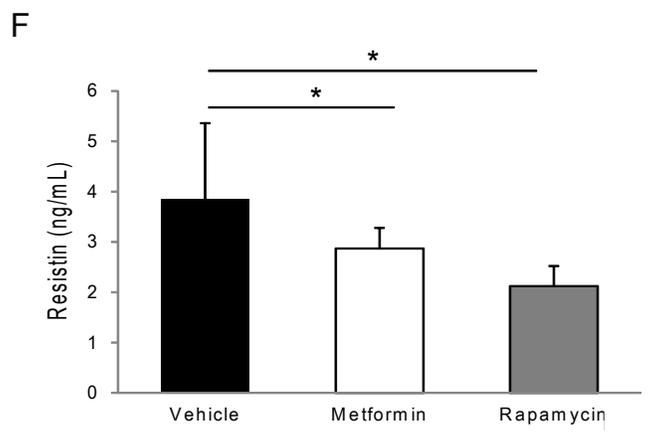
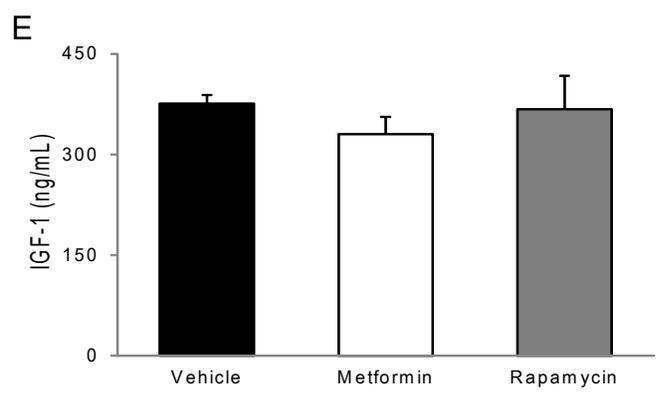
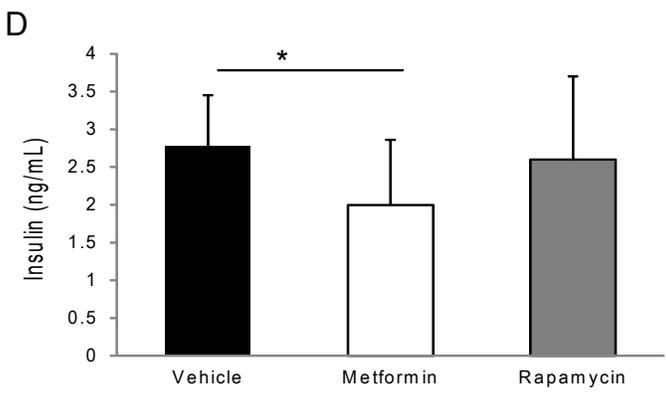
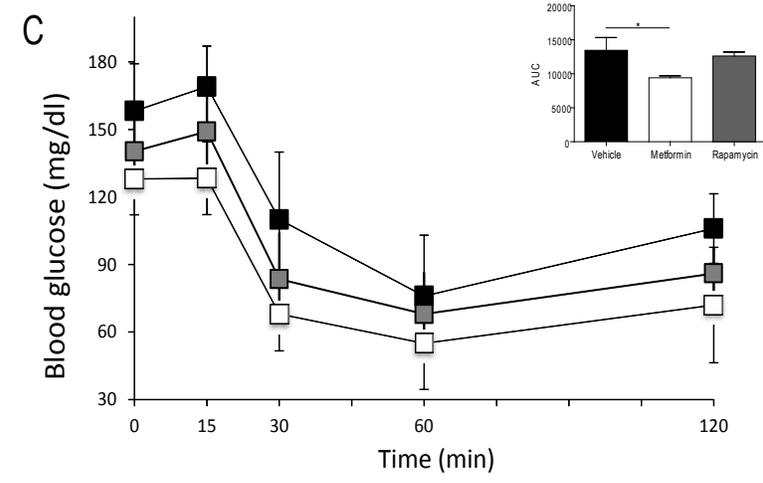
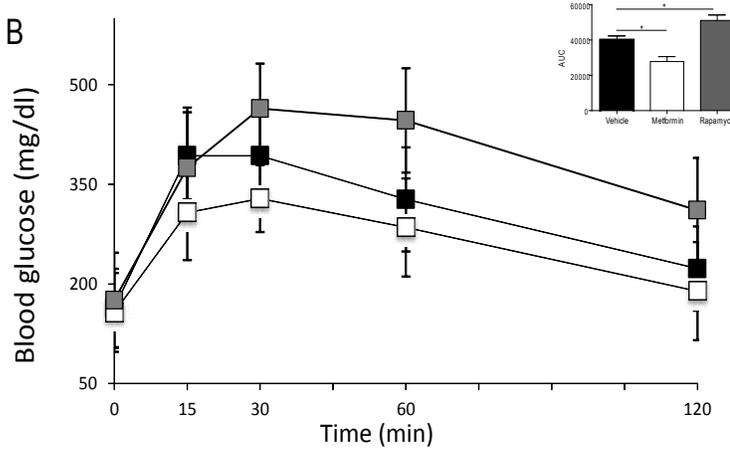
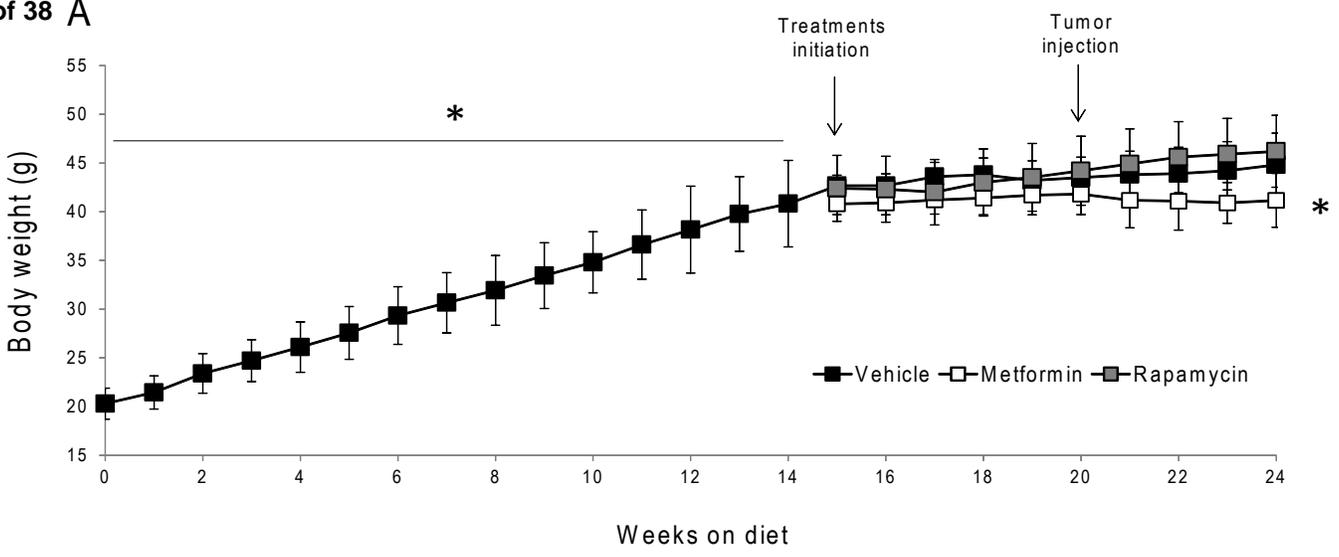


Figure 1

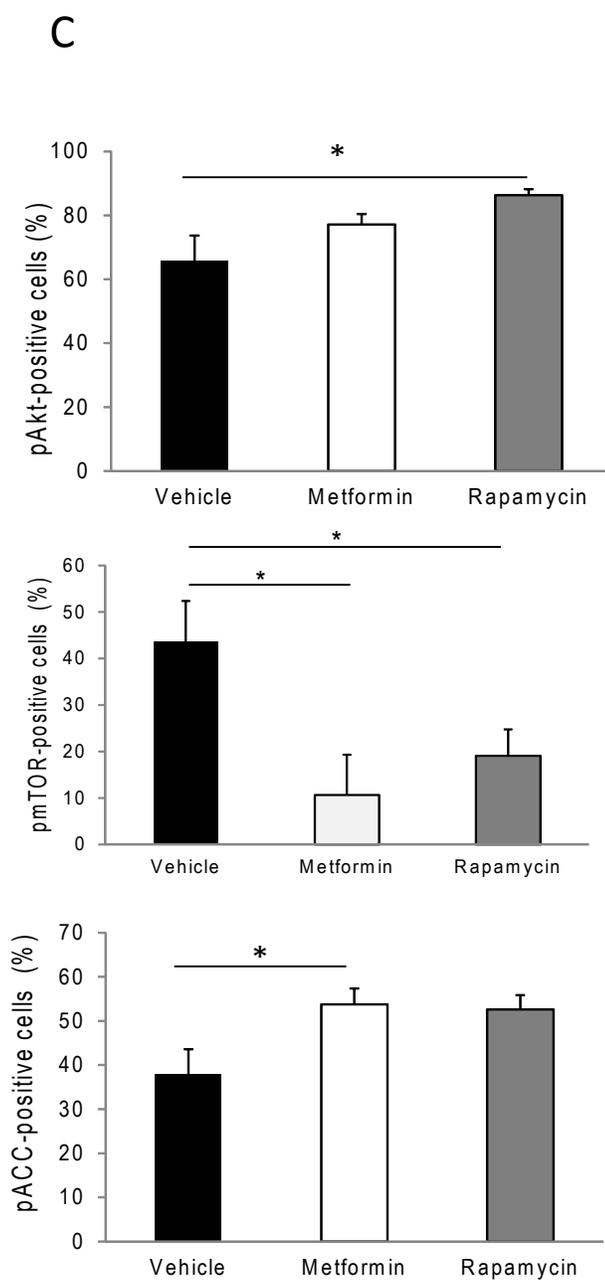
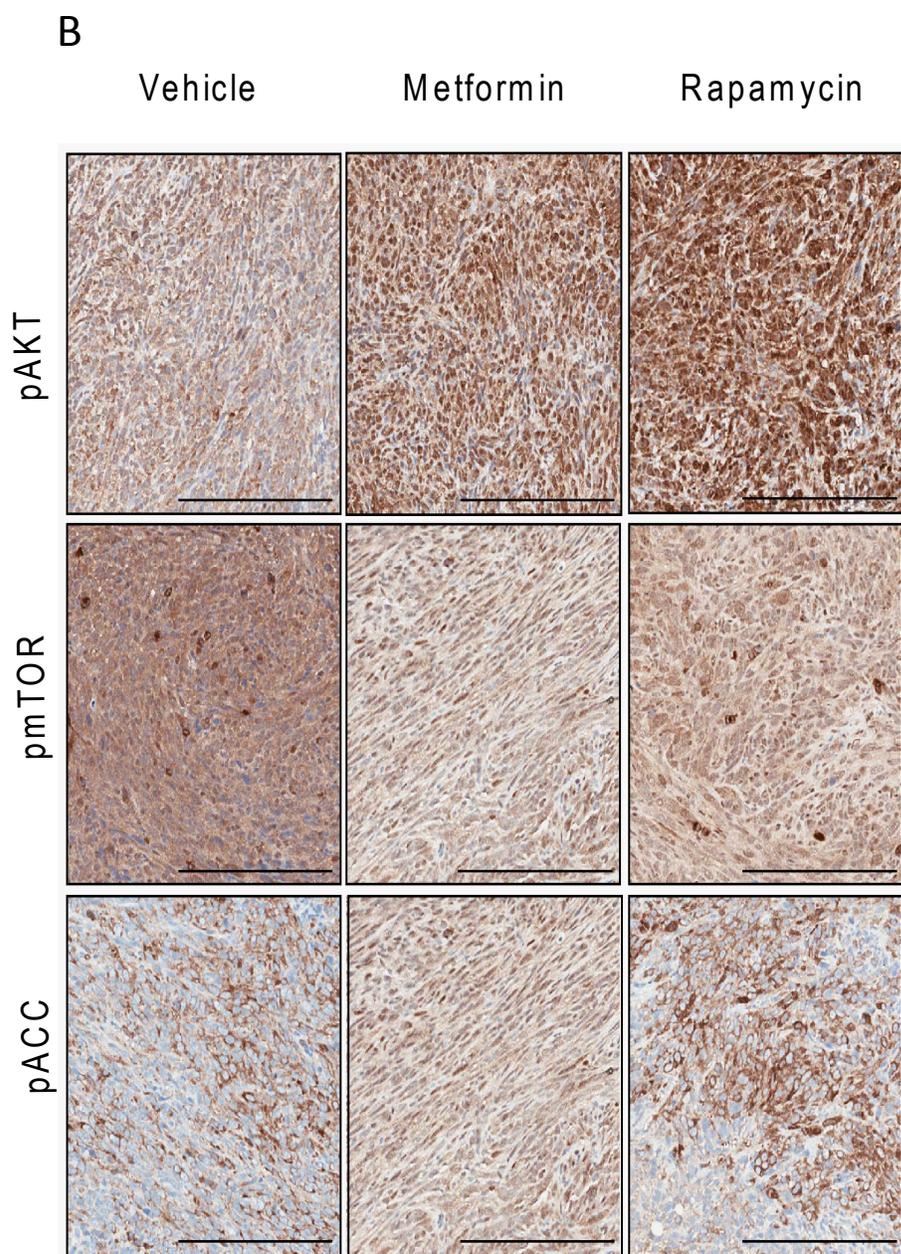
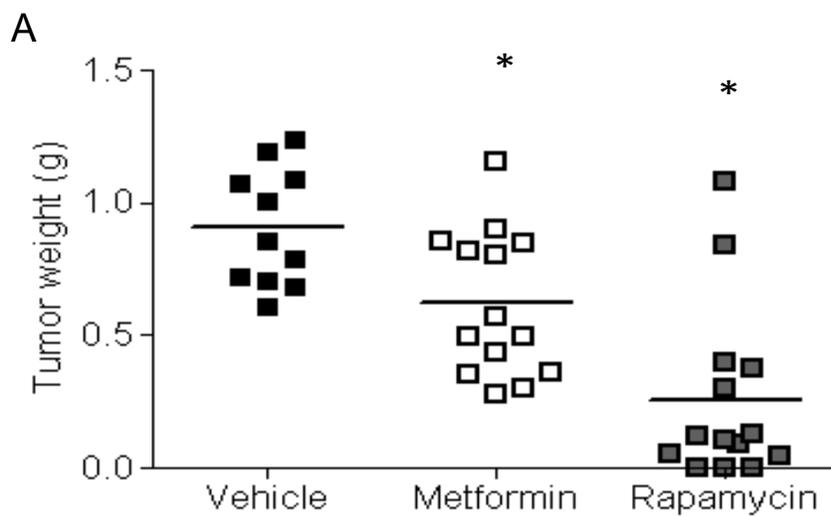


Figure 2

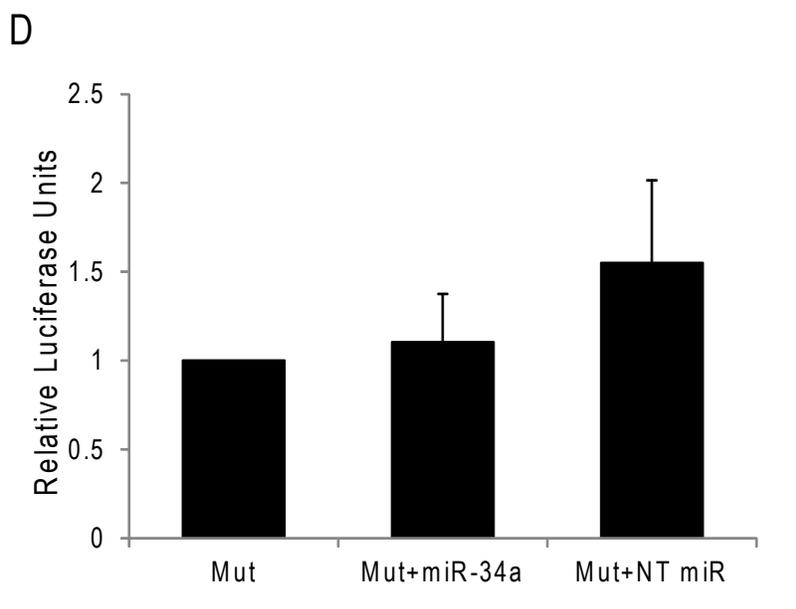
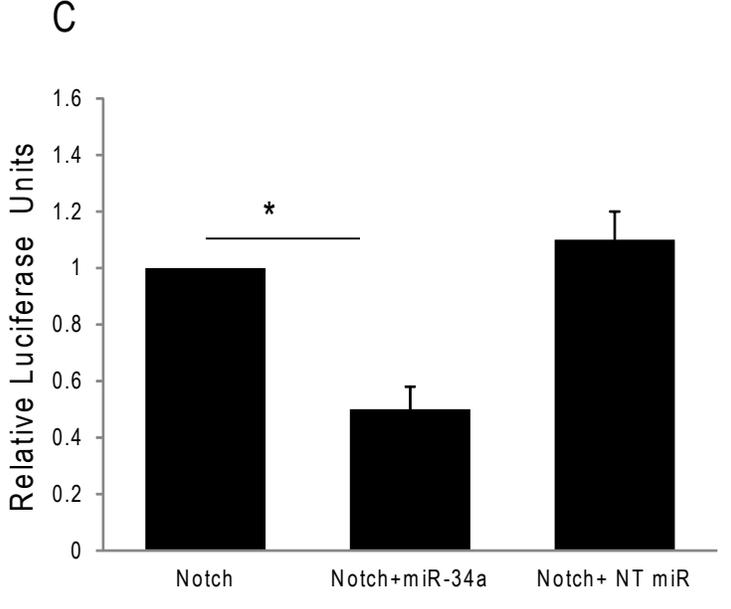
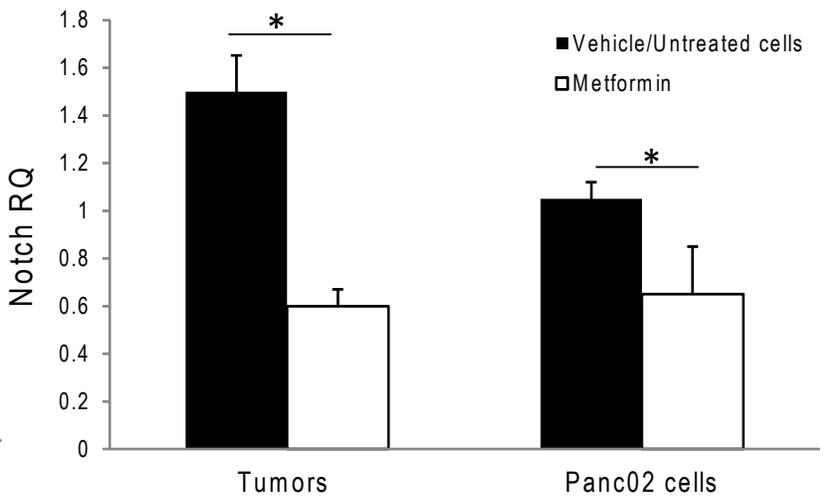
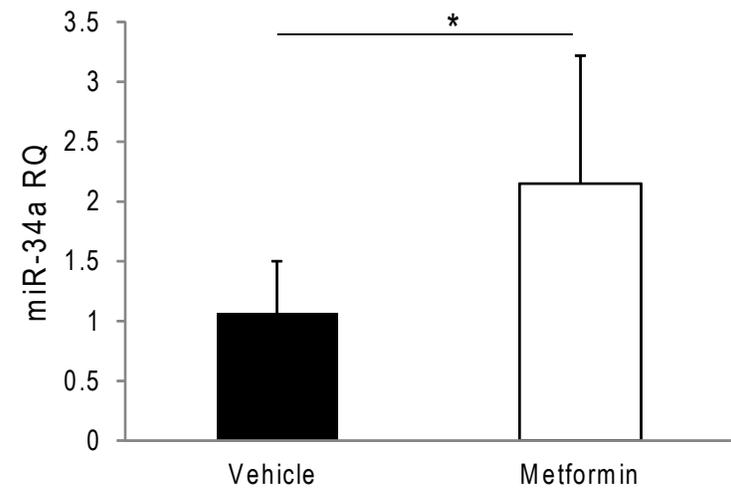


Figure 3

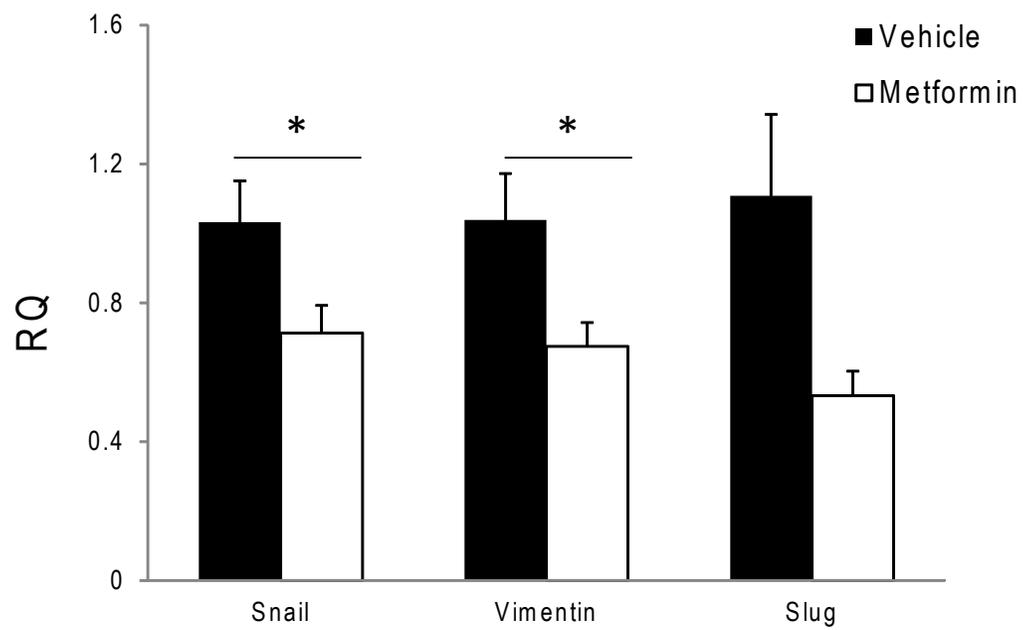


Figure 4

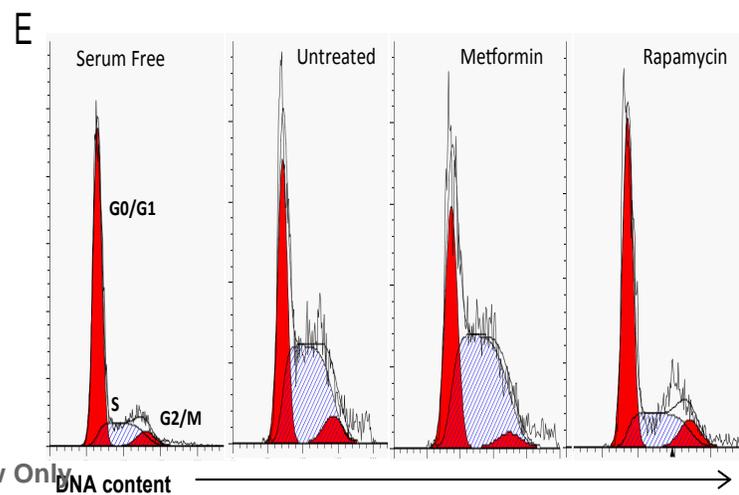
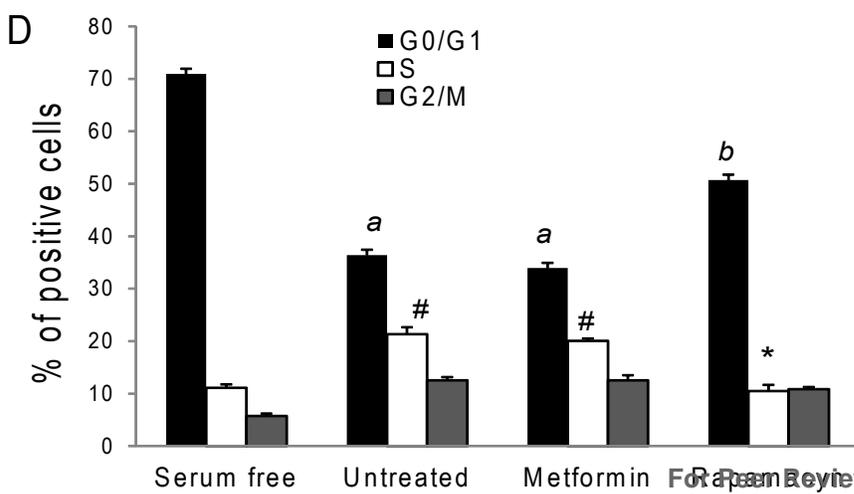
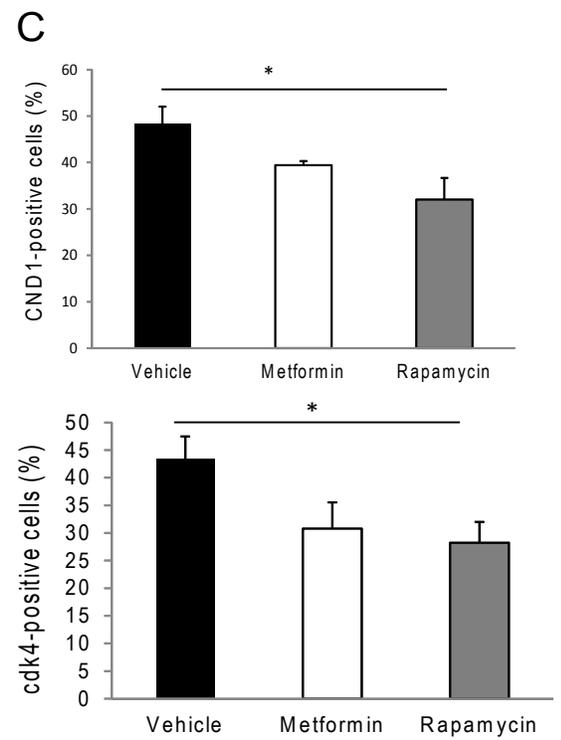
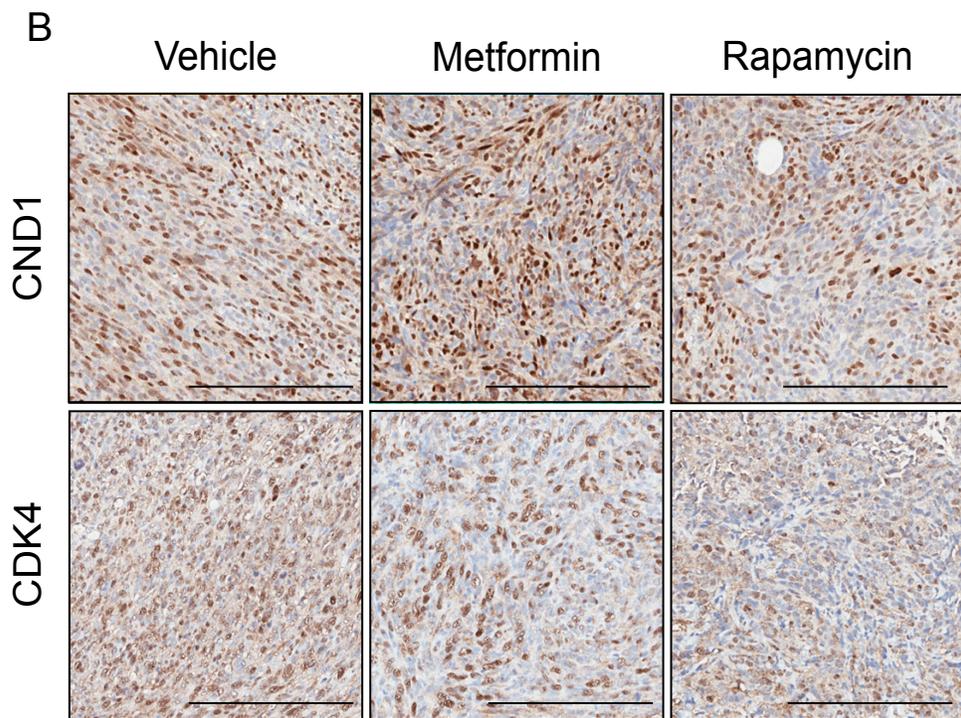
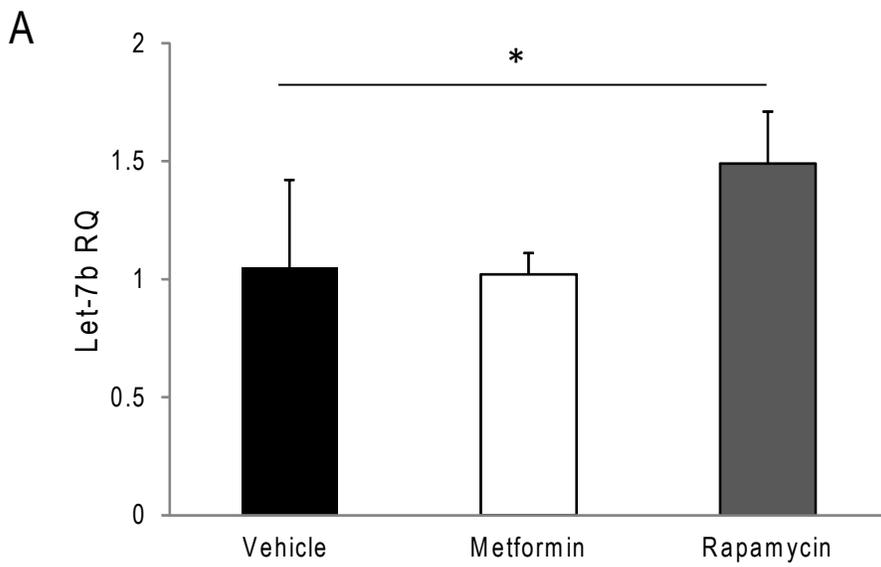
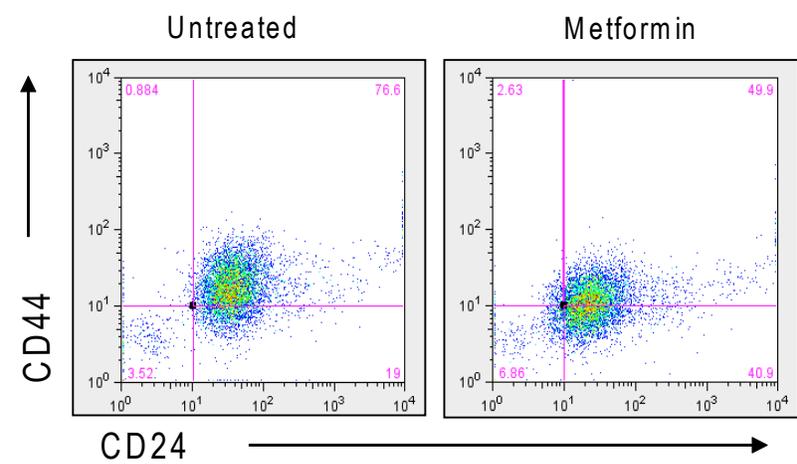
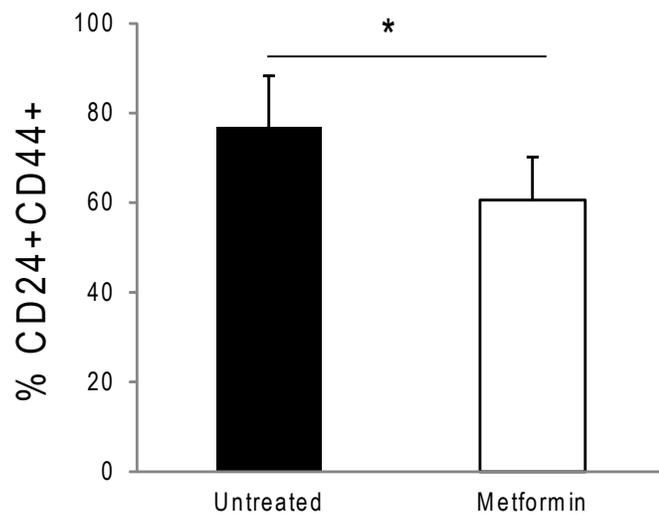


Figure 5

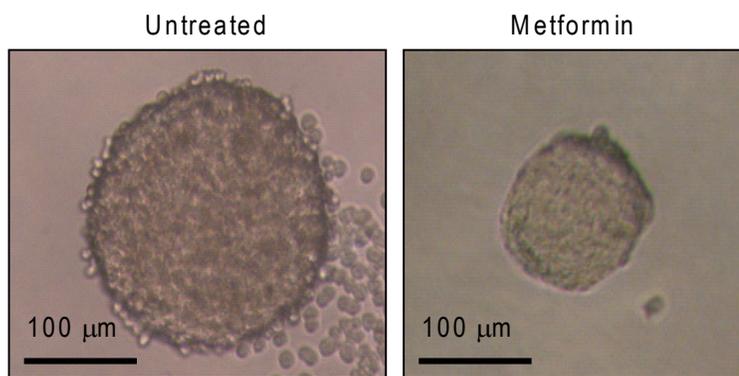
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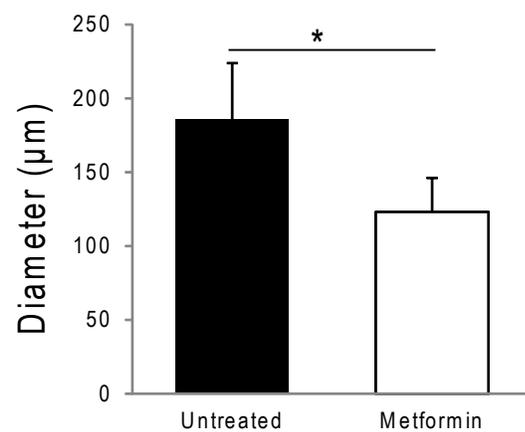
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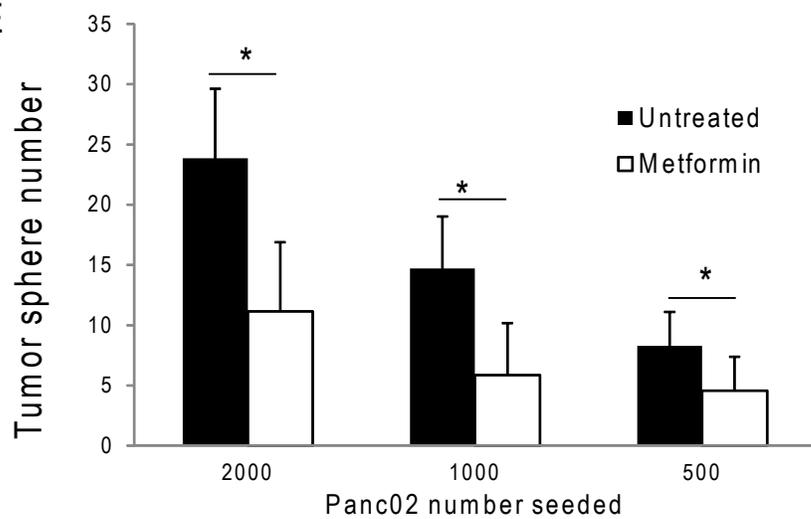
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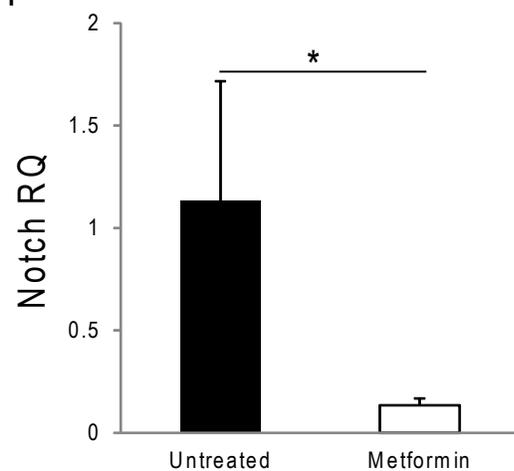
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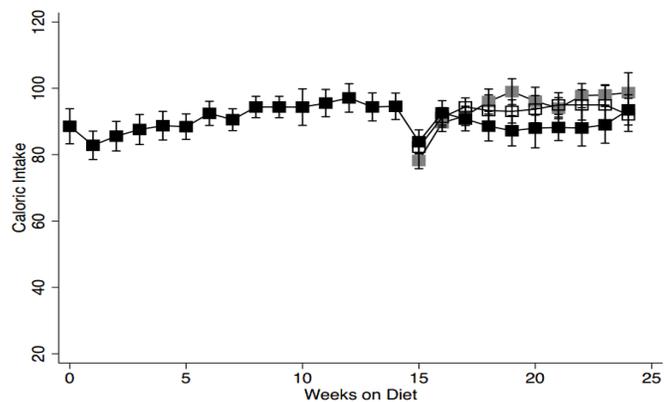
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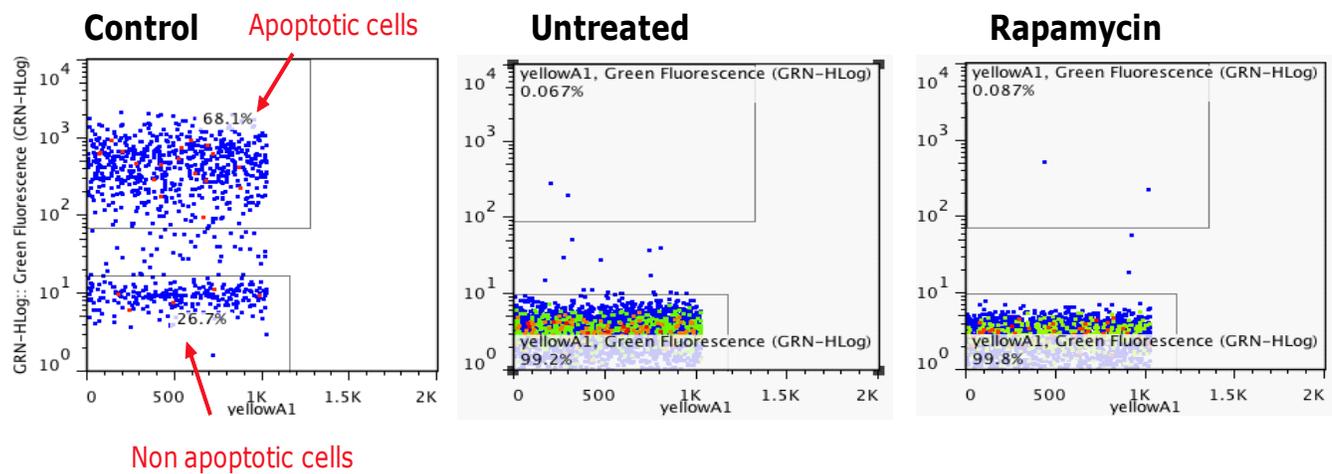


Supplementary Figure 1

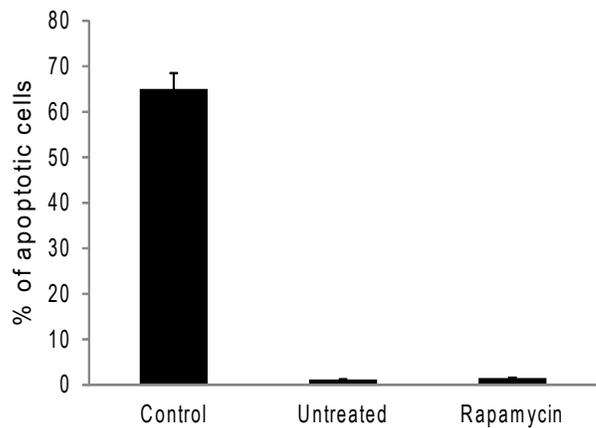


Supplementary Figure 2

A



B



Supplementary Table 1

miR	CONTROL	RAPAMYCIN		METFORMIN	
	Expression	Expression	<i>p-value</i>	Expression	<i>p-value</i>
let-7b	1.01 ± 0.09	1.17 ± 0.03	0.15	0.99 ± 0.05	0.88
miR-21	1.00 ± 0.03	1.1 ± 0.08	0.33	1.07 ± 0.03	0.22
miR-26a	1.00 ± 0.07	1.06 ± 0.06	0.55	0.97 ± 0.02	0.65
miR-34a	1.00 ± 0.05	0.91 ± 0.08	0.38	1.16 ± 0.04	0.08
miR-100	1.00 ± 0.07	1.12 ± 0.07	0.32	1.02 ± 0.07	0.92
miR-101	1.01 ± 0.08	0.81 ± 0.10	0.19	1.02 ± 0.09	0.9
miR-146a	1.07 ± 0.07	1.25 ± 0.17	0.42	1.24 ± 0.02	0.09
miR-200a	1.01 ± 0.04	0.84 ± 0.13	0.36	1.39 ± 0.78	0.65
miR-200b	1.01 ± 0.08	0.75 ± 0.14	0.19	1.21 ± 0.65	0.77
miR-200c	1.29 ± 0.66	1.03 ± 0.26	0.73	1.02 ± 0.56	0.78