

Insights into insulin-mediated regulation of CYP2E1: miR 132/212 targeting of CYP2E1 and role of PI3-K, Akt, mTOR signaling in regulating miR132/212 and miR 122/181a expression in primary cultured rat hepatocytes

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Abbreviations

Cytochrome(P450)2E1 – CYP2E1

Phosphatidylinositol 3-kinase – PI3-K

Mammalian target of rapamycin - mTOR

microRNAs – miRNAs, miRs

Dimethyl sulfoxide – DMSO

Quantitative real-time PCR – qRT-PCR

Target of rapamycin complex 1 – TORC1

Abstract

Several microRNAs (miRs) were selected for characterization of their response to insulin signaling based on in silico predictions of targeting Cytochrome (P450) 2E1 (CYP2E1) mRNA and previous reports implicating their role in hepatic metabolism and disease. CYP2E1 expression decreases with increasing insulin concentration and has been shown to be regulated by the phosphatidylinositol 3-kinase (PI3-K) / Akt / mammalian target of rapamycin (mTOR) signaling pathway. In primary cultured rat hepatocytes, insulin at 0.1, 1.0, 10 nM elevated miRNA 132 and 212 expression ~2- and 1.8-fold respectively, whereas expression of miRNAs181a and 122 increased ~1.6- and 1.4-fold, respectively. In contrast, insulin failed to significantly alter the expression of miRNA let-7a. Mechanistic studies employing inhibitors of PI3-K, Akt, and mTOR, were used to examine the role of the insulin signaling pathway on miR expression and resulted in significant suppression of the insulin-mediated elevation of miR-132, miR-212 and miR-122 levels, with a lesser effect observed for miR-181a. Targeting of the rat CYP2E1 3' untranslated region (UTR) by miR-132 and 212 was demonstrated with an in vitro luciferase reporter assay. These data show that insulin, which regulates CYP2E1 through the PI3-K, Akt, mTOR signaling pathway, also regulates the expression of miRs which target the 3'-UTR of CYP 2E1 mRNA and are involved in regulation of hepatic metabolism, and disease.

Introduction

MicroRNA's (miRNA's) are 20 - 25 nucleotides in length, endogenously expressed, noncoding RNAs that negatively regulate target messenger RNAs via inhibition of mRNA stability and translation (Hudder and Novak, 2008). One-third of all human gene RNAs are predicted targets of miRNA's, (Lewis et al., 2005) highlighting the abundance and importance of miRNA's in regulating mRNA translation and degradation and hence, serving as regulatory molecules. The regulation of genes by miRNA's is complex, with single miRNAs targeting multiple mRNAs and several miRNAs binding one mRNA, working co-operatively to silence gene expression. miRNAs have been associated with cell development and differentiation, apoptosis, fatty acid metabolism, hormone secretion, long term memory, limb regeneration as well as a multitude of other diverse pathophysiological events and conditions, including diabetes, oxidative stress, and cardiac disease. Importantly, recent studies have uncovered a fundamental role for miRNA's in many cancers. (Calin et al., 2004; Kerr et al., 2011)

The biosynthesis of miRNA's is a complex, multistage affair, beginning with the transcription of the miRNA gene to a primary miRNA (pri-miRNA), typically greater than 100 bp in length. The pri-miRNA is processed by the RNase III enzyme Drosha, resulting in a double stranded precursor miRNA (pre-miRNA) of 60 to 70 nucleotides. The pre-miRNA is exported from the nucleus into the cytoplasm and subjected to cleavage by another RNase III enzyme, Dicer, producing a double stranded miRNA duplex 20 – 20 nucleotides in length. The duplex is cleaved, and a single strand forms the mature miRNA (Hudder and Novak, 2008; Krol et al., 2010).

Insulin and its physiological effects have been extensively studied, and insulin and Insulin-like growth factor I (IGF-1) activation of signaling pathways have been well characterized (Esguerra et al., 2011). The effects of insulin and activation of the phosphatidylinositol 3-kinase (PI3-K) signaling pathway on miRNA expression have not yet been examined in primary cultured hepatocytes. However, insulin mediated regulation of miRNA's could constitute a significant element of its pathophysiological effects. Global profiling of miRNA's in human skeletal muscle revealed that insulin regulates the expression of muscle specific miRNA's, supporting the hypothesis that some of insulin's downstream effects are dependent on regulation of miRNAs (Gallagher et al., 2010). However, no study has yet been undertaken to examine insulin effects on miRNA expression in primary cultured hepatocytes. Such a study in hepatocytes could increase the scope of our understanding of the mechanism of insulin's activity on its target genes in the liver. Of particular interest is the expression of miRNA's implicated in liver disease and those predicted to target drug metabolizing enzymes which are regulated in response to insulin activation of the PI3-K signaling pathway.

The current study aims to determine the effects of insulin treatment on miRNA expression in rat primary cultured hepatocytes, as well as establish the role of PI3-K, Akt and mTOR in the regulation of targeted miRNAs levels. Four target miRNA's were chosen for investigation based on their previously reported role in regulating hepatic metabolism and/ or in hepatic disease, including obesity, diabetes, hepatitis, and hepatocellular carcinoma.(Guay et al., 2011; Hsu et al., 2012; Xu et al., 2011) or their predicted targeting of CYP2E1. Mir-122 has been shown to be involved in the regulation of gene networks, metabolic pathways, hepatic function, response to toxicants and disease (Guay et al.,

2007; Ji and Wang, 2009). Mir-181a has been implicated in the regulation of starvation and rapamycin-induced autophagy and was elevated in blood in Type 1 diabetes (Tekirdaq et al., 2013; Nielsen et al., 2012). Both miRs -212 and 132 were predicted by a consensus of multiple computational methods to target rat CYP2E1, a drug-metabolizing enzyme that is up-regulated in diabetes and has been implicated as a risk factor in hepatocellular carcinoma. The mechanism of CYP2E1 induction in response to impaired insulin signaling has remained elusive. Elevated CYP2E1 expression in subjects with metabolic disease is of concern due to the potential for increased xenobiotic metabolism and production of hepatotoxic metabolites. We hypothesized that microRNAs may be involved in insulin-mediated CYP2E1 regulation, and we focused our investigation on the two microRNAs having the greatest likelihood of regulating the gene based on rigorous computational predictions. Hence, mechanistic studies employing qRT-PCR analysis of target miRNAs were conducted using insulin and inhibitors of downstream serine and threonine kinases in the insulin signaling pathway to identify the role of this pathway and these kinases in the regulation of miRNA expression.

Materials and Methods

Materials: Modified Chee's medium and L-glutamine were obtained from Invitrogen (Carlsbad, CA). Insulin (Novolin R) was purchased from Novo-Nordisk (Princeton, NJ). Collagenase (type I) was purchased from Worthington Biochemicals (Freehold, NJ). PureCol (97% Type I collagen, 3% Type III collagen) was obtained from Advanced BioMatrix, San Diego, CA. LY294002, and rapamycin were obtained from Calbiochem (San Diego, CA). The AKT inhibitor A-443654 was obtained from Dr. Giranda, Abbott Laboratories, Abbott Park, IL. Reagents for reverse transcription PCR and real-time PCR

(Taqman microRNA reverse transcription kit, TaqMan fast advanced master mix 2X, TaqMan microRNA probe) were purchased from Applied Biosystems Inc (Foster City, CA).

Luciferase 3' UTR reporter assay: A Luc-Pair miR luciferase assay was used to demonstrate targeting of the rat CYP2E1 3' UTR by miR-132 and 212. A CYP2E1 3' UTR luciferase reporter construct (RmiT051147-MT01), rno-miR-132 and rno-miR-212 precursor expression plasmids (RmiR6036-MR04 and RmiR6108-MR04 respectively), and scrambled control expression vector (CmiR0001-MR04) were all obtained from Genecopoeia (Rockville, MD). Renilla luciferase is included on the 3' UTR CYP2E1 construct and was used to normalize for transfection efficiency. Cos-7 cells were used for the luciferase reporter experiment. DMEM (high glucose, Gibco, Life Technologies, Grand Island, NY, USA) with 10% FBS were used to plate cells. The cells were plated in a 96-well flat bottom white plate. After 24 hours cells were transfected with 3' UTR-luc reporter, and microRNA expression vector (or control vector) in the concentration ratio of 2:4 respectively, with Lipofectamine (Life Technologies, Grand Island, NY) per manufacturer's instructions. Transfections were performed using three replicates for each condition. After 48 hours of transfection, the Renilla and Firefly luciferase activities were quantified by the Luc-Pair miR luciferase assay system (Genecopoeia, Rockville, MD) and measured on a FlexStation 3 universal plate reader (Molecular Devices). Data analysis was performed using Softmax Pro. Expression of the 3'UTR reporter was measured by Firefly luciferase luminescence and normalized using the control Renilla-luc luminescence. Normalized expression values were compared between transfections with the scrambled control vector and those with each pre-miR vector. A two-tailed Student's T-Test with equal variance was used to determine statistical significance.

Primary rat hepatocyte culture: Hepatocytes were isolated from the livers of male Sprague-Dawley rats (200–300 g) using collagenase perfusion as described previously (Woodcroft et al., 2002). Hepatocytes were plated onto dishes covalently coated with Purecol and modified Chee's medium was fortified as described previously (Woodcroft et al., 2002) and supplemented with 1 μ M insulin 0.1 μ M dexamethasone, 12.5mg/ml transferrin, 50ug/ml gentamycin, and 2mM L-Glutamine. Cells were plated at a density of 3×10^6 cells/60-mm dish. Hepatocytes were incubated in a humidified 37 °C incubator with 95% air/5% CO₂ atmosphere. Four hours after the initial plating, cells were washed with insulin-free medium three times and cultured in insulin-free medium prior to initiation of treatment with insulin and/or inhibitors.

Treatment of primary cultured Hepatocytes: Hepatocytes were treated with insulin at 0.1, 1, and 10 nM concentrations 48 h after plating. The PI3-Kinase inhibitor LY294002, the mTOR (TORC1) inhibitor Rapamycin and the pan AKT inhibitor A-443654 were dissolved in DMSO and were added 1.5 h prior to insulin addition. LY294002, Rapamycin and A-443654 were used at the concentrations of 1 μ M, 500nM and 10 μ M respectively. Treatment of cells continued for 24 h prior to harvesting of cells and isolation of RNA. Total hepatocyte RNA was extracted by using Trizol (Invitrogen, Carlsbad, CA) according to manufacturer's protocol. The concentration (ng/ul) of the purified RNA was quantified using the Nano Drop, ver. 3.3 (Applied Biosystems, Foster City, CA) The quality of the RNA was assessed by Agilent RNA 6000 Nano kit (Agilent Technologies, Germany) which estimates the ratio of the band intensity of 28S and 18S rRNA. Statistical analysis was performed on triplicate preparations of total RNA from a single hepatocyte preparation. Reproducibility of results was confirmed in at least 3 hepatocyte preparations. The Wayne

State University Animal Investigation Committee approved all experimental procedures involving animals.

Real-time RT PCR analysis: The cDNA templates were prepared from total RNA using with the TaqMan microRNA Reverse Transcription kit, which utilizes the stem-loop reverse primers, according to manufacturer's protocol. (Applied Biosystems, Foster City, CA) qRT-PCR was performed to measure the relative expression of miRNAs among the treatments using an ABI 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA) The expression of mature miRNAs was assayed using the Taqman microRNA assay specific for hsa-mir-122, hsa-mir-132, hsa-mir-212, hsa-mir-181a and hsa-mir-let7a (hsa- Homo sapiens) and the expression level was normalized to 4.5s rRNA. The Taqman probes targeting human (hsa) and rat (rno) miRNA's were identical, and termed hsa assays by Taqman. Relative expression was calculated using the comparative CT method.

Statistics: Data is represented as mean +/- S.E., and statistical significance was determined with unpaired 2-tailed Student's T-Test.

Results

Identification of microRNAs predicted to regulate rat CYP2E1. To identify microRNAs likely to target CYP2E1 we utilized a computational approach that employs a consensus of multiple prediction methods. Computational methods are widely used to predict target transcripts of microRNAs. It is recognized that these methods often have high false positive rates, and one approach to reduce false positives and improve accuracy is to rely on the consensus of multiple, independent computational methods. Accordingly, we used

the web-based application miRWalk to perform consensus-based identification of microRNAs predicted to target CYP2E1 (Dweep H et al., 2011). Using the rat CYP2E1 transcript (NM_031543) we found that the two microRNAs predicted by the greatest number of computational methods to target CYP2E1 were miRs rno-miR-212 and 132. Each of these microRNAs were predicted by four independent methods (miRanda, miRDB, miRWalk, PITA) to target the CYP2E1 3' UTR. The next highest scoring candidates included one microRNA predicted by three methods to target CYP2E1, three microRNAs predicted by two methods to target the gene, and the remaining predictions were restricted to a single computational method. Figure.1A shows the target site in the rat CYP2E1 3' UTR for miRs- 212 and 132.

Rat CYP2E1 3' UTR is targeted by miR-132 and 212: To confirm computational predictions that miRs 132 and 212 target rat CYP2E1, we used a luciferase 3' UTR reporter assay. A vector construct was created with the firefly luciferase reporter gene and the 3'UTR of rat CYP2E1. Luc-Pair miR luciferase assays were performed by transfecting the vector into the Cos-7 cell line along with vectors expressing a pre-miR or scrambled control. The Cos-7 cell line has been widely used for microRNA target validation assays. Co-transfection of pre-miR-132 or 212 with the reporter plasmid containing 3'UTR CYP2E1 showed a dramatic reduction in the luciferase signal compared to transfection with a scrambled pre-miR control. Pre-miR -132 and 212 resulted in 75% and 90% decrease respectively (P=0.009 and 0.003) in expression of the reporter construct (Fig.1B), indicating direct targeting of rat CYP2E1 by miR-132 and 212.

Insulin effects on miRNA expression: MiR-132 and miR-212 are of the same miRNA family, target the same putative sequence in CYP2E1, and co-localize to the same genomic region in rats, humans, and mice and hence, are referred to as the miR- 132/212 cluster. (Remenyi et al.,2010). Primary rat hepatocytes either contained no insulin or were treated with low (i.e. diabetic plasma level insulin) (0.1 nM insulin), normal (1.0 nM insulin) or supra-physiologic (10 nM) insulin for 24 h, and miRNA expression was determined with qRT-PCR and data reported relative to untreated (no insulin) cells. Insulin treatment of hepatocytes increased miR-132 expression ~2-fold at 1.0 nM insulin, relative to controls, with no additional increase monitored at 10 nM insulin (Fig. 2A). Insulin progressively increased miR-212 levels in a concentration-dependent manner, with 1 and 10nM insulin producing statistically significant increases of ~ 1.5- and ~1.7-fold, respectively, relative to untreated cells (Fig. 2B).

miR-122 is highly expressed in hepatocytes, constituting up to 70 % of the level of all miRs present in the hepatocyte (Law and Wong, 2011). miR-122 has been implicated in a variety of liver-specific cellular processes, including lipid metabolism, and is considered one of the most vital miRNA's for normal hepatic function, with aberrant expression observed in a variety of altered liver-specific pathological conditions, including hepatocellular carcinoma (Gramantieri et al., 2007). Mice with streptozotocin-induced diabetes were shown to express lower levels of miRNA-122 (Fernandez-Valverde et al., 2011). Insulin treatment of hepatocytes increased miRNA-122 expression, with statistically significant elevations of ~1.5-fold monitored at 1 and 10nM concentrations relative to untreated controls or hepatocytes treated with 0.1 nM insulin (Fig. 2C).

miRNA-181a has been identified as being elevated in blood in Type 1 diabetics and as regulating starvation and rapamycin-induced autophagy, and has been implicated as playing a role in cellular differentiation, and effecting abnormal cellular proliferation and cancer pathogenesis (Tekirdaq, et al., 2013; Ji et al., 2009). The ability of insulin to regulate miRNA-181a levels was also examined. A significant, albeit lesser response was observed in response to insulin, with insulin elevating miRNA-181a ~ 1.4-fold at 1 and 10 nM insulin, respectively, relative to untreated cells or cells treated with 0.1 nM insulin (Fig.2 D).

Let-7a was one of the first experimentally discovered miRNA's, and was first isolated and identified as an element of developmental regulation in *C. elegans*. (Guay et al., 2011) It has since been implicated in a variety of cellular processes, including regulation of cell proliferation, cancer, and angiogenesis. (Ji and Wang, 2009) However, CYP2E1 mRNA is not a target of miR let-7a. Hence, the ability of insulin to alter the expression of miRNA let-7a in hepatocytes was examined as a positive control. Insulin treatment of cells failed to significantly alter miRNA let-7a expression over the concentrations examined (Fig. 2E).

Mechanistic studies involving insulin signaling through PI3-K: The signaling pathways involved in insulin's regulation of the miRNAs- 132/212, 122, 181a and let-7a were also examined through mechanistic experiments, employing various inhibitors of the kinase members of the insulin signaling pathway. The rationale behind these experiments resides in the PI3-K, Akt, mTOR signaling pathway and downstream kinases which have been implicated as playing a role in the storage and regulation of miRNA expression. The initial kinase of interest in the insulin signaling pathway is PI3-K.

Insulin-mediated elevation of miR-132 and miR-212 through the PI3-K signaling was examined. Inhibition of PI3-Kinase with LY294002 revealed that insulin effects on both miRNA-132 and miR-212 were mediated through PI3-K. LY294002 treatment significantly abrogated the insulin-mediated elevation of miR-132 and miR-212, with miR-132 and miR-181a suppressed to levels below, or equal to, respectively, the levels present in untreated controls (Figs. 3 A, B). The effect of LY294002 in decreasing the miR-132 levels is clearly more pronounced as compared to miR-181a. At 1.0 nM insulin, LY294002 significantly inhibited the insulin-mediated increase in expression of miRNA-132 by ~70% relative to the respective insulin-mediated increase, with suppression of miR-132 by inhibition of PI 3-K equal to, or below that of untreated controls (Fig. 3 A).

MiRNA-212 levels were less affected by inhibition of PI3-K, although miR-212 levels were nonetheless significantly decreased to levels present in untreated controls at 1 and 10 nM insulin (Fig. 3 B). In the presence of 10 nM insulin, LY294002, produced an ~ 60% decrease in miR-132 levels, relative to the respective insulin-treated cells, as compared to an ~25 % decrease in miRNA-212 at 1 nM insulin, relative to insulin-treated cells (Fig. 3 B). These data support the analysis that insulin-mediated up-regulation of miRNA's- 132 and 212 is PI3-K dependent.

Experiments utilizing the PI3-Kinase inhibitor LY 294002 revealed that the insulin-mediated increase in miRNA-122 appears to be associated with the insulin signaling PI3-K pathway (Fig. 3 C). Examination of miRNA 122 levels from cells treated with insulin alone, and cells treated with insulin in combination with the PI3-K inhibitor LY 294002 treatment, revealed a significant suppression of miR-122 expression. Inhibition of PI3-K with LY 294002 produced a significant decline of ~ 20 to 50% in miR-122 relative to the levels

detected in cells treated with the respective insulin concentrations, even at the lowest concentration of 0.1 nM insulin (Fig. 3 C). LY 294002 suppression of the insulin-mediated increase in miR-122 expression was maximal at 1 and 10 nM insulin, where a decline of ~50 and ~44% at 1 and 10 nM, respectively, was monitored relative to cells treated with insulin alone. These data reveal a pronounced effect of PI3-K inhibition on the suppression of miR-122 thereby showing the critical regulatory role of PI3Kinase in the insulin-mediated increase in miRNA-122 levels.

Inhibition of PI3-K also plays a critical role in effecting miRNA-181a expression (Fig. 3 D). Inhibition of PI 3-K with LY294002 produced an ~30% and ~50% decrease in miRNA-181 levels at 1 and 10 nM insulin, respectively, relative to the respective insulin-treated cells. Inhibition of PI3-K abrogated the insulin mediated increase in miRNA-181a levels at all concentrations of insulin and decreased miR-181a to levels below those detected in untreated cells.

It was previously shown that insulin did not appear to cause any significant change in miR let-7a expression. Nonetheless, to assess the possibility of PI3-K playing a role in the regulation of basal miR let-7a expression, as well as serving as a positive control, the effects of the PI3-K inhibitor LY 294002 treatment on miR let-7a expression in the absence and presence of insulin was examined. No significant change in miR let-7a expression was observed in cells treated with increasing concentrations of insulin in the presence LY294002 relative to insulin alone (Fig. 3 E).

Mechanistic studies involving Akt: There are three members of the Akt family and this serine threonine kinase is downstream of PI3-K. Akt regulates a variety of critical

cellular processes including mRNA storage, translation, gene transcription, cell proliferation and apoptosis and hence, plays a critical role in metabolic disease and tumorigenesis. Therefore, the role of Akt in regulating miR expression in primary cultured hepatocytes was also examined with the view that insulin-mediated up-regulation of the identified miRs is also mediated through Akt. Akt inhibition was achieved using a highly specific and efficacious pan inhibitor of Akt A-443654, (Han *et al.*, 2007), Cells were treated with insulin alone and insulin in combination with A-443654 and miR expression analyzed.

The Akt inhibitor A-443654 was used to examine the effects of Akt, on the insulin-mediated increase in miR-132 and 212 (Fig. 4 A, B). Akt inhibition by A-443654 ameliorated the insulin-mediated increase in miR-132 and miR-212 by ~60% and 75 %, at 0.1 and 1.0 nM, respectively, relative to the respective insulin-treated cells (Fig. 4 A, B), Inhibition of Akt significantly decreased the levels of miR-132 and 212 in insulin-treated cells to well below levels present in controls (Fig. 4 A, B). These data clearly establish a role for Akt in regulating mir-132 and miR-212 expression.

The role of Akt in regulating miR-122 expression was also examined with a view that insulin-mediated up-regulation of miRNA-122 is also mediated through Akt (Fig. 4 C). Cells were treated with insulin alone, and insulin in combination with A-443654. A significant difference in miR-122 expression was between cells treated with insulin and insulin in combination with the Akt inhibitor A-443654. Inhibition of Akt with A-443654 decreased the 1 and 10 nM insulin-mediated increase in miRNA-122 expression by 40 and 50%, respectively. Thus, Akt inhibition significantly prevented the insulin-mediated

increase in miR-122 expression, although the effect was not as pronounced as that for miR-132 or miR-212.

The effect of Akt inhibition on miRNA-181a expression is provided in Fig. 4 D. Although the increase in miRNA-181a levels associated with insulin treatment is relatively small, there was nonetheless a significant difference between cells treated with insulin alone and those with insulin and A443654 in combination at 1nM and 10nM insulin ($p < 0.01$) (Fig.4 D). An ~30% decrease miRNA-181a levels was monitored for at 1nM and 10nM insulin in the presence of A-443654 as compared to the respective 1 and 10 nM insulin treated cells. Inhibition of Akt diminished the insulin-mediated increase in miR-181a to levels present in controls.

Examination of the effects of Akt inhibition on miR let-7a revealed an inconsistent effect at 0.1, 1 and 10 nM insulin, which was statistically insignificant relative to the respective insulin-treated cells (Fig. 4 E).

Mechanistic studies on mTOR (TORC1): The serine threonine kinase downstream from Akt is mTOR and inhibition of mTOR (TORC1) was accomplished with rapamycin in order to examine the role of this kinase in regulating the expression of the target miRNAs.

The role of mTOR (TORC1) in the insulin-mediated increase of miRNA-132 and 212 levels was examined using rapamycin. Inhibition of mTOR (TORC1) with rapamycin significantly abrogated the insulin-mediated increase in either miR-132 or miR-212 expression by ~50% at 1 and 10 nM insulin relative to the respective insulin-treated cells and decreased the levels to those present in untreated cells.(Fig. 5 A, B). Thus, inhibition

of mTOR (TORC1) completely abrogated the insulin-mediated increase in miRNA-132 and 212 levels, decreasing the insulin-mediated increase in miR-132 and miR-212 to the level present in controls, demonstrating that insulin signaling through the PI3-K, Akt, mTOR pathway regulates expression of the miRNA-132/212 cluster.

The role of mTOR (TORC1), in the insulin-mediated elevation of miRNA-122 expression was examined using rapamycin. Treatment of cells with rapamycin resulted in a suppression of the insulin-mediated increase in miR-122 expression. Comparison of miR-122 expression in cells containing insulin, and insulin plus rapamycin, revealed a significant 33% and 50% inhibition of miR-122 expression at 1 and 10 nM insulin, respectively, relative to the respective insulin-treated cells (Fig.5 C). In summary, inhibition of mTOR appeared to decrease the insulin-mediated increase in miRNA-122 levels to those present in controls (untreated cells), suggesting that insulin signaling through PI3-K, Akt and mTOR regulates miR-122 expression.

Inhibition of mTOR (TORC1) with rapamycin however, failed to have a significant effect on miRNA-181a expression (Fig. 5 D), suggesting that mTOR may not play a major role in modulating the effect of insulin on miRNA-181a expression. Although rapamycin treatment appeared to slightly decrease miR-181a levels, this decrease was not statistically significant for any of the concentrations of insulin examined. Thus, PI3-K and Akt, but not mTOR, appear to play a role in the insulin mediated increase of miRNA-181a levels.

Inhibition of mTOR on miR let-7a expression however, revealed no inhibitory effect (Fig. 5 E). These results show that miR let-7a expression is unaffected by mTOR (TORC1).

Discussion

miRNA's have emerged as a new class of regulatory RNA's that appear to be ubiquitously distributed and involved in a variety of cellular processes (Murakami et al., 2006). Insulin is a major hormone involved in a multitude of biological responses, including gluconeogenesis, cell proliferation, differentiation, and metabolism. Although miRNA's have been implicated in many liver-specific activities, including glucose regulation and cell cycle control, insulin's potential to regulate these miRNA's has never been examined in primary hepatocytes. In human skeletal muscle cells insulin treatment was shown to influence the expression of multiple miRNA's, as determined from miRNA profiling, many of which contained putative target genes known to be relevant to insulin signaling. The present study utilized the sensitive and specific qRT-PCR assay to examine the expression of four microRNAs that were selected based on their role in regulating hepatic metabolism and disease, and because of predicted targeting of CYP2E1 mRNA. Selective inhibition of the insulin signaling components PI3-K, Akt and mTOR was accomplished to examine the role of the individual kinases in regulating the expression of the individual miRs. We utilized a luciferase reporter assay to establish the repression of rat CYP2E1 by two of the microRNAs that were induced by insulin.

The regulation of CYP2E1 expression is perhaps the most complex of the P450's, encompassing gene transcription, mRNA translation (Kim and Novak, 1990; Woodcroft et al., 2002) and post-translational events. CYP2E1 is up regulated in diabetes, starvation, and through chemical inducers including alcohols (Woodcroft et al., 2002; Yokoi and Nakajima, 2011) and nitrogen heterocycles, such as pyridine (Kim and Novak, 1990). Induction appears to occur through translational control based on polysomal distribution and half-life studies of CYP2E1 mRNA (Kim, et al., 1990; Abdelmegeed et al., 2005). Additional studies have established a role for insulin regulation of CYP2E1 expression through both transcriptional and translational mechanisms and which proceed through PI 3-K, and mTOR signaling, with no or low insulin elevating CYP2E1 mRNA in primary cultured hepatocytes, whereas normal or supraphysiologic levels of insulin markedly decrease CYP2E1 mRNA levels in primary cultured hepatocytes. Additional studies examining the various arms of the insulin signaling pathway revealed that the Ras-Raf, Erk- Mek MAP kinase pathway had no role in the insulin-mediated effects on CYP2E1 expression. In view of the regulatory effects of insulin and insulin signaling through PI 3-K, Akt, and mTOR and the potential for miRs which target CYP2E1, to play an important regulatory role, we hypothesized that insulin may indeed also regulate the expression of these miRs.

The possibility that microRNAs may be involved in the post-transcriptional regulation of human CYP2E1 has been established. Human CYP2E1 expression is regulated by miR-378, mainly via translational repression (Mohri *et al.*, 2009). The current studies focused on examination of miRs which have been implicated in the regulation of hepatic metabolism and disease and in the regulation of CYP2E1, which has been shown

to be down regulated by insulin through the PI 3-K, Akt, mTOR arm of the insulin signaling pathway. Several of these miRs have been identified as biomarkers of disease and can be detected in the blood.

MiR-122 comprises the most highly expressed miRNA in the liver. It has been shown to be involved in diverse liver-specific processes, including lipid metabolism and hepatocyte proliferation as well as hepatocellular carcinoma and chronic hepatitis (Ji and Wang, 2009). Additionally, miRNA-122 expression is significantly altered in models of diabetes (Han et al., 2007; Xu, et al., 2011). The miRNA-132/212 cluster is a family of miRNA's containing both miRNA-132 and miRNA-212. These miRNA's are located at the same genetic locus, and their transcriptional control appears to be concerted. Since they have identical seed regions, it is believed that these two microRNAs regulate a largely overlapping set of transcripts (Wanet et al., 2012). miR-132 and 212 have also been shown to be upregulated in isolated pancreatic islets in the obese phenotypes of both the diabetes-resistant and diabetes-susceptible mouse models (Zhao et al., 2009). Both miRNA's have been shown to play an important role in neuronal processes and angiogenesis, amongst other biological activities.

The elevation of the miRNA-132/212 cluster and miR-122 by insulin was shown to be dependent upon PI3-K and Akt/mTOR signaling, and its expression patterns in response to insulin treatment (dose-dependent) may contribute, in part, to the mechanism by which insulin regulates CYP2E1 expression in an inverse dose-relationship manner. Using in vitro luciferase reporter assay we have demonstrated that rat CYP2E1 expression is regulated by rno mir-132 and 212. Thus, we conclude that of the miRs examined, expression of the miR- 132/212 cluster and miR-122 were the most responsive, whereas

miR-181a was less responsive, to insulin and to inhibitors of the PI3-K, Akt and mTOR kinases. We also conclude that rat CYP2E1 is one of the direct targets of the miR-132/212 cluster.

MiR let-7a was the first discovered miR and is reported to play a role in cellular differentiation and proliferation (Boyerinas *et al.*, 2010). However, miR let-7a is not identified as a miR that targets CYP2E1. Let-7a expression was not elevated in response to insulin, nor was miR let-7a expression decreased by inhibition of PI 3-K, Akt, or mTOR.

Whether insulin alters the levels or activities of the the various proteins involved in miR production (e.g. dicer) or the cellular localization and storage of miRs remains to be established.

In summary, seminal evidence is provided for selective insulin regulation of miR expression in primary cultured hepatocytes through PI 3-K, Akt and mTOR (TORC1). We anticipate that subsequent *in vivo* studies will further characterize the role of this pathway in hepatic microRNA regulation and metabolic diseases. We hypothesize that PI3-K, Akt and mTOR are involved in regulating the expression of insulin-responsive miRs because of the regulatory role that this pathway plays in the storage and translation of mRNAs.

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Authorship Contributions:

Participated in research design: Shukla, Tumma, Gratsch and Novak.

Conducted experiments: Shukla, Tumma and Gratsch.

Performed data analysis: Shukla, Tumma and Dombkowski.

Wrote or contributed to the writing of the manuscript: Shukla, Tumma, Dombkowski and Novak

References

Abdelmegeed MA, Carruthers NJ, Woodcroft KJ, Kim SK, and Novak RF (2005)

Acetoacetate induces CYP2E1 protein and suppresses CYP2E1 mRNA in primary cultured rat hepatocytes. *J Pharmacol Exp Ther* **315**: 203-213

Boyerinas B, Park SM, Hau A, Murmann AE, and Peter ME (2010) The role of let-7 in cell differentiation and cancer. *Endocr Relat Cancer* **17**: 19-36.

Calin GA, Sevignani C, Dumitru CD, Hyslop T, Noch E, Yendamuri S, Shimizu M, Rattan S, Bullrich F, Negrini M, and Croce CM (2004) Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers. *Proc Natl Acad Sci USA* **101**: 2999-3004.

Dweep H, Sticht C, Pandey P, and Gretz N (2011) miRWalk-database: prediction of possible miRNA binding sites by "walking" the genes of three genomes. *J Biomed Inform.* **44**: 839-847.

Esguerra JL, Bolmeson C, Cilio CM, and Eliasson L (2011) Differential glucose-regulation of microRNAs in pancreatic islets of non-obese type 2 diabetes model Goto-Kakizaki rat. *PLoS One* **6**: e18613.

Fernandez-Valverde SL, Taft RJ, and Mattick JS (2011) MicroRNAs in β -cell biology, insulin resistance, diabetes and its complications. *Diabetes* **60**: 1825-1831.

Gallagher IJ, Scheele C, Keller P, Nielsen AR, Remenyi J, Fischer CP, Roder K, Babraj J, Wahlestedt C, Hutvagner G, Pedersen BK, and Timmons JA (2010)

Integration of microRNA changes in vivo identifies novel molecular features of muscle insulin resistance in type 2 diabetes. *Genome Med* **2**: 9.

Gramantieri L, Ferracin M, Fornari F, Veronese A, Sabbioni S, Liu CG, Calin GA, Giovannini C, Ferrazzi E, Grazi GL, Croce CM, Bolondi L, and Negrini M (2007) Cyclin G1 is a target of miR-122a, a microRNA frequently down-regulated in human hepatocellular carcinoma. *Cancer Res* **67**: 6092-6099.

Guay C, Roggli E, Nesca V, Jacovetti C, and Regazzi R (2011) Diabetes mellitus, a microRNA-related disease? *Transl Res* **157**: 253-264.

Han EK, Levenson JD, McGonigal T, Shah OJ, Woods KW, Hunter T, Giranda VL, and Luo Y (2007) Akt inhibitor A-443654 induces rapid Akt Ser-473 phosphorylation independent of mTORC1 inhibition. *Oncogene* **26**: 655-661.

Hsu SH, Wang B, Kota J, Yu J, Costinean S, Kutay H, Yu L, Bai S, LaPerle, K, Chivukula RR, Mao H, Min W, Reed Clark K., Mendell JR, Caligiuri MA, Jacob ST, Mendell JT, and Goshai K (2012) Essential metabolic, antoinflammatory and anti-tumorigenic functions of miR-122 in liver. *J Clin Invest.* **122**, 2871-2883.

Hudder A, and Novak RF (2008) miRNAs: effectors of environmental influences on gene expression and disease. *Toxicol Sci* **103**: 228-240.

Ji J, and Wang XW (2009) New kids on the block: diagnostic and prognostic microRNAs in hepatocellular carcinoma. *Cancer Biol Ther* **8**: 1686-1693.

Ji J, Yamashita T, Budhu A, Forgues M, Jia HL, Li C, Deng C, Wauthier E, Reid LM, Ye QH, Qin LX, Yang W, Wang HY, Tang ZY, Croce CM, and Wang XW (2009)

Identification of microRNA-181 by genome-wide screening as a critical player in EpCAM-positive hepatic cancer stem cells. *Hepatology* **50**: 472-480.

Kerr TA, Korenblat KM, and Davidson NO (2011) MicroRNAs and liver disease. *Transl Res*; **157**: 241-252.

Kim SG, and Novak RF (1990) Induction of rat hepatic P450IIE1 (CYP 2E1) by pyridine: evidence for a role of protein synthesis in the absence of transcriptional activation. *Biochem Biophys Res Commun* **166**: 1072-1079.

Krol J, Loedige I, and Filipowicz W (2010) The widespread regulation of microRNA biogenesis, function and decay. *Nat Rev Genet* **11**: 597-610.

Law PT, and Wong N (2011) Emerging roles of microRNA in the intracellular signaling networks of hepatocellular carcinoma. *J Gastroenterol Hepatol* **26**: 437-449.

Lewis BP, Burge CB, and Bartel DP (2005) Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell* **120**: 15-20.

Mohri T, Nakajima M, Fukami T, Takamiya M, Aoki Y, and Yokoi T (2010) Human CYP2E1 is regulated by miR-378. *Biochem pharm* **79**: 1045-1052.

Murakami Y, Yasuda T, Saigo K, Urashima T, Toyoda H, Okanoue T, and Shimotohno K (2006) Comprehensive analysis of microRNA expression patterns in hepatocellular carcinoma and non-tumorous tissues. *Oncogene* **25**: 2537-2545.

Nielsen LB, Wang C, Sorensen K, Bang-Berthelsen CH, Hansen L, Andersen ML M, Hougaard P, Juul A, Zhang CY, Pociot F, and Mortensen HB (2012) Circulating Levels

of MicroRNA from Children with Newly Diagnosed Type 1 Diabetes and Healthy Controls: Evidence That miR-25 Associates to Residual Beta-Cell Function and Glycaemic Control during Disease Progression. *Exp Diabetes Res*: 896362.

Remenyi J, Hunter CJ, Cole C, Ando H, Impey S, Monk CE, Martin KJ, Barton GJ, Hutvagner G, and Arthur JS (2010) Regulation of the miR-212/132 locus by MSK1 and CREB in response to neurotrophins. *Biochem J* **428**: 281-291.

Tekirdaq KA, Korkmaz G, Ozturk DG, Aqami R, and Gozuacik D. (2013) MIR181A regulates starvation- and rapamycin-induced autophagy through targeting of ATG5. *Autophagy* **9**: 374-385.

Wanet A, Tacheny A, Arnould T, and Renard P. (2012) miR-212/132 expression and functions: within and beyond the neuronal compartment. *Nucleic Acids Res.* **40**:4742-4753.

Woodcroft KJ, Hafner MS, and Novak RF (2002) Insulin signaling in the transcriptional and posttranscriptional regulation of CYP2E1 expression. *Hepatology* **35**: 263-273.

Yokoi T, and Nakajima M (2011) Toxicological implications of modulation of gene expression by microRNAs. *Toxicol Sci* **123**: 1-14.

Xu J, Wu C, Che X, Wang L, Yu D, Zhang T, Huang L, Tan W, Wang C, and Lin D, (2011) Circulating microRNAs, miR-21, miR-122, and miR-223, in patients with hepatocellular carcinoma or chronic hepatitis. *Mol Carcinog* **50**: 136-142.

Zhao E, Keller MP, Rabaglia ME, Oler AT, Stapleton DS, Schueler KL, Neto EC, Moon JY, Wang P, Wang IM, Lum PY, Ivanovska I, Cleary M, Greenawalt D, Tsang J, Choi

YJ, Kleinhanz R, Shang J, Zhou YP, Howard AD, Zhang BB, Kendziorski C, Thornberry NA, Yandell BS, Schadt EE, and Attie AD (2009) Obesity and genetics regulate microRNAs in islets, liver, and adipose of diabetic mice. *Mamm Genome* **20**: 476-485.

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Figure Legends

Figure 1A: The rat CYP2E1 transcript is predicted by a consensus of computational methods to be regulated by microRNAs miR-212 and 132. The first 75 nucleotides of the CYP2E1 3' UTR are shown, with the predicted target sites highlighted.

Figure 1B: Rat CYP2E1 3' UTR is targeted by miR-132 and 212. A luciferase reporter assay was used to confirm targeting of the rat CYP2E1 3' UTR by miR-132 and 212. A Luc-Pair miR luciferase reporter with the 3' UTR of CYP2E1 was co-transfected into Cos-7 cells with a vector expressing pre-miR-132, 212, or scrambled control. Expression levels are shown relative to control. Transfection with pre-miR-132 or 212 resulted in approximately 75% and 90% decrease of reporter signal respectively (P=0.013 and 0.002) demonstrating targeting of the 3' UTR by the microRNAs. Error bars are standard error.

Figure 2: Primary cultured rat hepatocytes either contained no insulin or were treated with 0.1, 1.0, or 10 nM insulin, RNA extracted, and miRNA expression quantified as described in Methods. The data show the concentration-dependent effects of insulin on: A) miRNA-132 expression, B) miR-212 expression; C) miR-122 expression; D) miR-181 expression, and E) miR let-7a expression. Whereas insulin produced a concentration dependent increase in miR-132 and miR-212 expression, the effects of insulin on miR-122 and 181a occurred at 1 and 10 nM, with little, if any increase noted at 0.1nM insulin, relative to

untreated cells (no insulin). No statistically significant or concentration-dependent effect of insulin on miR let-7a expression relative to untreated cells was observed, although a slight (~10%) increase was noted for 1 and 10 nM insulin.

¥Statistical significance was determined using a two-tailed Student's T-test. Statistically significant relative to untreated cells: * p< 0.05; **, p<0.01; ***, p<0.001. Statistically significant relative to 0.1nM insulin treated cells: #, p<0.1; ##, p<0.01; ###, p<0.001.

Figure 3: The role of PI 3K in the insulin-mediated elevation of miR expression in primary cultured rat hepatocytes was examined using the PI3-K inhibitor LY294002 at the concentration of 1µM, in the absence and presence of 0.1, 1.0, and 10 nM insulin. MiR expression was monitored as described in Methods. A) The effects of LY294002 on the insulin-mediated increase in miRNA-132 expression. B) The effects of LY294002 on the insulin-mediated increase in miRNA-212 expression. C) The effects of LY294002 on the insulin-mediated increase in miRNA-122 expression. D) The effects of LY294002 inhibition of PI 3K on the insulin-mediated increase in miRNA-181a expression. E) The effects of inhibition of PI3-K with LY294002 on miR let-7a expression. Inhibition of PI 3K significantly decreased the insulin-mediated increase in expression to levels below those monitored in untreated cells for miR-132 at all insulin concentrations, for miR- 212 at 1 and 10 nM insulin, and for miR-122 and miR-181a at all insulin concentrations, demonstrating a critical regulatory role for PI 3K in the expression of these miRs.

¥Statistical significance was determined with two tailed Student's t-Test between cells treated with insulin alone, and the respective inhibitor and insulin treated cells. Statistically

significant at: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Statistically significant relative to 0.1 nM insulin treated cells: #, $p < 0.1$; ##, $p < 0.01$; ###, $p < 0.001$.

Figure 4: The role of Akt in the insulin-mediated elevation of miRs in primary cultured rat hepatocytes was examined using the Akt inhibitor A-443654 at the concentration of 10 μ M in the absence or presence of 0.1, 1.0 and 10 nM insulin. MiR expression was monitored as described in Methods. A) The effects of A-443654 on the insulin-mediated increase in miRNA-132 expression. B) The effects of A-443654 inhibition of Akt on the insulin-mediated increase in miRNA-212 expression. C) The effects of A-443654 on the insulin-mediated increase in miRNA-122 expression. D) The effects of A-443654 on the insulin-mediated increase in miRNA-181a expression. E) The effects of A-443654 on miR let-7a expression. Inhibition of Akt produced a significant and dramatic suppression of miR-132, miR-212, and miR-122 expression to levels below that present in controls at 0.1, 1.0 and 10 nM insulin, whereas inhibition of Akt resulted in a less pronounced decrease in miR-181a. An inhibitory effect was observed with miR let-7a at 1.0 and 10 nM insulin, although there was no consistent pattern of miR let7a suppression.

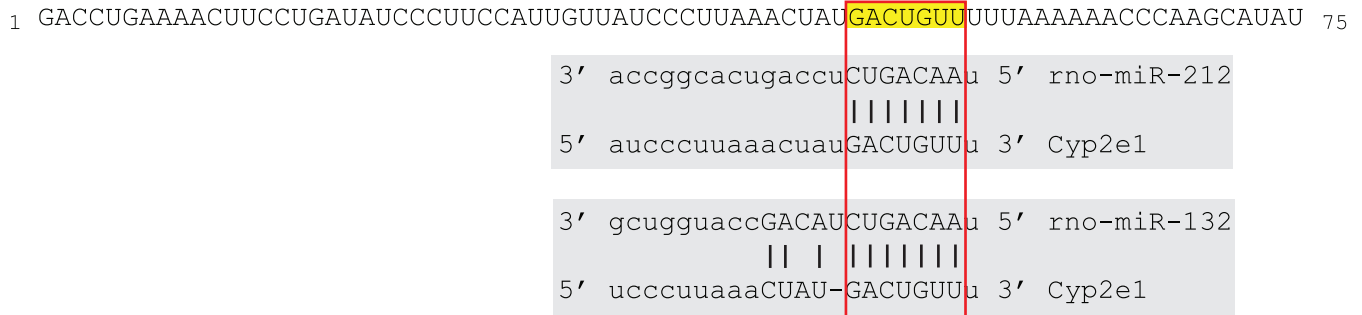
¥Statistical significance was determined using a two tailed Student's t-Test for analysis of significant differences between cells treated with insulin alone and the respective insulin plus inhibitor treated cells. Statistically significant at: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Statistically significant relative to 0.1 nM insulin treated cells: #, $p < 0.1$; ##, $p < 0.01$; ###, $p < 0.001$.

Figure 5: The role of mTOR (TORC1) in the insulin-mediated elevation of miRs in primary cultured rat hepatocytes was examined using the TORC1 inhibitor rapamycin at the concentration of 500nM in the absence and presence of 0.1, 1.0 and 10 nM insulin. Mir expression was monitored as described in Methods. A) Rapamycin effects on the insulin-mediated elevation in miR-132 expression. B) Rapamycin effects on the insulin-mediated elevation in miR-212 expression. C) Rapamycin effects on the insulin-mediated elevation of miR-122 expression. D) Rapamycin effects on the insulin-mediated elevation of miR-181a expression. E) Rapamycin effects on miR let-7a. Inhibition of mTOR (TORC1) produced a significant decrease in miR-132, miR- 212, and miR-122 and a marginal decrease in miR-181a which was not statistically significant.

¥Statistical significance between cells treated with insulin alone and with the respective inhibitor was determined using a two tailed Student's t-Test. Statistically significant at: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Statistically significant relative to 0.1nM insulin treated cells: #, $p < 0.1$; ##, $p < 0.01$; ###, $p < 0.001$.

Figure 1

A



B

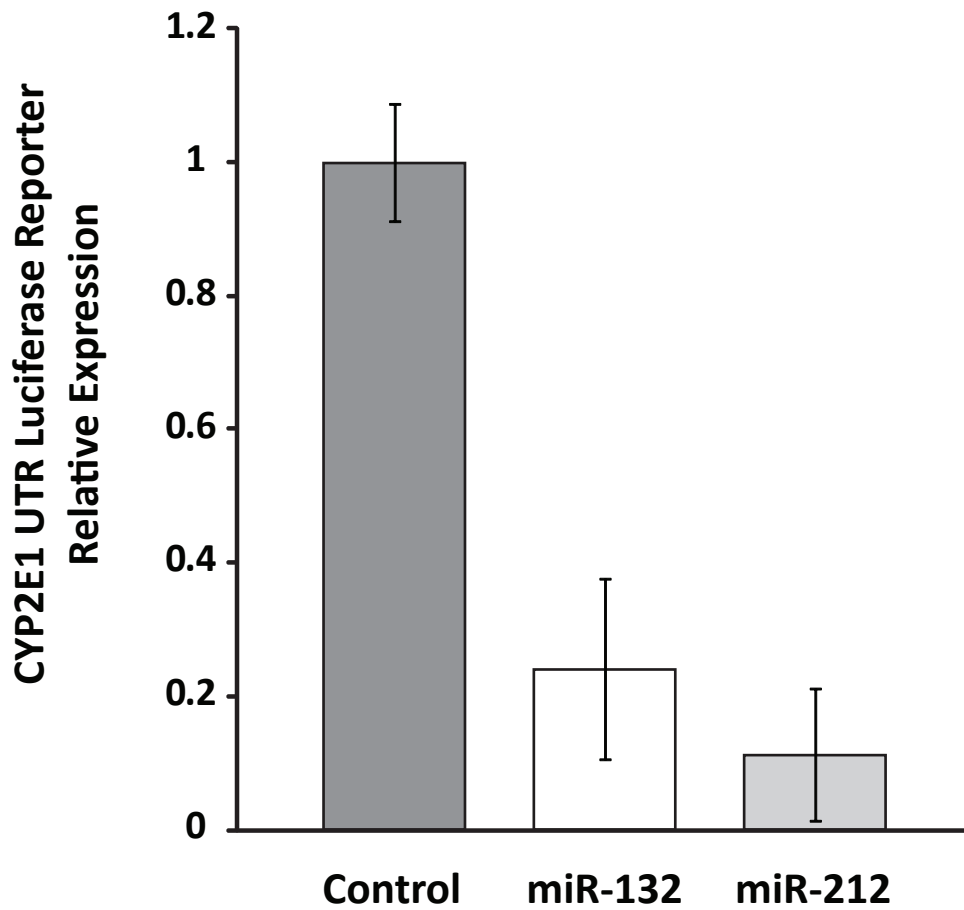


Figure 2

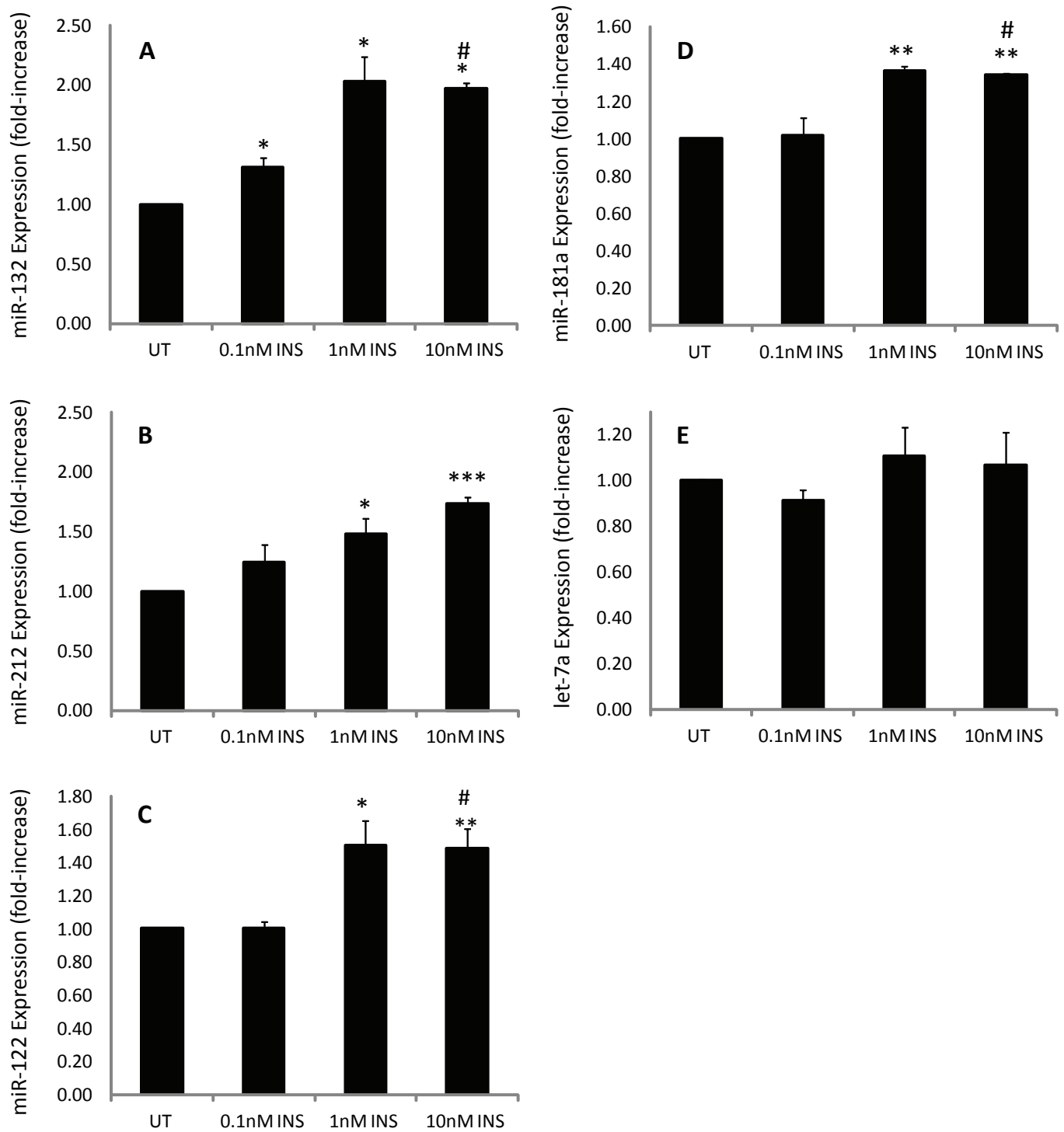


Figure 3

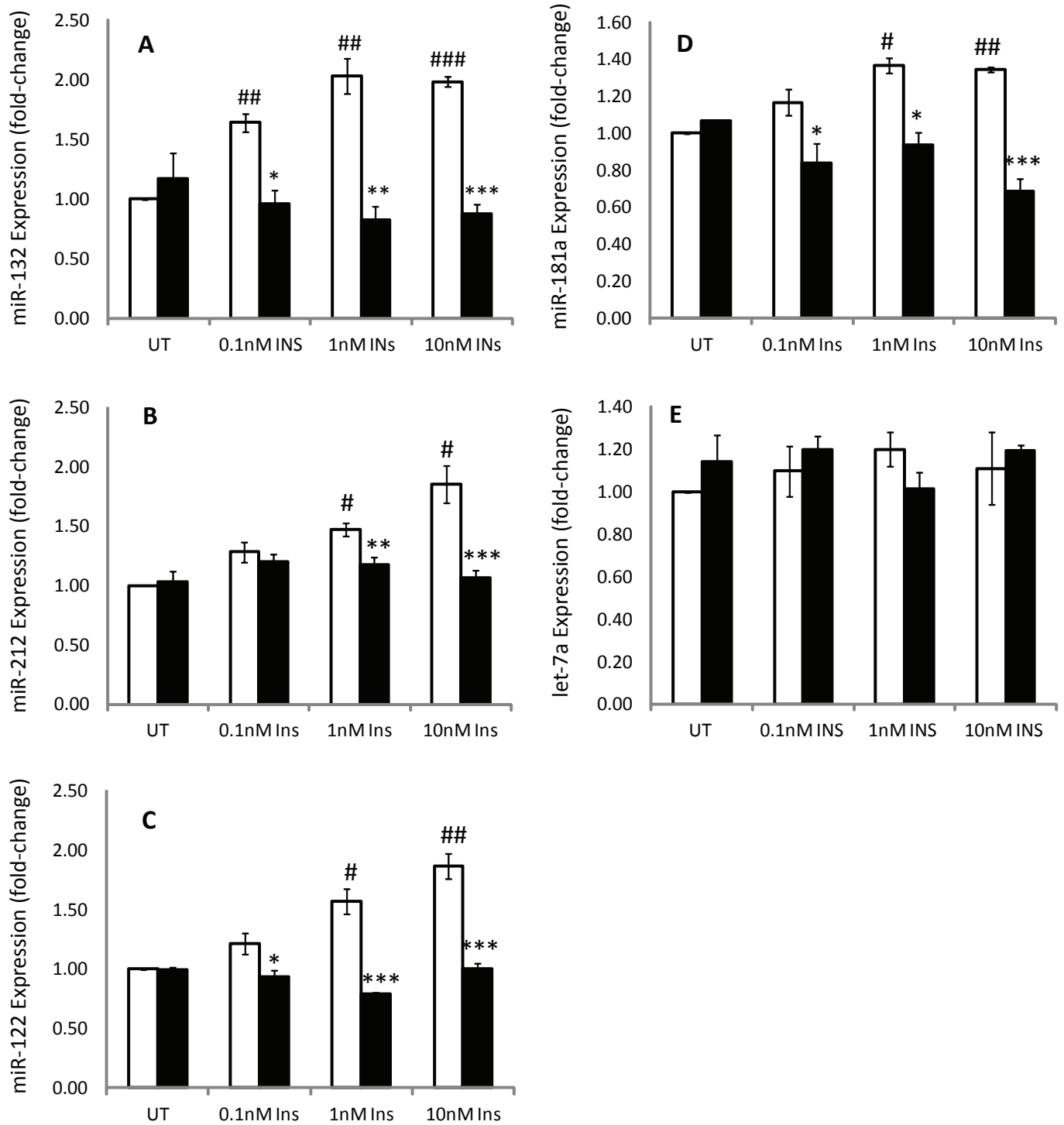


Figure 4

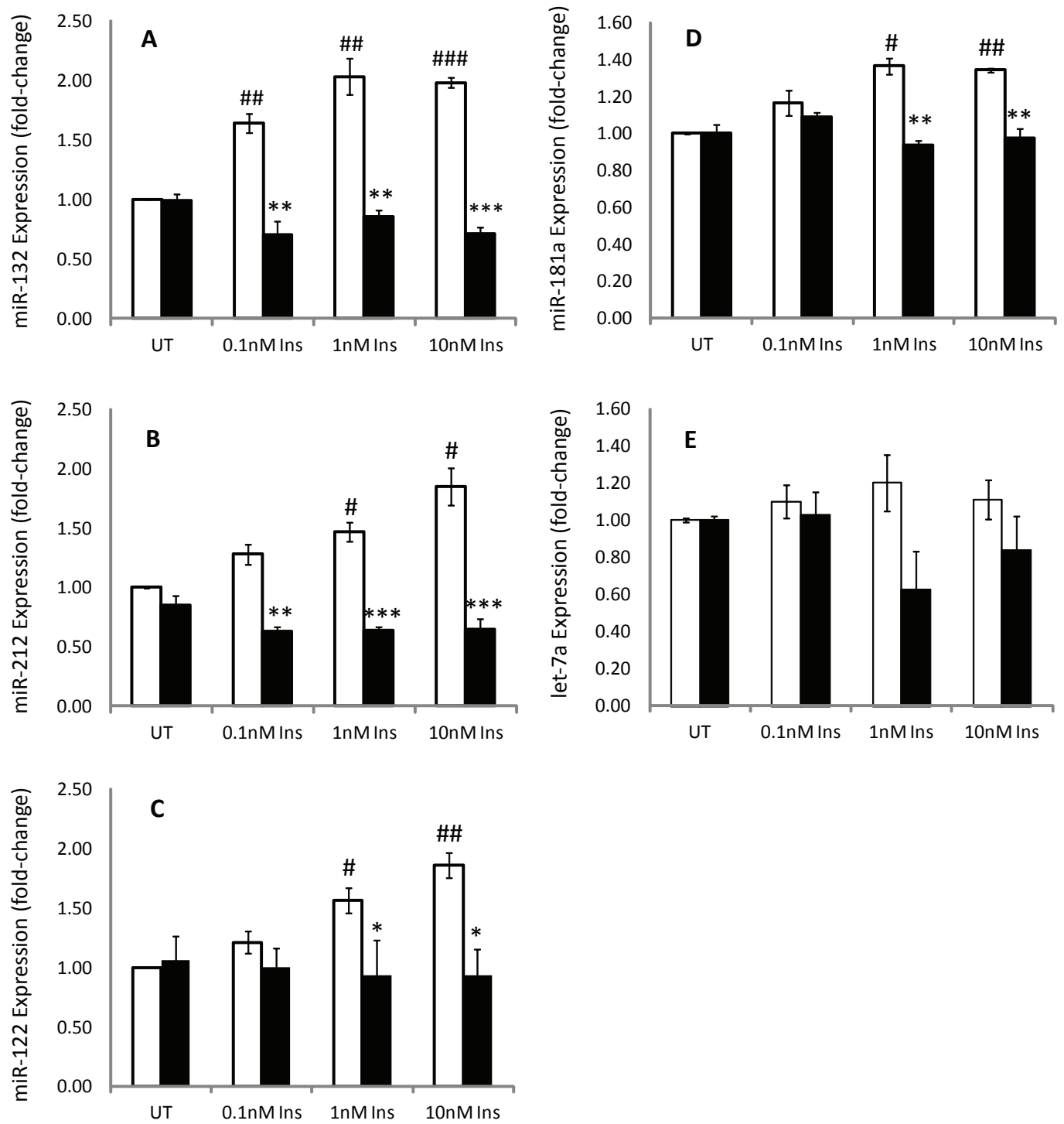


Figure 5

