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Human CYP2C8 is Post-transcriptionally Regulated by MicroRNAs 103 and 107 in Human Liver

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Running title MiRNAs 103/107 Regulate Human CYP2C8 Expression

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Abbreviations

miR, microRNA; miR-103, microRNA 103, miR-107, microRNA 107, MRE, miR-Response Element; AsOs, Antisense locked nucleic acid/DNA mixed oligonucleotides; 3'-UTR, 3'-untranslated region, CAR, Constitutively Activated Receptor; HNF4α, Hepatocyte Nuclear Factor 4 alpha; GR, glucocorticoid receptor; CYP, Cytochrome P-450; q-PCR, Quantitative polymerase chain reaction; CITCO, 6-(4-chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde O-(3,4- dichlorobenzyl)oxime

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ABSTRACT

The CYP2C genes are extensively regulated at the transcriptional stage. The present study shows for the first time that CYP2Cs are also regulated post-transcriptionally by microRNAs (miRNAs). Using an on-line search engine, we found potential recognition sites for miRNAs (MRE's) in the 3'-untranslated region (3'-UTR) of the CYP2C mRNAs. Among these were a MRE for miR-103/107 in the 3'- UTR of human CYP2C8. Moreover, CYP2C8 protein as measured by immunoblot analysis did not correlate with CYP2C8 mRNA measured by qPCR in human liver samples. Instead, the translational efficiency of CYP2C8 (protein/mRNA) was inversely correlated with the expression of miR-103 and miR-107. When three copies of the putative MREs from CYP2C8 were inserted downstream of a luciferase expression reporter, transfection with precursors for miR-103 or miR-107 into primary hepatocytes decreased luciferase activity while transfection with antisense oligonucleotides (AsOs) for miR-103/107 increased luciferase activity. As expected, there was no effect of the precursors or AsOs when three copies of the putative MRE's were in the reverse orientation. Moreover, when precursors for miR-103/107 were transfected into primary human hepatocytes, CYP2C8 protein was decreased, while AsOs increased the CYP2C8 protein levels. Neither precursors nor AsOs affected CYP2C8 mRNA, indicating the effect was post-transcriptional. Putative MRE motifs were also found in the 3'-UTR of CYP2C9 and CYP2C19, suggesting the same miRNAs could regulate translation of other members of CYP2C family albeit to a lesser degree than CYP2C8. These results clearly show that CYP2Cs are regulated post-transcriptionally by miR-103 and miR-107.

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Introduction

Cytochrome P450 monooxygenases provide crucial protection from xenobiotics and environmental toxins by metabolizing these hydrophobic compounds and converting them to more soluble, inactive compounds that are more readily excreted. In humans, the CYP2C subfamily of cytochrome P450 enzymes consists of CYP2C8, CYP2C9, CYP2C19, and CYP2C18 is an important subfamily of drug-metabolizing enzymes responsible for the metabolism of ~ 20% of all clinically prescribed therapeutics (Goldstein, 2001). They are found at highest levels in human liver (Goldstein and de Morais, 1994; Inoue et al., 1994; Klose et al., 1999; Nishimura et al., 2003), but CYP2C protein and/or mRNA expression has also been detected at lower levels in extra hepatic tissues such as kidney, lung, heart, endothelial tissue, adrenal, mammary gland and brain (Delozier et al., 2007; Deng et al., 2011; Klose et al., 1999; McFayden et al., 1998; Nishimura et al., 2003; Yasar et al., 2003).

Recently, numerous studies have described the transcriptional up regulation of the CYP2C genes (Chen et al., 2004; Ferguson et al., 2002; Pascussi et al., 2000a) by xenobiotics including clinically prescribed and over-the-counter drugs such as phenobarbital, rifampicin, St.John's Wort, and dexamethasone via the xenobiotic sensing receptors constitutive androstane receptor (CAR), pregnane X receptor (PXR) and the glucocorticoid receptor (GR) (Chen et al., 2003a; Chen et al., 2004; Ferguson et al., 2002; Rana et al., 2010; Rana et al., 2011; Surapureddi et al., 2011). The *CYP2C* genes are also upregulated by the liver enriched receptor hepatic nuclear factor 4α (Ferguson et al., 2005; Rana et al., 2010; Yue et al., 2010). However, to date there is no information concerning the possible translational regulation of these enzymes.

In recent years, microRNAs (miRNAs) have been discovered as a new class of small non-coding RNA genes (~22-nucleotides) that play important roles in the regulation of target

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genes, frequently through promoting mRNA degradation and repressing mRNA translation by binding to the 3'-untranslated regions (3'-UTR) or the coding region of target mRNAs (Bartel, 2004). At present ~ 1000 miRNAs have been identified in humans, and miRNAs are predicted to control 40-90% of the genes within the human genome (Lewis et al., 2005; Xie et al., 2005). MicroRNAs have been found to be involved in biological processes such as development, cell cycle, apoptosis, proliferation, differentiation, and carcinogenesis (Ambros, 2003; Carrington and Ambros, 2003; Gandellini et al., 2011; He and Hannon, 2004; Sempere et al., 2003). MicroRNAs can affect translation of multiple targets. Recently microRNAs have also been reported to affect expression of certain cytochrome P450 enzymes. Yokoi and coworkers (Tsuchiya et al., 2006) reported that miR-27b binds to a potential MRE in the 3'-UTR of CYP1B1 and affects expression of CYP1B1 in MCF-7 cells, a human breast cell line. Moreover, they found an association between expression of CYP1B1 protein and miR-27b in breast cancer tissue. Yokio and coworkers also found that CYP2E1 was regulated by miR-378; they established HEK293 cell lines stably expressing CYP2E1 mRNA with or without the 3'-UTR (Mohri et al., 2010). When these cells were treated with precursor for miR-378, CYP2E1 protein levels were decreased in the cell line that contained the 3'-UTR of CYP2E1 but not those lacking the UTR. Although there was some effect of miR-378 on CYP2E1 mRNA, the effect was primarily translational. Importantly, an MRE for miR-148 regulated the effects of the xenobiotic sensing receptor PXR, and miR-148a decreased the induction of PXR targets including CYP3A4 (Takagi et al., 2008).

Employing an on-line search using the miRBase Target database and Target Scan (Griffiths-Jones, 2004), we found several potential MRE's for miRNAs in the 3'-untranslated region (UTR) of the human CYP2C mRNAs including miR-103 and miR-107 in the 3'-

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untranslated region of CYPC8. miR-103/107 belongs to the miR-15/107 group of miRNAs, which contain the seed sequence AGCAGCA at or near the 5'-region of the mature miRNA. They are widely expressed in numerous human tissues including liver, brain, heart, and lung (Finnerty et al., 2010). They are transcribed from the introns of PANK genes, which are involved in regulation of acetyl CoA levels, cell stress, insulin sensitivity, lipid metabolism ischemic stress and hypoxia as well as angiogenesis and regulation of vascular endothelial growth factor (Trajkovski et al., 2011). Possibly, miR-103/107 may also be involved in regulation of some of these pathways. In the present study, we show that miR-103 and miR-107 affect the post-translational regulation of the human hepatic CYP2C family, particularly CYP2C8.

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Materials and Methods

Human Livers, Cell Culture Conditions and Reagents. Human primary hepatocytes and 31 liver samples representing excess tissue from the hepatocyte preparations individual donors were obtained from Cellzdirect, North Carolina (Invitrogen). Use of these hepatocytes was ruled exempt by the Office of Human Subjects Research (#3382) since they are from commercially available anonymous sources. HepG2 cells were grown in standard growth conditions. The pMIR-REPORT™ miRNA Expression Reporter Vector System was obtained from Ambion. Lipofectamine 2000 was from Invitrogen. Precursors for miR-103/107 and the negative controls were from Ambion (Austin, TX). Antisense locked nucleic acid/DNA mixed oligonucleotides (AsOs) for miR-103 (5'-CATAGCCCTGTACAATGCTGC-3'; Cat#414336-00) and miR-107 (5'-GATAGCCCTGTA CAATG-3'; Cat# 410524-00) and negative controls (5'-GTGTAACACGTCTATACGCCA-3' Cat#199004-00) were from Exiqon (Woburn, MA). All primers and oligonucleotides were commercially synthesized at Integrated DNA Technologies (Coralville, IA).

Reporter Plasmid Constructs. To construct luciferase reporter plasmids, target oligos were inserted between the MluI and HindIII site downstream of the luciferase gene in the pMIR-REPORT miRNA expression reporter vector. The sequence from 1670 to 1692 (~ 200 bp downstream of the stop codon) in the human CYP2C8 mRNA (5' AACACTTGTATTAATT GCTGCATATGCT-3') containing a putative miR-103/107 recognition element was termed CYP2C8MRE. Three copies of the CYP2C8MRE (5'AACACTTGTATTAATTGCTGCATA TGCTNNNNNNNAACACTTGTATTAATTGCTGCATATGCTNNNNNNNAACACTTGTAT TAATTGCTGCATATGCT-3'; 2C8 MRE is underlined) were cloned into the pMIR-Report expression reporter vector and termed pMIR3XMRE. The complementary sequence of the three

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copies of the CYP2C8MRE was also cloned into the pMIR-Report expression reporter vector as a negative control and termed pMIR3XMRE-Rev. Oligos containing the perfect matching sequences of the mature miR-103 (5'-TCATAGCCCTGTACAATGCTGCT-3') and miR-107 (5'-TGATAGCCCTGTACAATGCTGCT-3'), both containing the 5' seed sequence AGCAGC, were also cloned into the pMIR-Report expression reporter vector as positive controls. All the constructs were verified by sequencing.

Quantitative Real time PCR. Total RNA was isolated using the *mir*Vana miRNA isolation kit (Applied Biosystems). For quantification of mature miRNA, cDNA was reverse transcribed from total RNA using specific primers from the Taqman MicroRNA Assay (Applied Biosystems) and reagents from the Taqman MicroRNA Reverse Transcription Kit (Applied Biosystems). Amplification reactions were performed using the Taqman MicroRNA Assay and Taqman Universal PCR Master Mix (Applied Biosystems). RNU44 was used as the endogenous control. To determine the expression levels of CYP2C8 mRNA, cDNA was synthesized from total RNA by MultiScribe reverse transcriptase (Applied Biosystems) and quantified using Taqman gene expression assay (Applied Biosystems) with Taqman Universal PCR Master Mix. GAPDH was used as the endogenous control. Real time PCR was carried out on an Applied Systems 7900HT Sequence Detection System. The relative quantity was normalized to each endogenous control and calculated using the $2^{-\Delta\Delta Ct}$.

Luciferase Assays. A series of luciferase reporter plasmids or empty vector controls were transiently transfected along with the internal control pRL-TK plasmid into human primary hepatocytes and HepG2 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). miR-103/7 precursors (10 pmol) (Applied Biosystems) or the precursor control or miRCURY locked nucleic acid antisense nucleotides (AsOs) or the AsO control (10 or 50 pmol) to miR-103 and -107

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(Exiqon, Woburn, MA) were co-transiently transfected into cells. After 48h, the cells were resuspended in passive lysis buffer (Promega, Madison, WI). Luciferase assays were performed using a dual luciferase assay kit (Promega) and firefly luciferase activities were normalized with *Renilla* luciferase activities.

Antibodies. The CYP2C8-specific peptide DNQKSEFNIENLVGC was synthesized by ResGen (division of Invitrogen, Carlsbad, CA) and coupled to Keyhole Limpet Hemocyanin (KLH) through the C-terminal cysteine. A new polyclonal antibody specific for the 2C8 peptide (termed 1937) was produced by Covance Research Products Inc. (Denver, PA) by the standard NIEHS protocol as follows: At the start of production, a 1 mg/ml suspension of conjugated peptide was diluted 1:1 with Freund's Complete Adjuvant and each of two rabbits received 500 μg (1 ml) following pre-bleed. After 4 weeks, each rabbit was boosted with 250 μg of peptide diluted (1:1) with Freund's Incomplete Adjuvant, followed after 2 weeks with a test bleed. Rabbit(s) received 4 additional boosts at 4-week intervals, with production bleeds beginning after the third boost. Rabbits were terminated by exsanguination at completion of production. Antibodies used in this study were from the second production bleed. This antibody was specific for CYP2C8 on immunoblots. Antibody to CYP2C19 (termed 1590) was similarly raised in rabbits using bacterially expressed recombinant CYP2C19. This antibody recognizes all of the human CYP2C enzymes (CYP2C9>CYP2C19>>CYP2C8), but they can be distinguished by their electrophoretic mobility on 16 cm gels. Mouse monoclonal antibodies for HNF4α (PP-H1415-00, clone: H1415) and CAR (PP-N4111-00, clone N4111) were obtained from R&D systems (Minneapolis MN (formerly Perseus Proteomics Inc., Tokyo Japan) Anti GAPDH, GR (Sc-8992), HNF4α (Sc-8987) and CAR (Sc-13065) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

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Immunoblotting: 10 or 50 pmol precursors or AsOs for miRNAs or their respective controls were transiently transfected into human primary hepatocytes using Lipofectamine 2000. After 72h, the cells were harvested. Cell homogenates from human primary cells or human livers were prepared by homogenizing with lysis buffer. Samples were separated by 10% Tris/glycine/SDS gel electrophoresis (using 16 cm gels for the CYP2C19 antibody) and transferred to a nitrocellulose membrane (Invitrogen). The membrane was blocked with 5% nonfat milk blocking buffer and blotted with rabbit-anti-human CYP2C8 (peptide) or GAPDH (Cell Signaling, Danvers, MA) primary antibody. Goat anti-rabbit-HRP (Pierce Biotechnology, Rockford, IL) was used as a secondary antibody. The protein bands on the membranes were developed using a SuperSignal Western Femto kit (Thermo-Pierce). The blots were imaged on the Gnome (SynGene, Frederick Maryland), and bands were quantitated using GeneTools, which comes with the instrument).

For immunoblotting of the receptors HNF4α, GR or CAR, cell homogenates from three replicate wells were combined and immunoblotted with antibodies to HNF4α, GR, CAR or GAPDH as a loading control.

Statistical Analysis. Data are expressed as the means $\pm$ standard error of triplicate determinations or of three independent experiments. Correlation analysis was performed by Spearman's rank method. The statistical significance of the difference between the expression levels of miR-103/107 in human liver samples was determined by a paired, two tailed Student's *t* test. The relationship between the CYP2C8 protein level and the miR-103/107 levels was investigated by analysis of variance and Tukey's test. A value of $P < 0.05$ was considered statistically significant.

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Results:

Relationship between Human CYP2C8 Protein, mRNA, and MiRNA expression

Levels in 31 Human Liver Tissues. We measured human CYP2C8 protein and mRNA expression levels by Western blot analysis and by q-PCR in a panel of 31 human liver tissues representing excess tissue from hepatocyte preparations obtained from CellzDirect (Invitrogen). As shown in Fig. 1A, there was no statistically significant correlation between CYP2C8 mRNA and protein levels (Spearman ρ=0.09, P>0.05), which suggested the possibility that CYP2C8 might be post-transcriptionally regulated. We therefore employed the on-line search engines miR-Base (www.miR-base.org) and Target scan (www.targetscan.org, Release 6) that identified several potential recognition elements for microRNAs in the 3' UTR for the human CYP2C genes. Among these a potential miR-103/107 target site was found ~200 bp (197-219) down stream of the stop codon of human CYP2C8 mRNA with minimum free energy of -10.20 and a score of 141.00 (miR-base) on target scan scores were -0.24, seed match of 7mer1A for both the miRNAs. The alignment of miR-103 and 107 and that of the putative recognition element in CYP2C8 mRNA is shown in Fig 1B. These two miRNAs differ by only one nucleotide at the 2nd position from the 3' end, where a nucleotide G in miR-103 is different from a C nucleotide in miR-107. We termed the putative recognition element (2C8MRE103/ 2C8MRE107). To investigate the possible role of miR-103/107 in the translational regulation of CYP2C8 in human liver tissue, we first compared the expression levels of miR-103/107 with those of CYP2C8 protein using the same panel of 31 human livers. The expression levels of miR-103/107 were variable in human livers. CYP2C8 protein/mRNA ratios were calculated as an index of translation efficiency of CYP2C8 and compared with levels of miR-103 and miR-107. The translational efficiency of CYP2C8 was inversely correlated with miR-103 with Spearman ρ=-

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0.4, $P<0.05$ and miR-107 with Spearman $\rho=-0.5$, $P<0.05$ (Fig 2A and Fig 2B) in human livers consistent with the possibility that CYP2C8 may be negatively regulated by miR-103 and miR-107.

Expression Levels of Mature miR-103 and 107 in Human Primary Hepatocytes and HepG2 Cells. CYP2C8 mRNA is expressed at considerably higher levels in human liver than HepG2 cells, and we have been unable to detect CYP2C8 protein in this hepatic cancer cell line. To quantify the levels of miRNA in primary hepatocytes representing human liver samples and HepG2 cells representing tumor cell lines, total RNA was isolated using the *mir*Vana miRNA isolation kit (Ambion). Quantification of miRNA was performed as described in methods. RNU44 was used as endogenous control as shown in Fig 3A. Human mature miR-103/107 expression levels were determined both in primary hepatocytes as well as in HepG2 cell lines as $2^{-\Delta\Delta Ct}$ values normalized to human RNU44 levels. Expression of both mature miR-103 and miR-107 were found to be higher in HepG2 cells (2.5 and 4-fold higher) compared to primary human hepatocytes. The relative expression of miR-107 appears to be moderately higher in HepG2 cells compared to miR-103 (Fig 3A).

Regulation of CYP2C8 by miR-103 and miR-107 Recognition Element (MRE) in the 3' UTR of CYP2C8 in Primary Human Hepatocytes. To determine whether the putative CYP2C8MRE (103/107) is functionally recognized by miR-103/107, luciferase assays were performed in human primary hepatocytes (Fig 3B). pMIR/P-Seq-miR103 and pMIR/P-Seq-miR-107 constructs in which the miR-103 and miR-107 complementary sequence was inserted individually downstream of the luciferase gene driven by SV40 promoter were used as positive controls. Luciferase activation of both constructs was significantly decreased ($p<0.05$) by the co-transfection with their respective individual precursors (miR-103 and miR-107)(Fig 3B).

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Moreover, the luciferase activity of pMIR/3XMRE constructs (containing three copies of the putative miR-103 or miR-107 CYP2C8 recognition site downstream of the luciferase gene) was significantly decreased by ~ 40% (*p<0.05)* when pMIR3XMRE (103) was co-transfected with 10 pmol of the precursor for miR-103 or miR-107 individually. The negative control pMIR/3XMRE-Rev construct containing three copies of the inverted putative recognition site had no effect when precursors for miR-103 or miR-107 were co-transfected individually. Human primary hepatocytes express significant levels of miR-103 and miR-107, and the luciferase activities of pMIR/P-Seq-miR103 and pMIR/P-Seq-miR107 were significantly increased when miR-103 or miR-107 expression was silenced by transfection of 10 pmol of the specific AsOs for either miRNA (Fig 3C). The luciferase activity of the pMIR/3XMRE construct was significantly increased (*p<0.05)* when it was co-transfected with 10 pmol of AsOs for miR-103 or miR-107. As expected, silencing miR-103 and miR-107 with AsOs had no effect on the luciferase activity of the negative control constructs pMIR/3XMRE-Rev. Taken together these results indicate that both miR-103 or miR-107 functionally recognize CYP2C8MRE (103/107) to negatively regulate the expression of CYP2C8.

We then examined the effects of the precursors of miR-103/107 on pMIR-Luc constructs in HepG2 cells (Fig 4A). As seen earlier in Fig 3A, HepG2 cells express miR-103/17 at higher levels than primary hepatocytes. Ten pmol of the precursors for miR-103/107 were effective in decreasing luciferase activity of both the positive control pMIR-Luc constructs and the pMIR/3xMRE construct. In contrast, 10 pmol of AsOs for miR-103/107 did not have any significant effect on the positive pMIR/c-103 or 107 constructs in preliminary experiments presumably due to the abundance of precusors for miR-103/107 in HepG2 cells. However, when miR-103 and miR-107 were silenced with 50 pmol of either AsO, we observed a marked

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increase in luciferase activity with the positive control luciferase constructs as well as the construct containing three copies of the CYP2C8 MRE (Fig 4B). A higher dose of 100 pmol had deleterious effects on the cell viability (data shown below). Taken together these results indicate that both miR-103 and miR-107 functionally recognize CYP2C8MRE (103/107) to negatively regulate the expression of CYP2C8 in both HepG2 cells and primary human hepatocytes.

Determination of Optimum Concentrations of miR-103/107 Precursors and AsO Silencers on Function and Cell Viability. To ascertain whether we were using the optimum concentrations of precursors and AsOs for miR-103/107 in HepG2 cells and to determine whether the levels of precursors and AsOs we were using were toxic to primary hepatocytes and HepG2 cells, we performed the following experiments. HepG2 cells and primary human hepatocytes were transfected with different doses of the precusors for miR-103 and compared to Lipofectamine 2000 in treated control cells and untransfected cells. There was a small 5% decrease in viability in Lipofectamine 2000 in treated cells vs nontreated cells, but no effect of the precursor for miR-103 on viability in HepG2 cells at any of the doses tested (10, 50 and 100 pmols) and only a minimal decrease ($\leq$5%) of viability in primary human hepatocytes treated with the miR-103 precursor compared to the precursor control (Fig. 5A and B). When miR-103 was inhibited with locked nucleic acids (AsOs) in HepG2 cells, we saw no effect on viability at doses below 100 pmol which significantly decreased viability for both the miR-103 AsO or its control. In primary hepatocytes doses of 10 and 50 pmol of the AsO control and AsO for miR-103 were tolerated relatively well (~10-15% decrease in viability) but 100 pmol was more toxic (24% decrease in viability) (Fig 5A and 5B).

We then tested the effectiveness and toxicity of varying doses of the precursor and AsO for miR-103 using the positive control luciferase construct pMIR/c-103 in HepG2 cells (Fig. 5C

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and D). As seen in Fig 5C, 10 pmol of precursor produced a maximum effect with minimum toxicity. There was little effect of the precursor control at 10 pmol compared to HepG2 cells that were transfected with Lipofectamine 2000 and the pMIR/c-103 construct alone (sham). On the other hand, 10 pmol of the AsO for miR-103 was not effective in HepG2 cells presumably due to the high concentrations of miR-103/107 in HepG2 cells. Maximum increases in luciferase activity were seen with 50 pmol of AsO. Further increases of AsO concentrations to 100 pmol did not have any added benefit, most likely due to the side effects on cell viability.

Effects of miR-103/107 on Human CYP2C8 Protein Expression in Human Primary Hepatocytes. We then examined the effects of ectopic over expression or silencing of miR-103 and miR-107 on CYP2C8 protein expression in primary hepatocytes. The transfection of the precursors for miR-103 or miR-107 into primary hepatocytes, which express low amounts of both, the miRNAs increased miR-103 or -107 several hundred fold (Fig. 6A). Ectopic expression of miR-103 and miR-107 at the 10 and 50 pmol level significantly decreased CYP2C8 protein levels on as measured by immunoblot analysis with a specific peptide antibody to CYP2C8 compared to control GAPDH (Fig. 6 B). The decrease appeared greater at 50 pmol than 10 pmol. Conversely, transfecting with 10 pmol of specific AsOs for miR-103 and miR-107 individually decreased the detectable levels of the two miRNAs by 70-80% (Fig. 6C) and increased endogenous CYP2C8 protein levels by 30-60% in primary human hepatocytes compared to GAPDH with a somewhat greater increase at the 50 pmol level(50-80%) (Fig. 6 D). Thus, these results confirm that miR-103 and -107 regulate human CYP2C8 protein content in primary human hepatocytes.

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Do miR-103/107 Affect the Expression of Other CYP2C Enzymes? The *CYP2C* genes are closely related with a high degree of amino acid and nucleic acid homology (Lewis et al., 2003). They are often transcriptionally regulated by many of the same receptors (Chen et al., 2003b; Chen and Goldstein, 2009; Ferguson et al., 2005; Ferguson et al., 2002). A scan of the 3'-UTR regions of 2C8, 2C9 and 2C19 showed that all three contain putative elements similar to the MRE of 103/107. In CYP2C9 cDNA, the putative MRE site is CATACTTATCT**AATGTAG**AG with the core AATGCTG of miR-103 (miRBase), with a mismatch of 2 nucleotides is 240-260 bp downstream from the stop codon in the 3'UTR. In 2C19 there are two possible MRE sites, the first located 222-242 bp and the second 138 - 152 bp downstream of the stop codon. The MRE TGATACTTGTCT**AATGTTG**AG contains the core AATGCTG of miR-103 with a mismatch of only 1 nucleotide (miRbase). With putative MREs located in the UTR regions of 2C9 and 2C19, we examined the effects of 50 pmol of the precursors for miR-103 and -107 on expression of CYP2C9 and CYP2C19 proteins in primary human hepatocytes (Fig. 7A) or 50 pmol AsOs for miR-103 or -107 (Fig. 7B) in cells from three donors. The crude membranes from hepatocyte lysates were separated on large 10% SDS-PAGE gels and immunoblotted with polyclonal antibody 1590, which recognizes all the human CYP2C proteins (CYP2C9>CYP2C19>>CYP2C8). The individual CYP2C proteins are distinguished by the differences in their electrophorectic mobility on large SDS-PAGE gels when compared to recombinant proteins expressed in yeast. Antibody 1937 is the specific CYP2C8 peptide antibody, which recognizes only CYP2C8. The precursors for miR-103/-107 down regulate CYP2C8 (Fig. 7A) to almost negligible levels (<90%) compared to precursor controls as judged by densitometric quantification (Fig. 7C). In addition, knockdown of miR-103 and 107 by transfection with AsOs upregulated 2C8 protein levels by more than 60-80% (Fig. 7B and

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Fig. 7D). Quantification of CYP2C9 expression from an immunoblot with antibody 1590 showed only a ~40% decrease in CYP2C9 protein levels after transfection with the precursors for miR-103 and -107 (Fig 7A and 7E), and the knockdown of miR-103/107 expression with specific AsOs had no significant effect ($p, \leq 0.5$) on the expression of CYP2C9 protein (Fig 7B and 7F. These data suggest that miR-103/107 have a minimal role in regulating CYP2C9 in human hepatocytes. When the polyclonal antibody 1590 was used to quantify the CYP2C19 protein levels, the both the precursors miR-103 and -107 down regulated CYP2C19 levels by ~ 60% as judged by the densitometric quantifications (Fig. 7A and 7G). The AsOs for miR-103 and -107 increased CYP2C19 protein levels by about 30% but this increase was not statistically significant (Fig.7B and H). These results suggest that miR-103/107 may regulate CYP2C19> CYP2C9 in human hepatocytes, albeit to a much lesser degree than CYP2C8. Results with a Gentest antibody to a 2C19 peptide were not specific for CYP2C19 and yielded similar results to antibody 1590 (data not shown).

Immnunoblotting of Hepatocytes Extracts for the Receptors HNF4*α*, CAR, and GR. Since the CYP2Cs are regulated by CAR, GR, and HNF4α, we examined the cellular content of these receptors 72 hours after transfection of primary human hepatocytes with precursors for miR-103 or miR-107 and after transfecting with AsOs to miR-103/107 to decrease expression of these miRNAs in primary hepatocytes (Fig. 8A and B). These hepatocytes were from the study of the effect of miR-103/107 on CYP2C protein expression (Fig. 6). There was no effect of miR-103 or miR-107 expression on CAR, GR, and HNF4α content of primary hepatocytes, suggesting the effect on CYP2C expression was a direct effect on the translational regulation of the CYP2C proteins rather than an indirect effect through changes in these receptors known to regulate the *CYP2C* genes.

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miR-103 and -107 do not Change CYP2C8 mRNA Levels. MiRNAs most frequently affect the translational regulation of the gene, although some microRNAs also affect mRNA levels. Total RNA was isolated from primary hepatocytes that had been transfected with either the precursors for miR-103 and miR-107 (Fig. 9A) or with AsOs for 103 and 107 individually (Fig. 9B) or with their respective controls. Precursors for miR-103 and -107 did not have any effect on CYP2C8 mRNA levels as quantified by qPCR. Moreover, AsOs for miR-103 and 107 did not increase the CYP2C8 mRNA levels, suggesting that the site of action of these miRNAs on CYP2C8 regulation is at the translation level and not at the transcriptional level.

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Discussion

CYP2C8 detoxifies a variety of important xenobiotic compounds primarily in liver but also in extra hepatic tissues (Goldstein, 2001; Totah and Rettie, 2005). CYP2C8 metabolizes endogenous substrate arachidonic acid to produce various EETs (epoxyeicosatrienoic acids) in liver and extra hepatic tissues viz. heart, and endothelial cells and is important for the regulation of vascular tone and cardiovascular homeostasis (Dai et al., 2001; Edin et al., 2011). CYP2C8 plays a central role in the metabolism of a number of therapeutic drugs including the anticancer drug taxol (Cresteil et al., 2002; Rahman et al., 1994), the cholesterol lowering drug cerivastatin (Ogilvie et al., 2006; Wang et al., 2002), the antimalarial drug amodiaquine(Li et al., 2002), the antidiabetic drugs troglitazone (Yamazaki et al., 1999) and rosiglitazone (Baldwin et al., 1999), the antiarrhythmic drug amiodarone (Ohyama et al., 2000), and calcium channel blocker verapamil (Tracy et al., 1999).

Previous studies have shown that the CYP2C genes are transcriptionally up regulated by rifampicin (Ferguson et al., 2005), phenobarbitol (Pascussi et al., 2000b; Raucy et al., 2002) and dexamethasone (Gerbal-Chaloin et al., 2001) through a number of xenosensing nuclear receptors, PXR, CAR and GR (Ferguson et al., 2005) and by HNF4α (Rana et al., 2010). This is the first study to show that CYP2C8 is regulated post-transcriptionally by microRNAs. We show that there is no significant correlation between the mRNA levels and the protein levels in human liver samples, which is consistent with the possible translational regulation of CYP2C8. In a panel of 31 liver samples, miR-103 and -107 were inversely correlated with the CYP2C8 protein expression suggesting that CYP2C8 could be negatively regulated by miR-103/107. miR-103 and miR-107 are paralogs and universally expressed in essentially all mammalian tissues, particularly brain, liver, and spleen in humans. They are transcribed from the intronic regions of

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the PANK1 and PANK2 genes (Pantothenate kinase enzyme) on two different chromosomes (Chr 10 and 20). Their expression is thought to parallel that of the two PANK genes. Ontogeny, distribution and functional significance of these miRNAs recently been an active field of investigation and these two miRNAs have been reported to be involved in the regulation of insulin sensitivity, glucose tolerance, cellular metabolism, lipid metabolism, angiogenesis, stress, and neuronal development, migration and various diseases reviewed previously (Moncini et al., 2011; Trajkovski et al., 2011).

In silico analysis identified a target site for miRNAs (103/107) which we termed CYP2C8MRE (103/107) in the 3' UTR in CYP2C8 mRNA. In primary hepatocytes, ectopic expression of precursors for miR-103 and -107 inhibited luciferase constructs containing three copies of the putative MRE in CYP2C8 (3XMRE) but did not affect constructs containing the reverse complementary 3XMRE, showing the putative MRE was functional. Even stronger evidence was provided by the fact that the AsOs for miR-103 and 107 restored the luciferase activity of 3XMRE but did not have affect on the reverse complementary 3XMRE. These results suggest that the CYP2C8MRE (103/107) is functionally recognized by the miR-103/107. Though both miR-103 and miR-107 recognize the CYP2C8MRE, they only differ by a single nucleotide (G to C). The overall complementarity of the recognition site in the CYP2C8 UTR and the miRNAs is quite high, allowing CYP2C8 to be effectively regulated by these miRNAs. Furthermore, the endogenous CYP2C8 protein level was decreased by the ectopic expression of the precursors for these individual miRNAs and blocking miR-103 and miR-107 expression by their respective AsOs increased CYP2C8 protein. There was no effect of miR-103 or miR-107 or their respective AsOs on CYP2C8 mRNA, clearly indicating that miR-103 and 107 post-transcriptionally regulate human CYP2C8.

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Interestingly, a putative MRE is also found in CYP2C9 and CYP2C19 albeit with a two nucleotide mismatch for CYP2C9 and two MREs with a one nucleotide mismatch in CYP2C19. On ectopic expression of individual precursors for miR-103 and 107, endogenous protein levels of the three enzymes were reduced compared to controls in the order of CYP2C8>CYP2C19>CYP2C9. Conversely when specific AsOs inhibited these miRNAs, the endogenous protein levels of CYP2C19 increased, although to a lesser degree than CYP2C8. The lesser effect of the miRNAs on CYP2C9 is consistent with the 2 base mismatch. However our results suggest that these miRNAs could be regulating not only CYP2C8 but also CYP2C19.

miRNAs are implicated in many biological processes. Recently, they have been implicated in the field of drug metabolism (Nakajima and Yokoi, 2011; Ingelman-Sundberg et al., 2007). Various drug metabolizing enzymes including CYP3A4 (Pan et al., 2009), CYP2E1 (Pan et al., 1992), CYP7A1 (Song et al., 2010), CYP1B1 (Tsuchiya et al., 2004), CYP2A3 (Kalscheuer et al., 2008) and CYP24 (Komagata et al., 2009) have been identified as direct targets for miRNAs. miRNAs have also been shown to regulate the receptors PXR (Takagi et al., 2008) and HNF4α (Takagi et al., 2010), thus affecting the expression of downstream targets such as CYP3A4 and CYP7A1 respectively. These data indicate that there are multiple targets of miRNAs which affect both the constitutive expression and the upregulation of drug metabolizing enzymes.

In conclusion, we found that the human CYP2C family is post-transcriptionally regulated by miR-103/107 affecting the CYP2C8 level in human liver. This study provides new insights into the mechanism of the large inter-individual variability in the expression CYP2C8 and other members of the CYP2Cs family in liver and other tissues. It also provides insight into various physiological or pathological stresses that may regulate these enzymes. Future studies should

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examine conditions that vary the expression of the PANK genes and the miR-103/107 family and thus alter expression of CYP2C8 and CYP2C19 in human tissues.

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Authorship Contributions

*Participated in research design***:** Zhang, Surapureddi, Goldstein

Conducted experiments: Zhang, Coulter

Contributed new reagents or analytical tools and discussions: Ferguson

Performed data analysis: Zhang, Surapureddi, Goldstein

Wrote or contributed to the writing of the manuscript: Zhang, Surapureddi, Goldstein

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Footnotes

The authors Shu-Yun Zhang and Sailesh Surapureddi contributed equally to this paper.

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Legends for Figures

Fig.1. Correlation between the CYP2C8 mRNA and protein levels in 31 human liver samples. (A) CYP2C8 mRNA levels were determined by real time quantitative PCR and were normalized with GAPDH mRNA levels. Microsomal CYP2C8 protein content was determined by immunoblots using a specific peptide antibody for CYP2C8 and was normalized with the GAPDH protein levels. As judged by the Spearman correlation analysis, there was no significant correlation between CYP2C8 mRNA levels and the protein levels. (B). Predicted target sequence of miR-103/107 in the human CYP2C8 mRNA. The numbering refers to the ATG in translation starting with A as 1, and the coding region is up to +1470 with the stop codon at 1471-1473. The sequence of the putative 2C8MRE (gray box) is located 197-219 bp downstream from the stop codon in the 3'-UTR of human CYP2C8 mRNA.

Fig. 2. Inverse relationship between the expression levels of miR103 (A) and miR-107 (B) and the translation efficiency of human CYP2C8 protein in human liver samples. Expression levels of miR-103 or miR-107 levels were inversely correlated with the translation efficiency of human CYP2C8 protein/mRNA ratios. Spearman analysis showed an inverse correlation (ρ=-0.4 for miR-103 and ρ=-0.5 for miR-107; with a $P<0.05$).

Fig. 3. Human CYP2C8 regulation by miR-103/107. (A) Relative endogenous expression levels of miR-103 and miR-107 in primary hepatocytes and HepG2 cells were determined by real time qPCR using a Taqman microRNA assay and kit, the values of the mature miR-103 and miR-107 were normalized with the RNU44 levels using the $2^{-\Delta\Delta Ct}$ method (B) Luciferase assays were performed to investigate whether miR-103 and -107 functionally regulate CYP2C8MRE. The

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reporter constructs were transiently transfected into primary human hepatocytes with (B) 10 pmol of the precursors for either miR-103, miR-107 or control precursors or (C) with 10 pmol of AsOs for miR-103, miR-107, or AsO controls. pMIR driven firefly luciferase activities were normalized with renilla luciferase activities and were plotted relative the pMIR control vector. Each column represents the mean of $\pm$ S.E of three independent experiments *, Significantly different from precursor or AsO control, $p<0.05$.

Fig. 4. Regulation of CYP2C8 MRE by miR-103/107 in HepG2 cells. (A). HepG2 cells were transfected with pMIR, pMIR/3xMRE, pMIR/3xMRE-Rev and their respective complementary sequences pMIR/c-103 or pMIR/c-107, and with 10 pmol of precursor control or precursors for miR-103 and miR-107 as shown above. (B) pMIR constructs were transfected with AsO inhibitors for miR-103 and miR-107 at a concentration of 50 pmol. Each column represents the mean of $\pm$ S.E of three independent experiments *, Significantly different from controls, $p<0.05$.

Fig. 5. Ectopic expression of miR-103 at higher concentrations effect cell viability and functionality. (A) Human Primary hepatocytes or (B) HepG2 cells were transfected with precursors and AsO inhibitors for miR-103 with Lipofectamine 2000 and compared their viability to untransfected cells or cells treated with the transfection agent alone. There was an ~5% decrease in viability when cells were treated with Lipofectamine 2000. However, precursors at the 10, 50 and 100 pmol range did not affect viability compared to the precursor controls. AsOs, on the other hand, did not affect viability in HepG2 cells (B) at concentrations of 10 and 50 pmol but both the AsO control and AsO for miR-103 decreased viability by ~10-15% at 100 pmol. (A) In primary hepatocytes AsOs and their controls affected the viability by

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~10% at all dose levels but more (~15%) at the highest dose of 100p mol. (C) To judge the effectiveness of the doses of the precursors and (D) AsOs for miR-103 on the activity of the luciferase constructs , the pMIR/c-103 positive control was used as a representative and transfected into HepG2 cells in the presence of miR-103 precursors (C) at 10, 50 and 100pmol range and compared to pMIR/c-103 transfected cells alone. Ectopic expression of precursors for miR-103 inhibited luciferase activity significantly at 10 pmol levels and higher does of the precursor (50 and 100 pmol) did not have any further effect. (D) On the other hand, Inhibition of miR-103 with its specific AsO increased pMIR/c-103 driven luciferase activity at 50 pmol but was not effective at 10 pmol levels compared to controls. The higher doses of AsO at 100 pmol did not show any significant increase over the level seen at 50 pmol concentration. The values represented are the mean of $\pm$S.E for triplicate observations *Significantly different from precursor or AsO control, $p<0.05$

Fig. 6. Ectopic over expression and silencing of miR-103 and miR-107 regulates human CYP2C8 protein levels in primary human hepatocytes. Cells were transfected with 10 or 50 pmol of precursor for miR-103 and -107 individually and 10 or 50 pmol AsOs or their respective controls. After 72h, total RNA and microsomal membranes were prepared as described earlier. Mature miRNA levels were determined by real time RT-PCR analysis after transfection with precursors (A) or AsOs (C). Values were normalized to RNU44 levels and then values expressed relative to controls. Microsomal human CYP2C8 levels along with GAPDH were determined by immunoblot analysis. Primary hepatocytes from one donor were transfected with (B) 10 and 50 pmol precursors and (D) 10 and 50 pmol AsOs individually; Lanes represent one of three replicate wells (Con) Precursor or AsO control (103) miR-103/AsO, (107) miR-107/AsO. The

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relative quantity of CYP2C8 protein level was analyzed by densitometric image analysis and expressed relative to GAPDH. The values are the mean of ± S.E for triplicate observations *, significantly different from controls at $p<0.05$.

Fig. 7. The effect of miR-103/107 precursors or AsOs on the expression of other CYP2C proteins. Randomly selected primary hepatocytes from three separate donors were transfected with (A) 50 pmol of precursor miRNAs for control, miR-103 and miR-107 or (B) 50 pmol of AsOs specific for control, miR-103 and miR-107 to silence these miRNAs for 48h and microsomal membranes were prepared from each set of donor hepatocytes. The membrane proteins were separated on a large 10% SDS-PAGE along with Gentest HLM (human liver microsomes) and recombinant CYP2Cs expressed in yeast as controls and transferred onto nitrocellulose membranes. The membranes were probed with in-house generated antibodies for CYP2C19 (1590), a specific CYP2C8 peptide (1937) and GAPDH. Antibody to CYP2C19 also recognizes CYP2C9 and weakly CYP2C8, thus the individual proteins could only be identified by their electrophoretic mobilities on large gels. (C-H) The images were scanned and quantitated by densitometry analysis and represented as mean of three donor samples, (C) effect of precursor miRNAs on CYP2C8 protein; D) effect of AsOs on CYP2C8 protein; (E) effect of precursor miRNAs on CYP2C9; and (F) effect of AsOs on CYP2C9; (G) effect of precursor miRNAs on CYP2C19 and (H) effect of AsOs on CYP2C19. **, Significantly different from precursor or AsO control at $p<0.01$.

Fig. 8. miR-103/107 do not affect the expression of the receptors HNF4α, CAR, or GR in primary human hepatocytes. Hepatocytes were treated with 10 or 50 pmol of precursors for

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miR-103 or miR-107 (A) or the precursor control or with 10 or 50 pmol of AsOs to miR-103/107 (B). These are the same cells described in Fig. 6. Total cell lysates from 3 replicate wells were pooled and immunoblotted with antibodies for HNF4α, GR, CAR, or GAPDH as a control.

Fig. 9. There was no effect of (A) ectopic over expression or (B) silencing of miR-103/107 with AsOs on human CYP2C8 mRNA levels in human primary hepatocytes. 50 pmol of (A) precursors or (B) AsO inhibitors or control were transfected into cells. After 72h, the cells were harvested, total RNA was isolated, and CYP2C8 mRNA levels were determined by real time RT-PCR analyses. Data represent the means ± S.E. (n=3).

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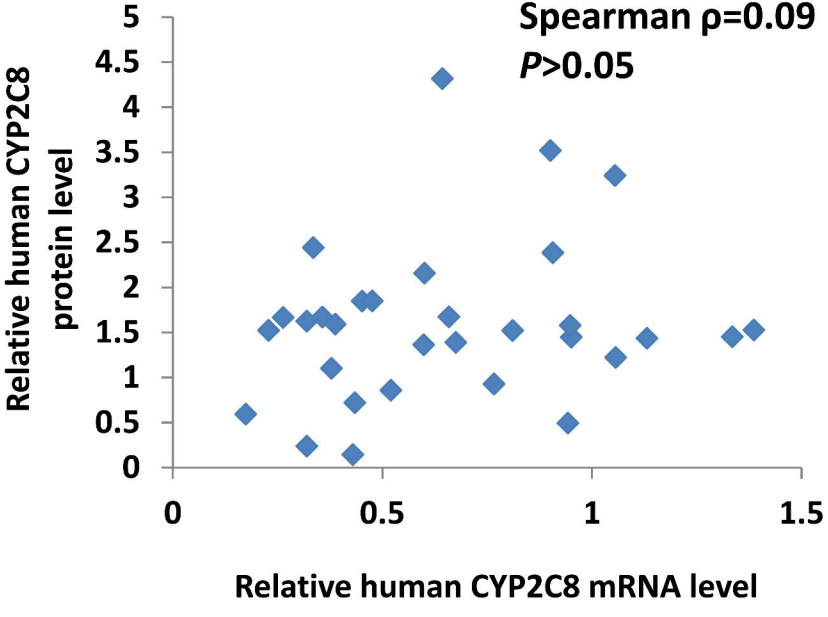
Fig 1

A



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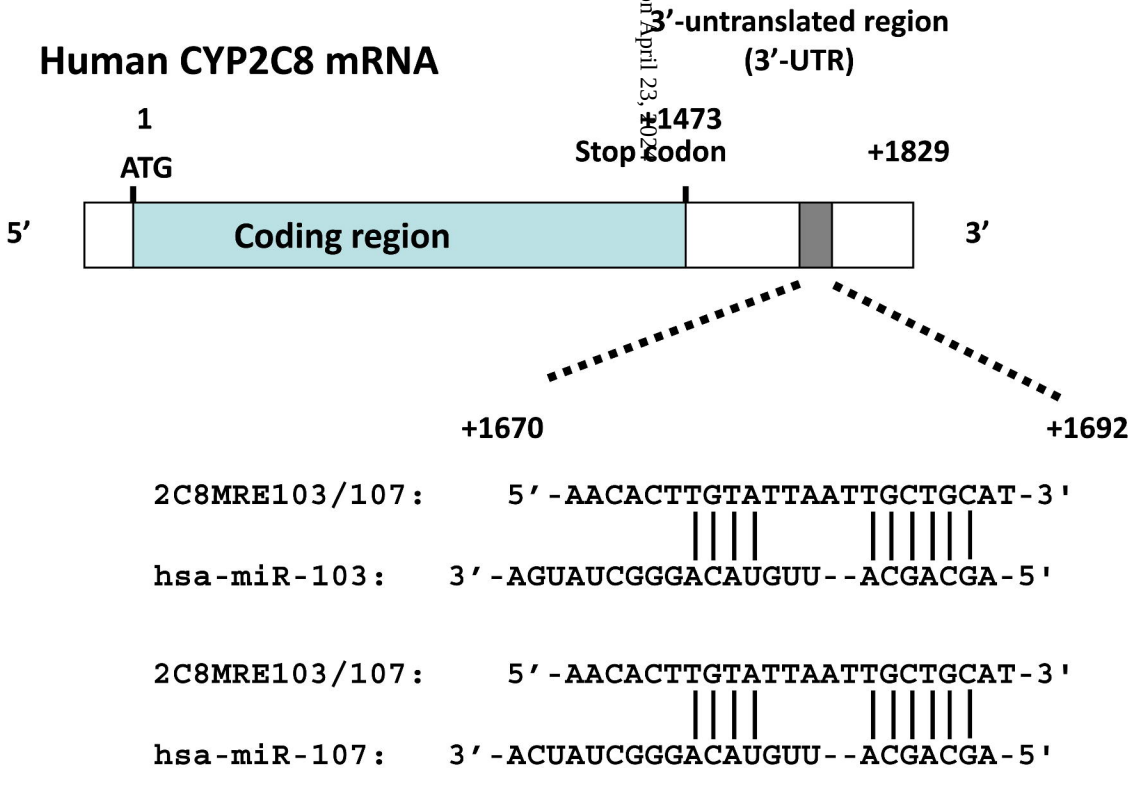

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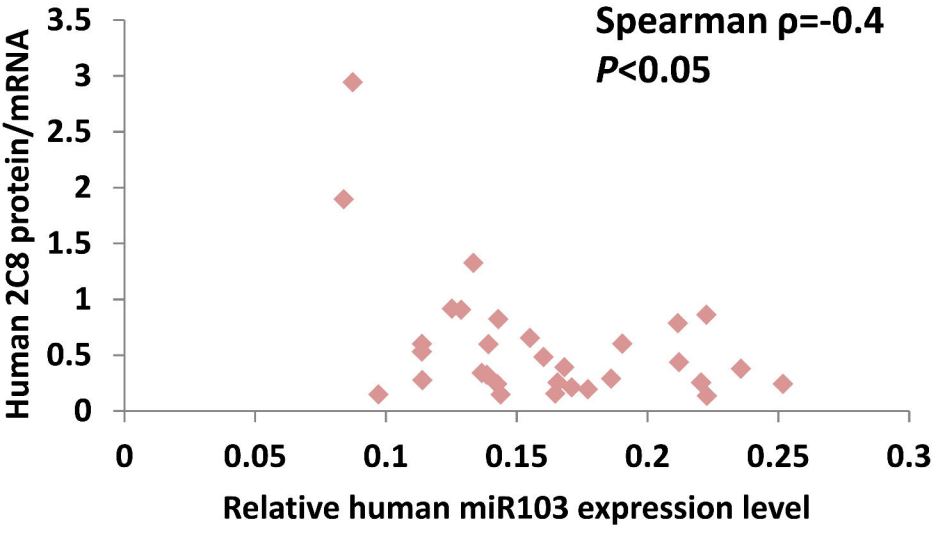
Fig 2

A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Human 2C8 protein/mRNA
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3
2.5
2
1.5
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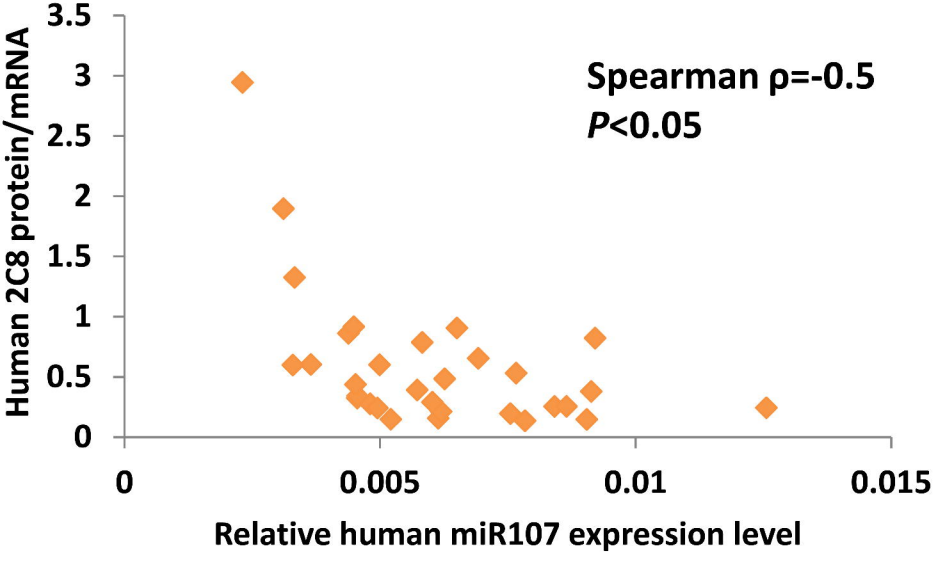
0
0
0.05
0.1
0.15
0.2
0.25
0.3
Relative human miR103 expression level

B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0.015
Relative human miR107 expression level

Fig 3

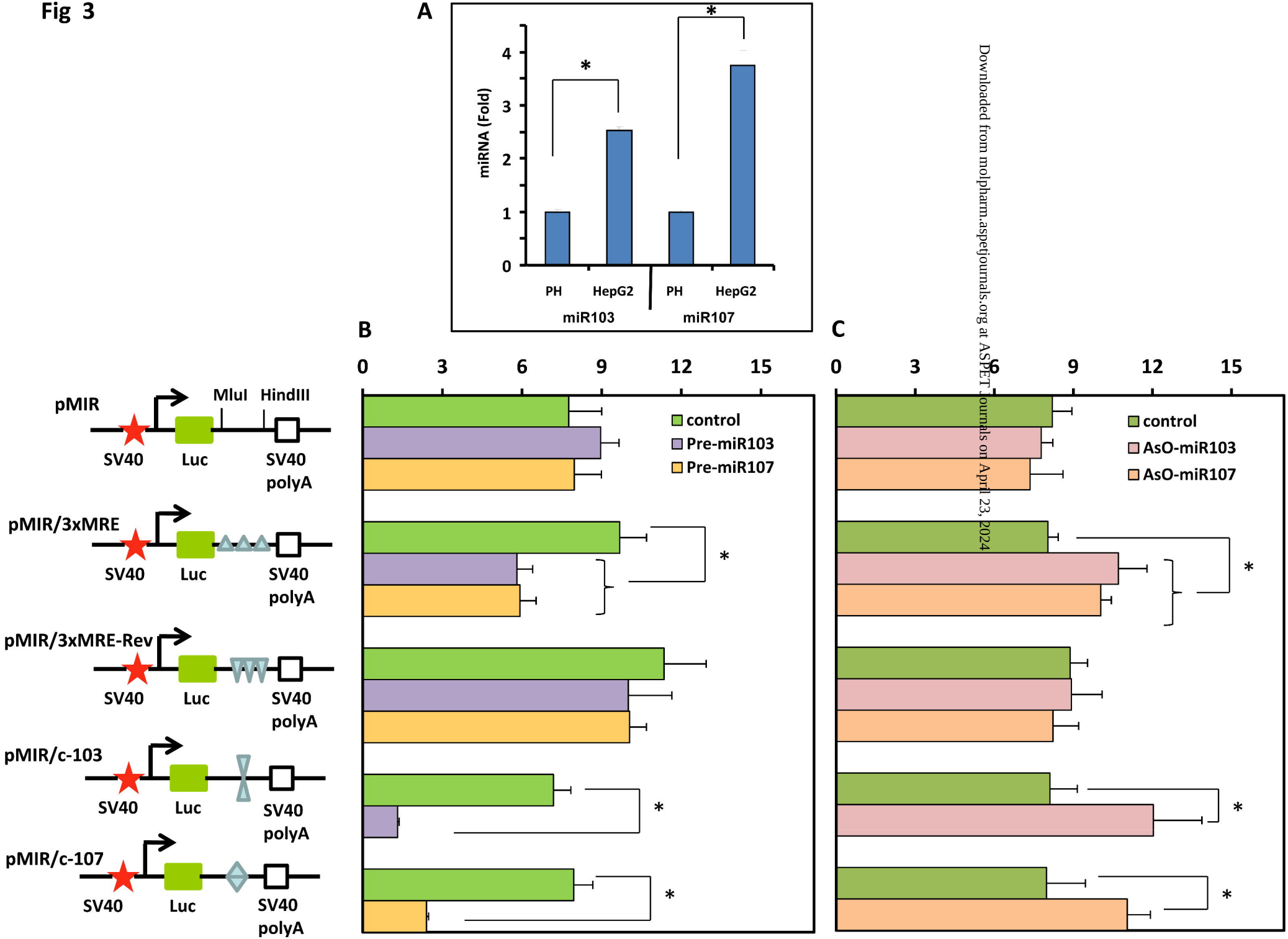

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Fig 4

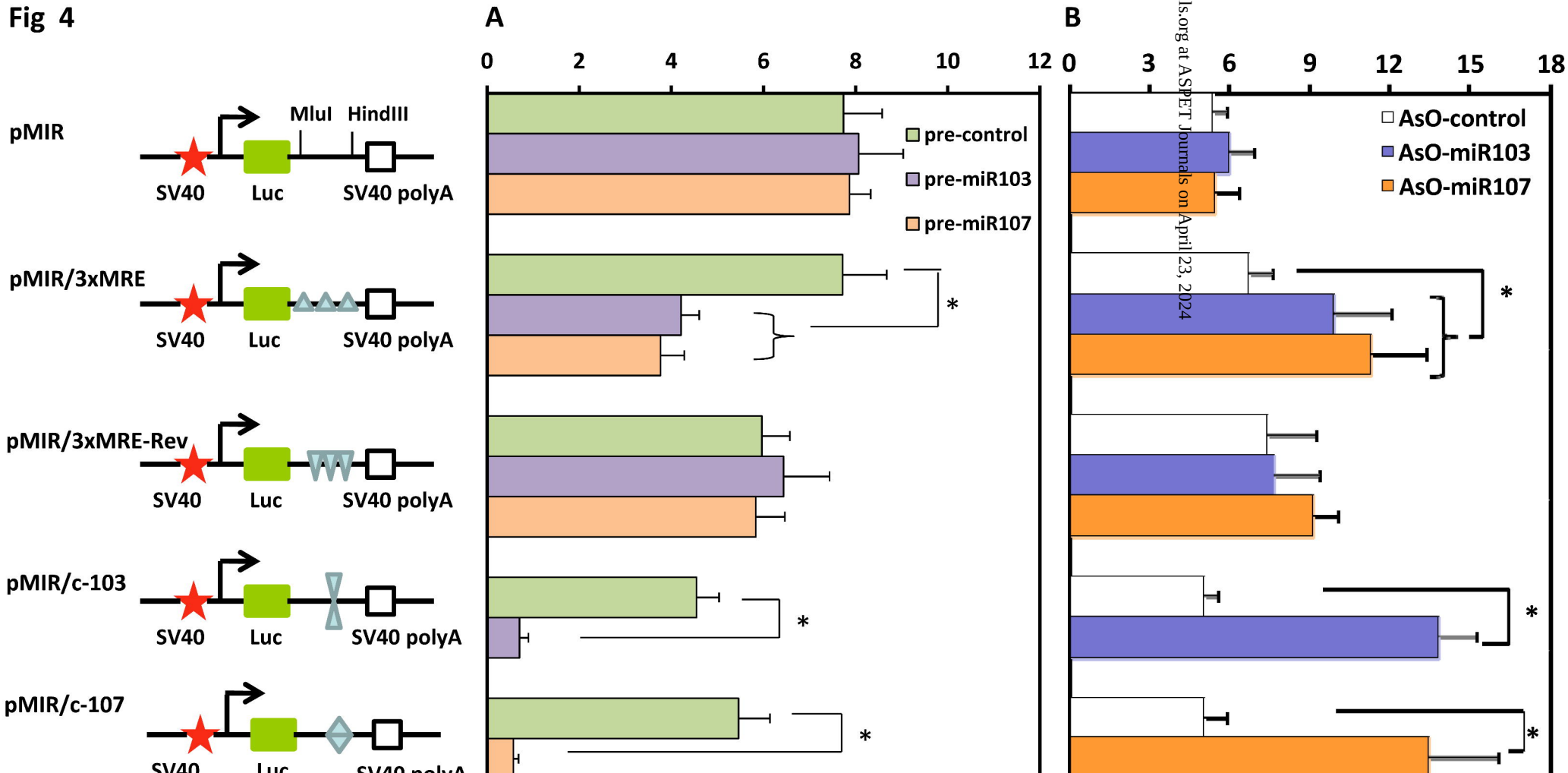

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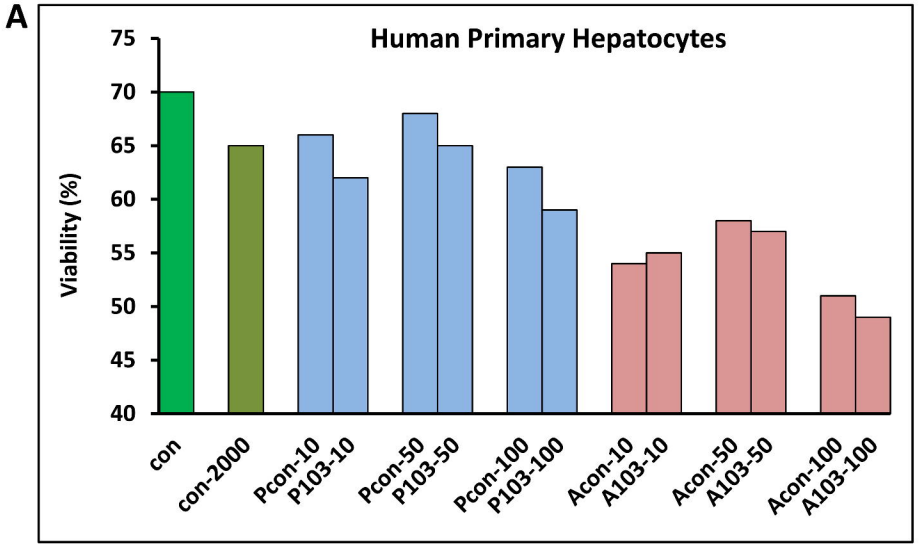
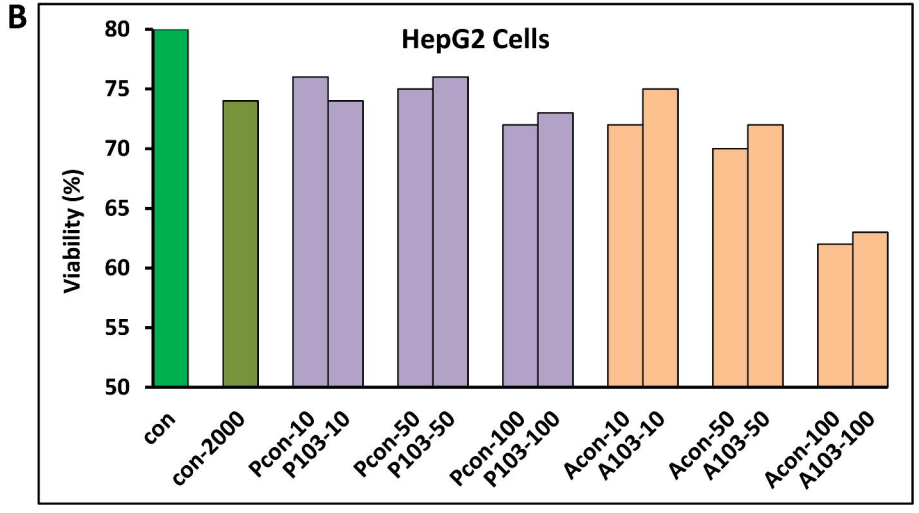
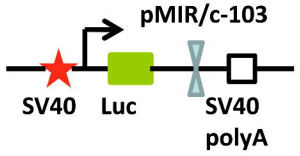
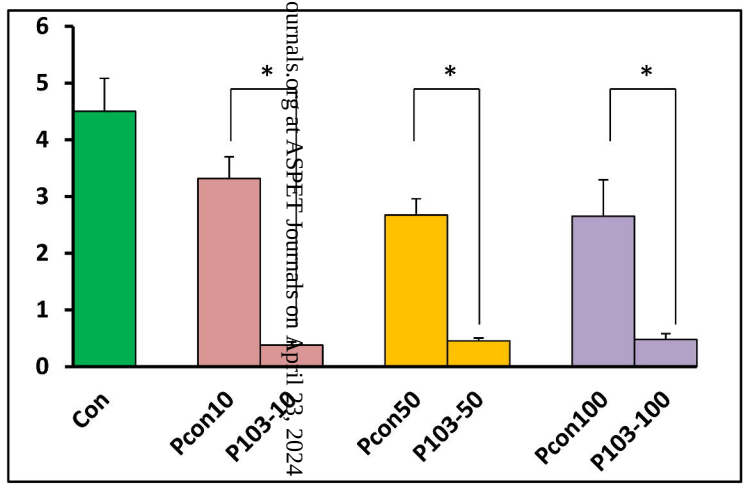
Fig 5









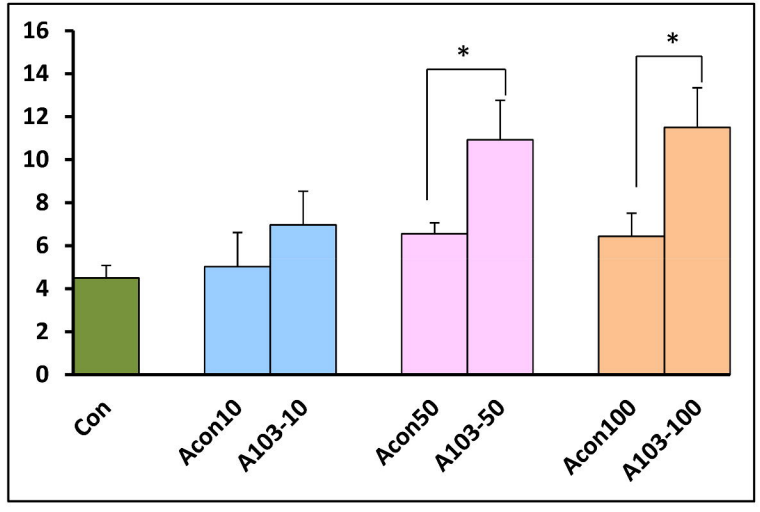


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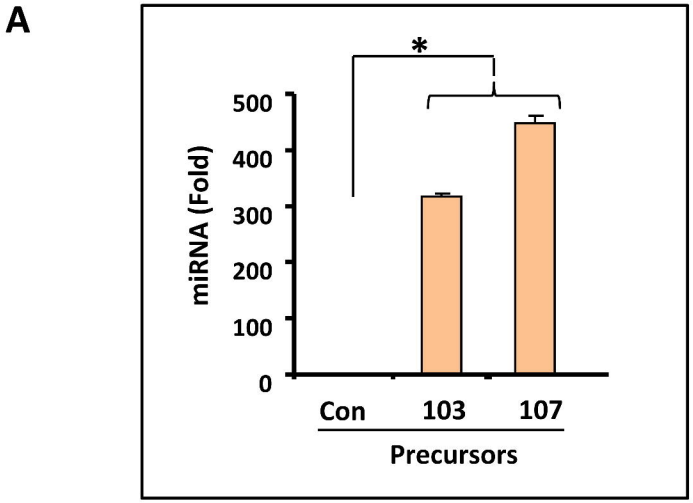
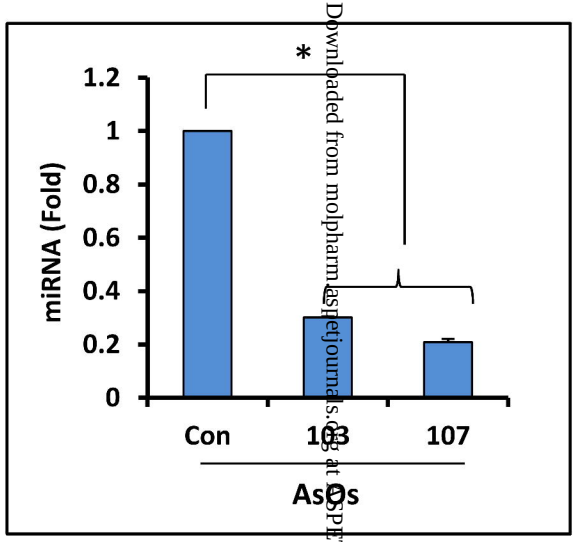
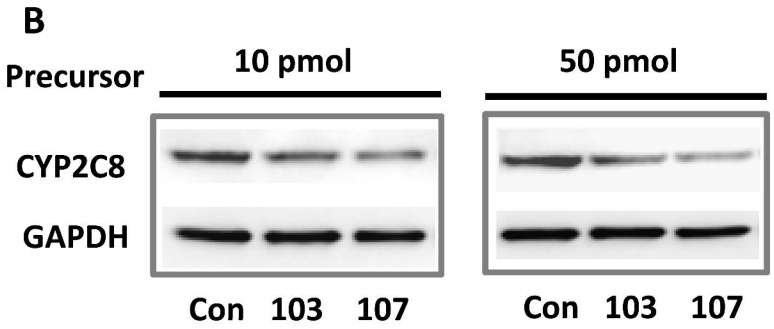
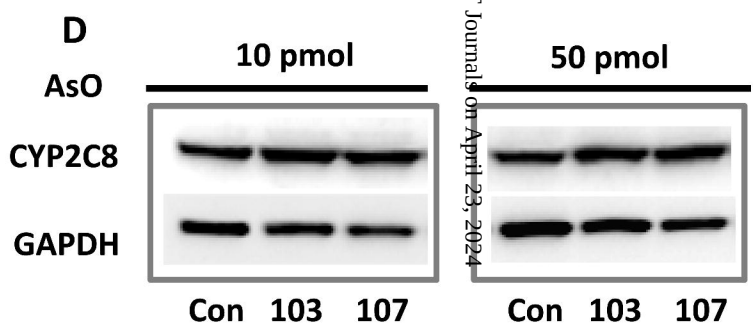
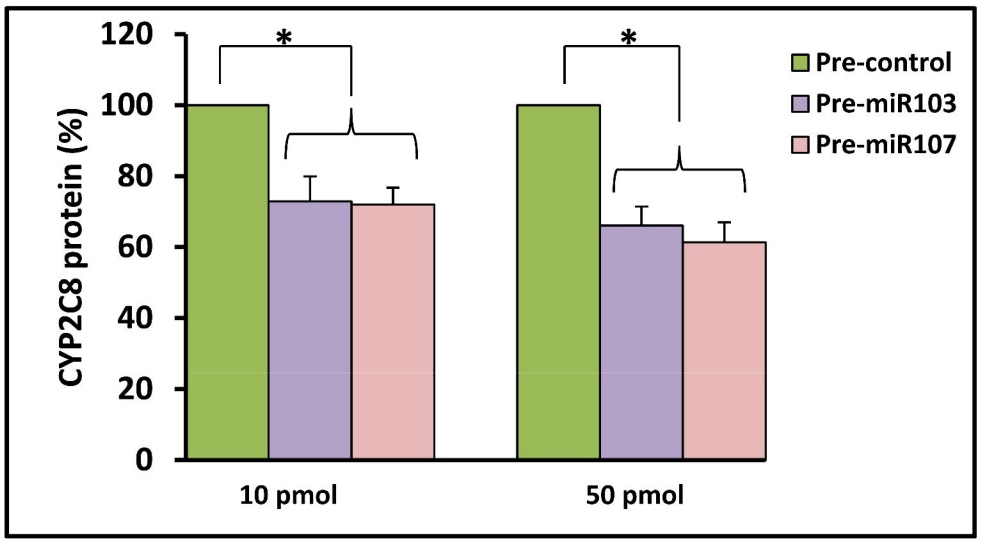
Fig 6











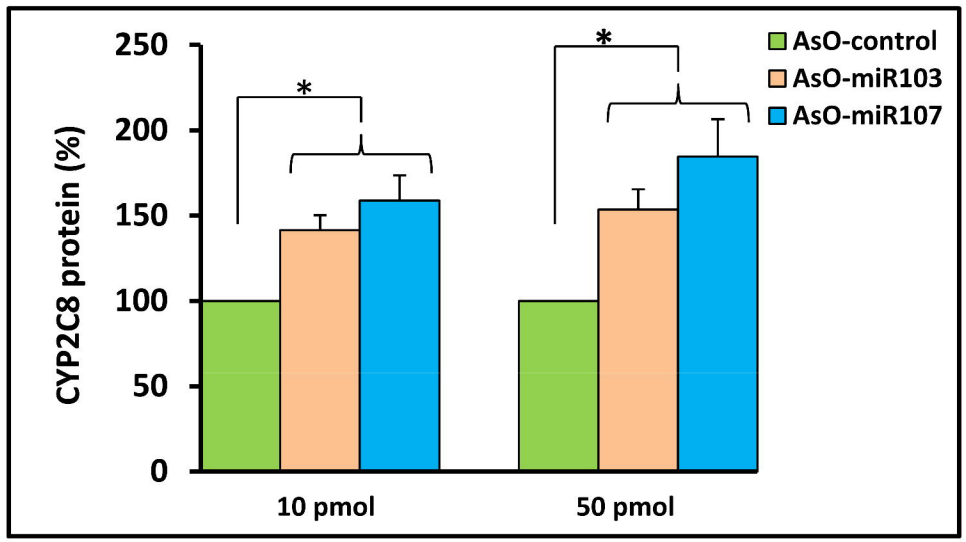


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Fig 7

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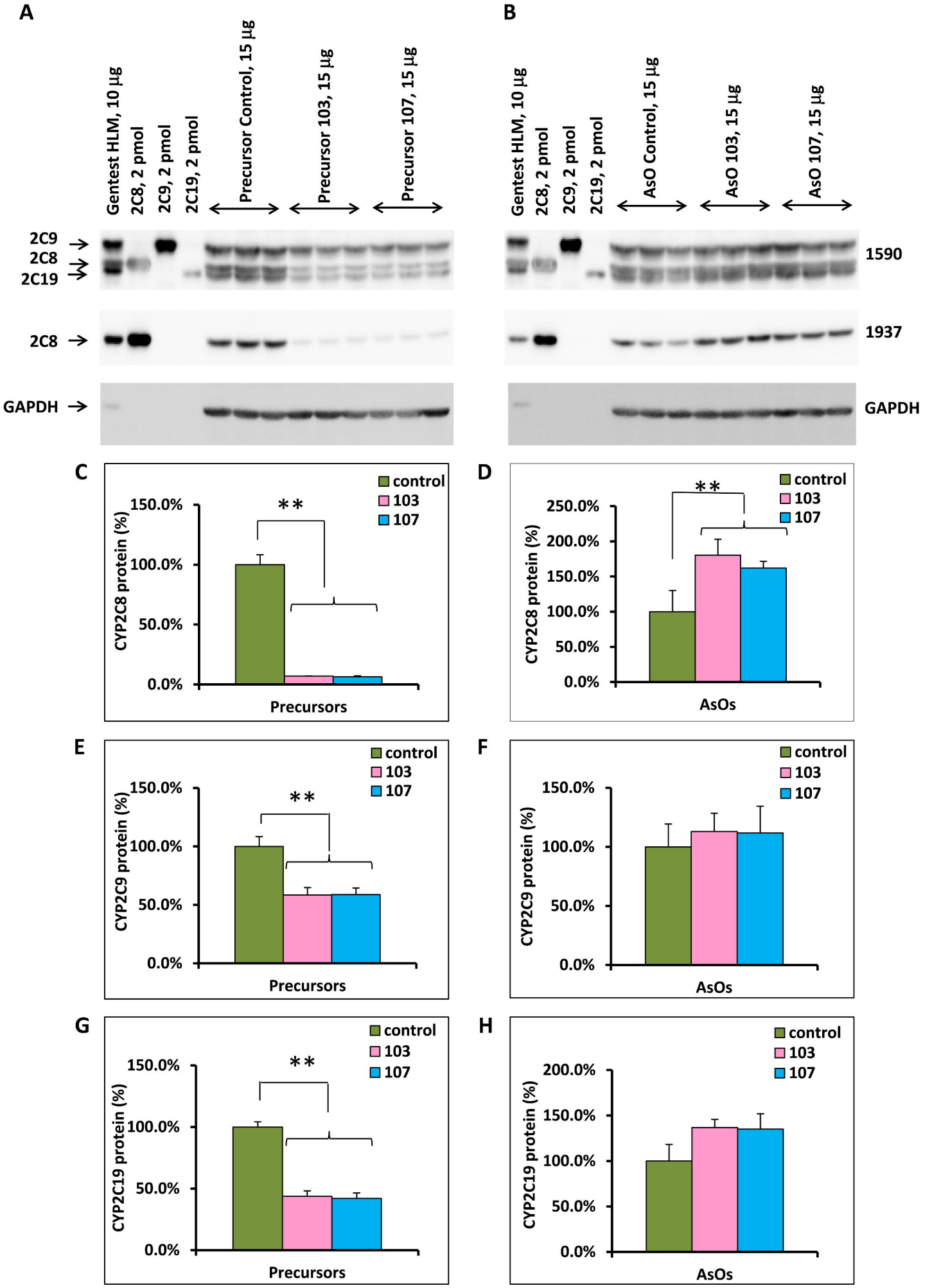

Fig 8

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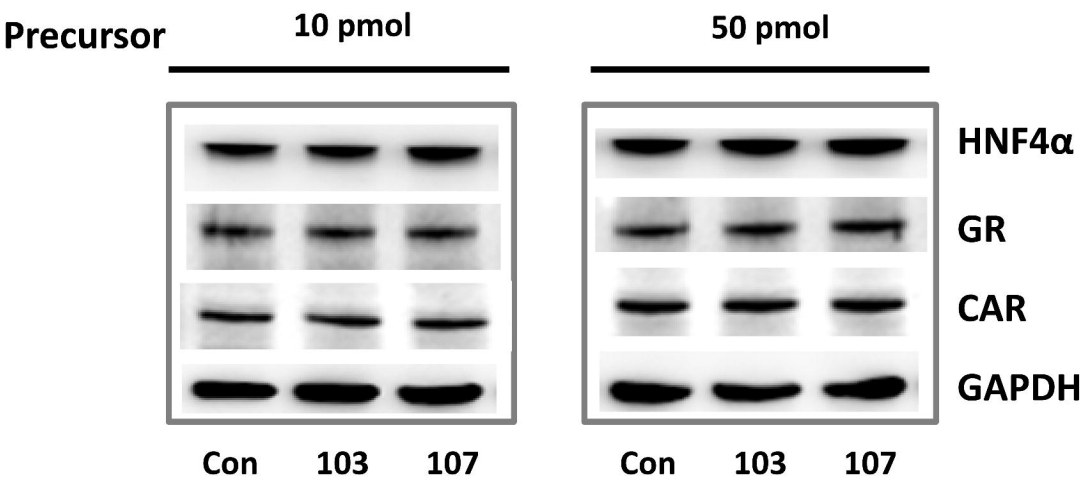
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50 pmol
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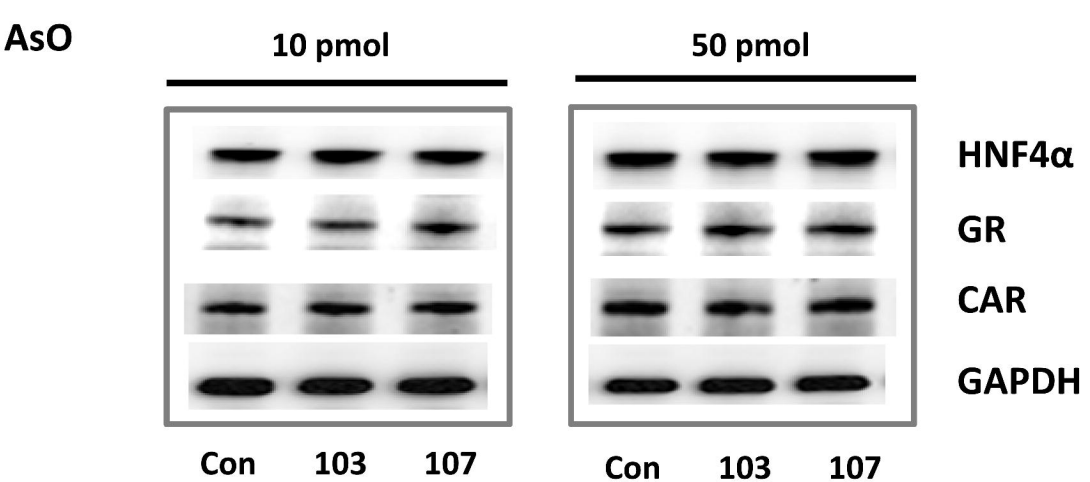
GR
CAR
GAPDH
Con
103
107
Con
103
107

B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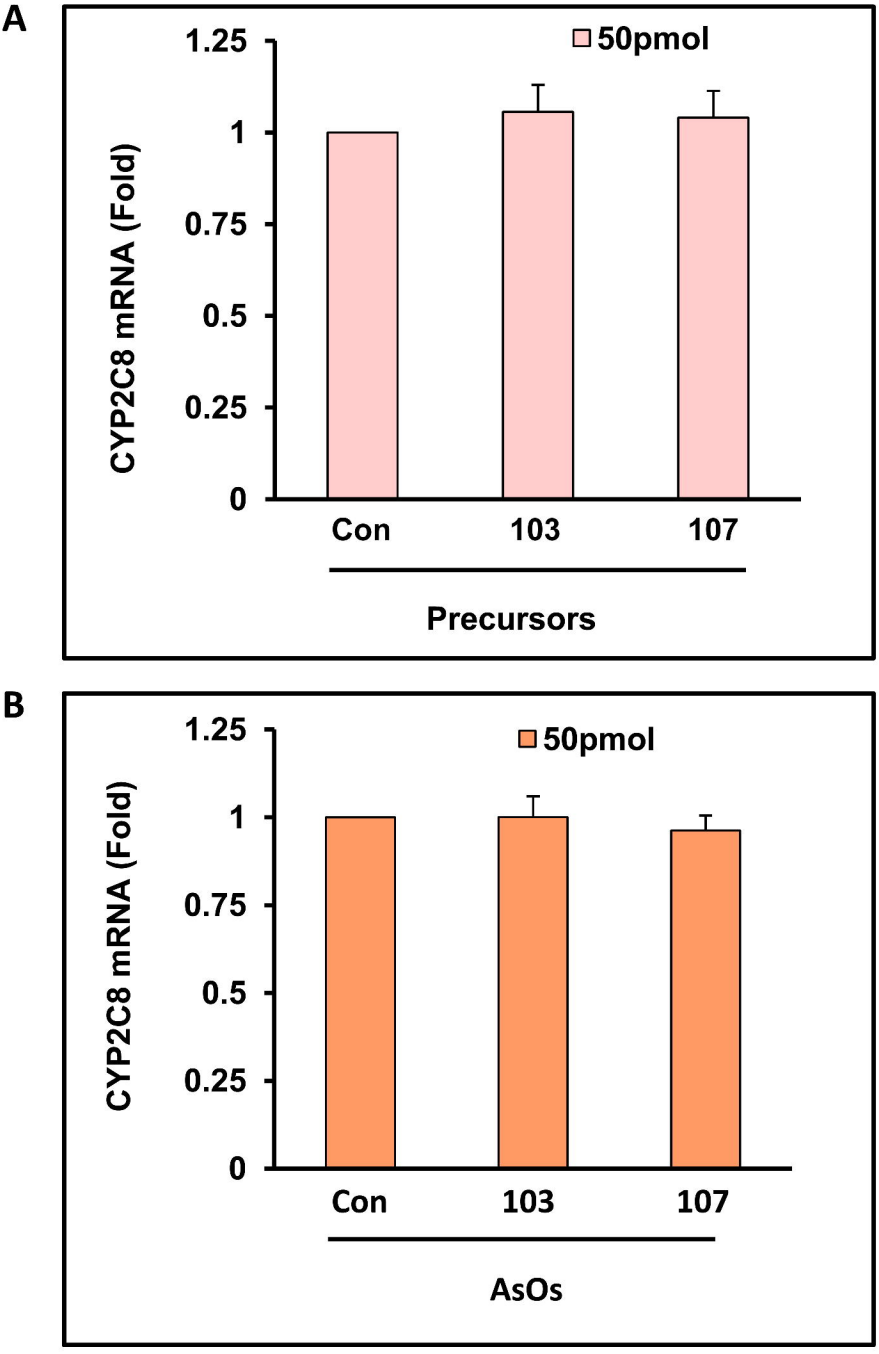
50 pmol
HNF4α
GR
CAR
GAPDH
Con
103
107
Con
103
107

Fig 9


A
1.25
1
0.75
0.5
0.25
0
CYP2C8 mRNA (Fold)
50pmol
Con
103
107
Precursors
B
1.25
1
0.75
0.5
0.25
0
CYP2C8 mRNA (Fold)
50pmol
Con
103
107
AsOs