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In silico and *in vitro* identification of microRNAs that regulate HNF4A expression.

Anuradha Ramamoorthy, Lang Li, Andrea Gaedigk, L. DiAnne Bradford, Eric A. Benson, David A. Flockhart, and Todd C. Skaar

Department of Medicine, Division of Clinical Pharmacology, Indiana University School of Medicine, Indianapolis, IN., USA (AR, LL, DAF, TCS); Division of Pediatric Pharmacology & Medical Toxicology, The Children's Mercy Hospital and Clinics, Kansas City, MO., USA (AG), and Morehouse School of Medicine, Atlanta, Georgia (LDB).

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MicroRNA regulation of HNF4A.

Todd C. Skaar

1001 W. 10th Street

WD Myers Building W7123

Indianapolis, IN. 46202.

Phone: 317-630-8795

FAX: 317-630-8185

Email: tskaar@iupui.edu

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A list of nonstandard abbreviations used in the paper:

µg	microgram
3'-UTR	3'-untranslated region
bp	base pairs

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cel-miR	*Caenorhabditis elegans* microRNA
CYP	cytochrome P450
HNF4A	hepatic nuclear factor 4α
hsa-miR	*Homo sapiens* microRNA
miRNA	microRNA
miRSNP	microRNA SNP
mRNA	messenger RNA
ng	nanogram
SNP	single nucleotide polymorphism

ABSTRACT

Hepatic nuclear factor 4α (HNF4A) is a nuclear transcription factor that regulates the expression of many genes involved in drug disposition. To identify additional molecular mechanisms that regulate HNF4A, we identified microRNAs (miRNAs) that target HNF4A expression. *In silico* analyses suggested that HNF4A is targeted by many miRNAs. We conducted *in vitro* studies to validate several of these predictions. Using an *HNF4A* 3'UTR luciferase reporter assay, five out of six miRNAs tested significantly down-regulated (~20–40%) the luciferase activity. In HepG2 cells, miR-34a and miR-449a also down-regulated the expression of both the HNF4A protein and an HNF4A target gene, *PXR* (~30-40%). This regulation appeared without reduction in HNF4A mRNA expression, suggesting that they must be blocking HNF4A translation. Using additional bioinformatic algorithms, we identified polymorphisms that are predicted to alter the miRNA targeting of HNF4A. Luciferase assays indicated that miR-34a and miR-449a were less effective in regulating a variant (rs11574744) than the wildtype *HNF4A* 3'UTR. *In vivo*, although not statistically significant (p=0.16), subjects with the variant *HNF4A* had lower CYP2D6 enzyme activity. In conclusion, our findings demonstrate strong evidence for a role of miRNAs in the regulation of HNF4A.

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INTRODUCTION:

HNF4A is an important transcriptional 'master regulator' that regulates the expression of many genes involved in drug disposition. They include phase I enzymes, phase II enzymes, transporters, and additional transcriptional factors that also regulate these genes (Kamiyama et al., 2007). Hepatic HNF4A expression appears to be regulated by a complex set of positive and negative transcription factors in the *HNF4A* promoter (Hatzis and Talianidis, 2001). Since there remains substantial unexplained variability in the expression of HNF4A and its downstream targets (e.g. cytochrome P450 genes; (Wortham et al., 2007)), we sought to identify additional mechanisms that regulate HNF4A that may ultimately contribute to the variable drug disposition. In these studies, we focused on identifying microRNAs (miRNAs) that regulate HNF4A expression.

MiRNAs are small (18-25 nucleotides), non-coding RNAs that regulate gene expression post-transcriptionally. In animals, miRNAs typically bind to the 3'-untranslated region (3'-UTR) of the messenger RNAs (mRNAs) and negatively regulate gene expression by one of two mechanisms: by blocking protein translation or by degrading the mRNA (Ambros et al., 2003; Olsen and Ambros, 1999). As more miRNAs are identified and studied, new target sites and functions are being recognized. For example, it has now been shown that miRNAs can also bind to coding regions and repress gene expression (Duursma et al., 2008); this mechanism may explain some of the differential expression of mRNA splice variants. MiRNAs also appear to be involved in the induction of gene expression; this induction occurs through binding to complementary regions in the promoter (Place et al., 2008) and the 5'-UTR (Orom et al., 2008).

In the human genome, 1424 mature miRNAs have been reported so far (miRBase Registry; version 17.0; (Griffiths-Jones et al., 2006)). The bioinformatic algorithms predict that miRNAs can regulate 20-90% of the human transcripts (Lewis et al., 2005; Miranda et al., 2006; Xie et al., 2005). Each miRNA can regulate multiple genes and each gene can be regulated by multiple miRNAs; therefore, these miRNAs form a broad and complex regulatory network.

MiRNAs are involved in a wide range of biological activities including cell differentiation, cell death, cancer and non-cancerous human diseases (John et al., 2004). Emerging evidence indicates that these miRNAs also regulate genes involved in drug metabolism and disposition. At least twelve of the cytochrome P450 (*CYP)* genes are predicted by bioinformatic algorithms to be targets of miRNAs (Ramamoorthy and Skaar, 2011). *In vitro* studies have validated several of these predictions and shown that several other drug disposition genes are also targets of miRNAs. Those studies have focused on genes such the ATP-binding cassette xenobiotic transporter *ABCG2* (To et al., 2008), pregnane X receptor (Takagi et al., 2008), *CYP1B1(*Tsuchiya et al., 2006) and *CYP3A4* (Pan et al., 2009). Others have speculated that interindividual variability in CYP expression and drug response may be due to the action of miRNAs (Ingelman-Sundberg et al., 2007).

The activity of miRNAs can be affected by single nucleotide polymorphisms (SNPs) that occur either in the miRNA or in the miRNA target site on the mRNA. Such SNPs are called miRSNPs (Mishra et al., 2007). These miRSNPs can alter miRNA gene processing and/or the normal mRNA-miRNA interactions. Thus, these SNPs can create new miRNA target sites or destroy old target sites. Hence, these miRSNPs may also contribute to the interindividual variability in the enzyme expression and activity.

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In this study, we hypothesized that endogenous miRNAs regulate the expression of HNF4A. To test this hypothesis, we first performed a comprehensive bioinformatic analyses to predict miRNAs that target HNF4A. Based on the bioinformatic analyses, we selected miRNAs that target HNF4A to perform further *in vitro* functional validation studies. Lastly, we identified polymorphisms in the miRNA target sites on the *HNF4A* 3'-UTR that are predicted to alter the mRNA-miRNA interactions. Collectively, these results suggest that miRNAs are likely to play an important role in the regulation of drug metabolism.

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METHODS:

In vitro Studies:

Cell culture: HeLa and HepG2 cells that were used in our *in vitro* transfection assays were obtained from ATCC (Manassas, VA, USA). All tissue culture reagents were purchased from Invitrogen (Carlsbad, CA, USA). HeLa and HepG2 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum and 1% Penicillin-Streptomycin. The cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2.

Generation of luciferase reporter gene constructs: The pIS-0 vector ((Yekta et al., 2004); Addgene plasmid #12178) was used to study 3'-UTR function. The 3'-UTR of *HNF4A* (1448 bp of the full length 1724 bp; nucleotides 1558-3005 of NM_000457) was amplified using genomic DNA from the Coriell panel using primers (Integrated DNA Technologies, Coralville, IA, USA) with *Nhe*I and *Sac*I restriction sites (FP: 5'-GGTGTT*GAGCTC*CTAAGAGAGCACCTGGTGA-3' and RP: 5'-GGGTTT*GCTAGC*GGAGACCTGGGTTCAAG-3'; the restriction sites are italicized and the *HNF4A* sequence is underlined). The PCR product was cloned into the TOPO TA vector (Invitrogen, Carlsbad, CA, USA) and the insert sequence was verified by DNA sequencing. The sequencing data revealed that the clone had the "variant" rs322210 (C allele); since the reported frequency of this allele is 55% (dbSNP), it appears to be the more common allele. Therefore, we used this clone. The insert was then subcloned into pIS-0 vector using the *Nhe*I and *Sac*I restriction sites and transformed into DH5α competent cells. Colonies with inserts were identified by restriction digestion and the sequence was verified by direct DNA sequencing.

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Plasmids were purified using the Plasmid Maxi Kit (Qiagen, Valencia, CA, USA). The DNA concentrations were determined using QuantIT DNA Broad Range kit (Invitrogen, Carsbald, CA, USA). The control vector is referred to as pIS-0 and the vector with *HNF4A* 3'-UTR sequence inserted is referred to as pIS-HNF4A. A plasmid with the variant HNF4A 3'-UTR (rs11574744) was created by site-directed mutagenesis (GenScript, Piscataway, NJ, USA). Presence of this mutation was confirmed by resequencing the plasmid. This mutant plasmid is referred to as pIS-HNF4A_SNP.

Transfection: For luciferase assays, $0.9x10^5$ HeLa cells were seeded into each well of a 24-well plate. The cells were transfected with 200ng of either pIS-0 or pIS-HNF4A plasmid. *Renilla* luciferase reporter plasmid pGL4.74 was used as a transfection control; the ratio of pIS:pGL4.74 was 50:1. Transfection was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions using Opti-MEM (Invitrogen, Carlsbad, CA, USA) and culture media without any antibiotics. At 24 hours after transfection, the cells were harvested and dual luciferase assays were performed as per manufacturer's instructions (Promega, Madison, WI, USA). Transfections for the site-directed mutagenesis experiments were performed using the same protocol using 200 ng of pIS-0, or pIS-HNF4A, or pIS-HNF4A_SNP plasmids.

For luciferase and miRNA co-transfection experiments, $1.5x10^6$ HeLa cells were seeded in a T-25 flask. At 24 hours, the cells were then transfected with 4 μg of pIS-0 or pIS-HNF4A plasmid, along with 80 ng of *Renilla* luciferase reporter plasmid as transfection control. At 24 hours after transfection, the cells were trypsinized and $1x10^5$ cells were seeded into each well of a 24 well plate. The cells were reverse transfected (i.e., plated onto the well containing the transfection mix) with either 30 nM of the miRNA, or combinations of miRNAs or negative

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control (miRIDIAN Mimics from Dharmacon, Lafayette, CO, USA) using Lipofectamine 2000. At 24 hours post miRNA transfection, the cells were harvested and dual luciferase assays were performed. Co-transfections for the site-directed mutagenesis experiment were performed with the same protocol using the pIS-0, or pIS-HNF4A, or pIS-HNF4A_SNP plasmids and the specific miRNAs. All transfections and luciferase assays were performed in triplicates on three different days.

In order to verify that the transfected synthetic miRIDIAN Mimics (Dharmacon, Lafayette, CO, USA) were efficiently taken up by the cells, we transfected HeLa cells (2×10^5 cells per well in 96-well plates) with 30nM of either hsa-miR-34a Mimic or negative control Mimic (cel-miR-67). RNA isolation and reverse transcription was performed using microRNA Cell-to-Ct kit (ABI, Forest City, CA, USA) and specific TaqMan MicroRNA Assays (ABI, Forest City, CA, USA) for hsa-miR-34a, U6 snRNA (endogenous control) and hsa-miR-449a (as a non-specific control) following the manufacturer's instructions. Specific details of the PCR are in the 'Quantification of miRNAs' section. The relative quantities of miRNA were calculated using the $\Delta\Delta$Ct method using U6 snRNA and negative control (cel-miR-67) transfections as controls. The relative miRNA expressions are reported as $2^{-(\Delta\Delta Ct)}$ (Kreuzer et al., 1999). The miRNA transfections were replicated on three separate days and the RT-PCR was performed in triplicates for each of the transfections. In an additional control experiment, we tested the effect of the transfection of the luciferase plasmids on the miRNA expression. The transfections did not alter the expression of either hsa-miR-449a or hsa-miR-34a (data not shown).

RNA isolation: Fresh human hepatocytes were isolated by Vitacyte LLC, (Indianapolis, IN, USA) from liver specimens that were collected after Indiana University's Institutional Review Board (IRB) approval. These hepatocytes were flash frozen until RNA isolation. Total

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RNA, including small RNAs, was isolated using the miRNeasy kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions with the exception that phase separation was performed using maXtract tubes (Qiagen, Valencia, CA, USA). The on-column DNase treatment step was included in the RNA isolation procedure and was done using the DNase set (Qiagen, Valencia, CA, USA). The RNA yield was determined using the Quant-iT RNA Broad Range assay kit (Invitrogen, Carlsbad, CA, USA). RNA quality/integrity was assessed with a RNA 6000 Nano Labchip and BioAnalyzer 2100 (Agilent Technologies, Palo Alto, CA, USA).

Quantification of miRNAs: The miRNA expression levels were determined using specific TaqMan MicroRNA Assays (ABI, Forest City, CA, USA) following the manufacturer's instructions in a StepOne Plus real time PCR instrument (ABI, Forest City, CA, USA). The PCR included a 10 min polymerase enzyme activation step at 95°C, 50 cycles each of denaturation at 95°C for 15 sec, and annealing/extension at 60°C for 1 min. Relative quantities of miRNA were calculated as $2^{-(\Delta Ct)}$ using U6 snRNA as endogenous control and was multiplied by 10^3 to simplify data presentation.

Quantification of HNF4A protein and mRNA: In a 6-well plate, $1x10^6$ HepG2 cells/ml were reverse-transfected with 100 nM of synthetic miRNA (hsa-miR-34a, hsa-miR-449a, hsa-miR-493* and control cel-miR-67; miRIDIAN microRNA Mimics from Dharmacon, Lafayette, CO, USA) using siPORT NeoFX transfection reagent (Ambion, Austin, TX, USA) following the manufacturer's specifications. We also used a Genome-Wide siRNA for human HNF4A (Hs_HNF4A_9) and an AllStars Negative Control siRNA (Qiagen, Valencia, CA, USA) as described by Iwazaki et al., (Iwazaki et al., 2008) as process controls. At ~72 hours after transfection, the cells were harvested for HNF4A protein and RNA analyses. The transfections were repeated on three separate days.

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For protein analyses, nuclear protein extract was isolated using the NucBuster protein extraction kit (Novagen, Madison, WI, USA). Protein concentrations were determined with the bicinchoninic acid (BCA) reagent protein assay kit (Pierce, Bradford, IL, USA). The nuclear protein lysate (20 µg) was electrophoresed using a 4-20% Tris-Glycine gel (Invitrogen, Carlsbad, CA, USA) and transferred to a PVDF transfer membrane (Millipore, Bedford, MA, USA) employing a Novex semi-dry blotter (Invitrogen, Carlsbad, CA, USA). The membranes were blocked with 3% milk and then incubated overnight at 4ºC with mouse anti-human HNF4A mouse monoclonal antibody (Catalog no. H1415, Perseus Proteomics, Tokyo, Japan) at a dilution of 1:5000, followed by horseradish peroxidase (HRP) conjugated ImmunoPure goat anti-mouse secondary antibody (Pierce, Bradford, IL, USA) at a dilution of 1:10000 for 1 hour. As described previously for their HNF4A study by Iwazaki et al., (Iwazaki et al., 2008), beta-actin was used as the internal control; a HRP conjugated beta-actin antibody (Catalog no. ab20272, Abcam, Cambridge, MA, USA) was used at a dilution of 1:5000. All three antibodies were diluted in Starting Block (T20) blocking buffer (Pierce, Bradford, IL, USA). Protein bands were developed using a SuperSignal enhanced chemiluminescence kit (Pierce, Rockford, IL, USA). The protein bands were visualized on a LAS-1000 plus system (Fujifilm, Tokyo, Japan).

For RNA analyses, total RNA, including small RNAs, was isolated and quantified as described above. The cDNA was generated from 1 µg of total RNA with the Reverse Transcription System (Promega, Madison, WI, USA) following the manufacturer's instructions. The expression of HNF4A, PXR and GAPDH (endogenous control) mRNAs was analyzed using specific TaqMan Gene Expression Assays (ABI, Forest City, CA, USA) following the manufacturer's instructions in a StepOne Plus real time PCR instrument (ABI, Forest City, CA, USA). Relative quantity of HNF4A mRNA was calculated using the ΔΔCt method using

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GAPDH and negative controls (cel-miR-67 and negative control siRNA) as reference controls. The RT-PCR was performed in triplicates for each of the three transfections.

Bioinformatics studies:

Bioinformatic analysis to predict miRNAs: To predict miRNAs that target the *HNF4A* gene, we used six different programs: miRanda (John et al., 2004), miRBase Targets (Griffiths-Jones et al., 2006), TargetScan (Lewis et al., 2003), PicTar (Krek et al., 2005), RNA22 (Miranda et al., 2006) and PITA (Kertesz et al., 2007). The parameter settings were either default or those used in the publications describing the programs. For RNA22, which is a downloadable program with user defined mRNA and miRNA sequences, we used the UCSC Genome Browser (http://genome.ucsc.edu) to identify the *HNF4A* 3'-UTR sequence. The mature miRNA sequences (version 10.0) were downloaded from the miRBase Sequence database (Griffiths-Jones et al., 2006).

Identification and bioinformatic analysis of SNPs located in the *HNF4A* 3'-UTR: SNPs from the *HNF4A* 3'-UTR were obtained from NCBI SNP database (dbSNP; http://www.ncbi.nlm.nih.gov/projects/SNP/) and UCSC Genome Browser database (http://genome.ucsc.edu/). The minor allele frequencies were obtained from dbSNP database and Seattle SNPs database (http://pga.gs.washington.edu). We used two programs, PolymiRTS (Bao et al., 2007) and Patrocles (Georges et al., 2006) to predict the effect of miRSNPs in the *HNF4A* 3'-UTR on the mRNA-miRNA interaction.

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In vivo human studies:

Genotyping of *HNF4A* 3'-UTR SNP: A custom TaqMan SNP genotyping assay (ABI, Forest City, CA, USA) was developed for *HNF4A* 3'-UTR SNP (rs11574744). The primer and probe sequences were: CCCGAGAACATGGCCTAAGG as forward primer, CCAGAGCAGGGCGTCAA as reverse primer, VIC-ATCCCACAGCCACCC as probe 1 and FAM-ATCCCACTGCCACCC as probe 2 (variant sequence is underlined). Genotyping was performed in a StepOne Plus Real-Time PCR System (ABI, Forest City, CA, USA) using the recommended genotyping PCR conditions. That is, an initial denaturation for 10 min at 95°C, followed by 40 cycles of denaturation for 15 sec at 92°C and annealing and extension for 1 min at 60°C. Since this was a custom assay, we confirmed the genotyping results by resequencing 96 DNA samples from the Coriell biorepository (48 Caucasian and 48 African-American samples). DNA resequencing was performed by Polymorphic DNA Technologies Inc (Alameda, CA, USA).

In addition, 151 Caucasian and 243 African American subjects previously phenotyped with the CYP2D6 probe drug dextromethorphan and genotyped for *CYP2D6* (Gaedigk et al., 2008) were screened for the presence of the SNP. In order to determine the effect of the *HNF4A* SNP on CYP2D6 enzyme activity, the urinary dextromethorphan metabolic ratio was compared after taking into account the CYP2D6 activity score. The *CYP2D6* gene score was assigned based on the expected enzyme activity (Gaedigk et al., 2008, Borges et al., 2010). The fully functional *CYP2D6* alleles (**1* and **2*) were assigned a score of 1, alleles associated with reduced enzyme activity (**10* and **41*) were assigned a score of 0.5 and the nonfunctional alleles (**3*-**6*) were assigned a score of 0. The CYP2D6 activity score was the summation of the two values assigned to the individual alleles.

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Statistical analysis: Statistical analyses were carried out as described in the figure legends, using SAS 9.1 software. The comparisons of relative luciferase activities (RLUs) and ΔCt among different treatment groups were analyzed with linear mixed models, in which between-day and within-day variances are treated as random effects. A p-value of < 0.05 was considered statistically significant.

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RESULTS:

***HNF4A* 3'-UTR has repressive luciferase activity *in vitro*:** In our first functional study, we cloned the 3'-UTR of the *HNF4A* gene into the 3'-UTR of the luciferase gene of the pIS-0 luciferase reporter construct, pIS-HNF4A (Supplemental Figure 1A). The pIS-0 reporter system has been used by others to study the miRNA regulation of several other target genes (Adams et al., 2007; Yekta et al., 2004). The pIS-HNF4A plasmid had significantly lower luciferase activity (~60 %; $p < .001$) when compared to the pIS-0 (control) plasmid (Supplemental Figure 1B). The effect of *HNF4A* 3'-UTRs was consistent across two other concentrations of transfected plasmids that we tested (100 ng and 400 ng; $p < .001$; data not shown). This indicated that the 3'-UTR of the *HNF4A* gene has repressive activities, possibly through miRNA targeting.

Bioinformatic predictions to identify miRNAs predicted to target *HNF4A*: We utilized six bioinformatic algorithms to identify miRNAs that are predicted to target *HNF4A*. The *HNF4A* gene has a 1,724 bp long 3'-UTR and was predicted to be targeted by 350 different miRNAs (Supplemental Table 1 and 2). Candidate miRNAs for functional testing were selected based on two criteria: (1) the miRNA predicted by two or more of the bioinformatic algorithms, and (2) there was a predicted favorable energy of binding ($\Delta G \leq -25$ kcal/mol) between the miRNA and the target sequence on the mRNA. Among those that fit these criteria, we initially selected hsa-miR-34c-5p, hsa-miR-449a, and hsa-miR-766, based on the number of predicting algorithms and their ranking energy of binding. As a negative control, we chose hsa-miR-493* (formerly referred to as hsa-miR-493-5p) that was not predicted to target *HNF4A*.

MiRNAs regulate *HNF4A* 3'-UTR luciferase activity *in vitro*: The next set of studies focused on testing the effect of individual miRNAs on the pIS-HNF4A plasmid. The selected

miRNAs were co-transfected with either pIS-0 or pIS-HNF4A. Relative to the negative control miRNA (cel-miR-67), hsa-miR-34c-5p and hsa-miR-449a reduced the luciferase activity of the pIS-HNF4 plasmid by 40% ($p < .001$) and 35% ($p < .001$), respectively (Figure 1A). In contrast, hsa-miR-766 did not have an impact on the expression of pIS-HNF4A and neither did the non-specific control, hsa-miR-493*.

In another set of transfections, we tested three additional miRNAs for activity against the pIS-HNF4A including hsa-miR-34a (same family as hsa-miR-34c-5p), hsa-miR-34b* (which is a part of a single large precursor miRNA transcript that makes hsa-miR-34c-5p) and hsa-miR-765. These miRNAs were selected because they were predicted by more than two bioinformatic algorithms and predicted energy of binding was favorable. When compared to the negative control miRNA (cel-miR-67), hsa-miR-34a, hsa-miR-34b* and hsa-miR-765 reduced the pIS-HNF4A luciferase activity by 23% ($p < .001$), 22% ($p < .001$), and 24% ($p < .001$), respectively (Figure 1B). Since multiple miRNAs can simultaneously interact with a target mRNA (Krek et al., 2005), we co-transfected hsa-miR-34c-5p with hsa-miR-34b* and hsa-miR-34c-5p with hsa-miR-765. These combinations of miRNAs significantly down-regulated ($p < .001$) pIS-HNF4A luciferase activity. However, we did not observe any additive or synergistic effects of the miRNAs when transfected together. In the case of hsa-miR-34c-5p co-transfection with hsa-miR-34b*, the lack of additional or synergistic effect may be because of competition, as they are predicted to share one target site in common.

Expression of miRNAs in human hepatocytes: To determine if the miRNAs that appear to target *HNF4A* are expressed in hepatocytes, we measured their expression in primary hepatocytes isolated from three individual subjects. Additionally, we also measured their expression in HeLa and HepG2 cells. We performed quantitative Real-Time PCR using specific

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TaqMan miRNA assays. U6 small nuclear RNA (snRNA) was used as endogenous control. Hsa-miR-34a was easily detectable in all 3 hepatocyte preparations, as well as in HeLa and HepG2 cells (Figure 2A). The other three hsa-miRNAs (hsa-miR-34b*, hsa-34c-5p and hsa-miR-449a) were also expressed in hepatocytes, although at a lower level (Figure 2B). Neither hsa-miR-34b* nor hsa-miR-34c-5p were detectable in HeLa or HepG2 cells. Hsa-miR-449a was expressed in HepG2, but not detectable in HeLa cells. Based on their expression, hsa-miR-34a and hsa-miR-449a were selected for further *in vitro* studies.

Effect of miRNAs on HNF4A mRNA and protein expression: In order to investigate whether these miRNAs regulate HNF4A mRNA and protein expression, we transfected HepG2 cells with hsa-miR-34a, hsa-miR-449a, hsa-miR-493*, and the negative control cel-miR-67. Control experiments demonstrating successful miRNAs transfection into the cells are shown in Supplemental Figure 2. Both hsa-miR-34a and hsa-miR-449a were predicted to target *HNF4A* at two positions and they both target the same two locations: (1) positions 164-171, and (2) positions 254-260 of *HNF4A* 3'-UTR corresponding to the miRNA seed sequence. Hsa-miR-493* is not predicted to target *HNF4A* and hence was used as a negative control. We also used HNF4A siRNA and a negative control siRNA as described by Iwazaki et al., (Iwazaki et al., 2008) as process controls.

HNF4A protein expression was down-regulated by hsa-miR-449a, hsa-miR-34a and the positive control HNF4A siRNA (Figure 3). The HNF4A mRNA expression was down-regulated by the HNF4A siRNA, but not by the miRNAs (Figure 4A), indicating that these miRNAs must be blocking HNF4A translation and not causing degradation of the HNF4A mRNA. To determine whether the down regulation of the *HNF4A* gene resulted in altered expression of downstream *HNF4A* target genes, we determined the effect of the miRNAs on PXR mRNA

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expression. *PXR* is a target of *HNF4A* that has been used as a marker of HNF4A activity (Iwazaki et al., 2008). The expression of PXR mRNA was significantly down-regulated ($p < .001$) by ~30% by hsa-miR-449a, ~40% by hsa-miR-34a and ~40% by HNF4A siRNA (Figure 4B). In contrast, hsa-miR-493* did not alter HNF4A mRNA, or HNF4A protein expression. Surprisingly, hsa-miR-493* appeared to up-regulate PXR mRNA expression ($p < .001$); the mechanism, however, is unclear.

SNPs in the miRNA target sites: Since SNPs in the miRNA target sites of mRNA 3'-UTRs can alter normal miRNA-mRNA interactions, we searched for SNPs in the 3'-UTR of *HNF4A* that may disrupt or create miRNA-mRNA interactions. The dbSNP database indicated that many SNPs are present in the 3'-UTRs of *HNF4A*. We used two programs: PolymiRTS Database (Bao et al., 2007) and Patrocles (Georges et al., 2006) to determine if any of these 3'-UTR SNPs that exists in miRNA target sites are predicted to affect the base-pairing between HNF4A mRNA and the target miRNAs. There were 5 SNPs in the *HNF4A* gene that are predicted to destroy six miRNA target sites and create two new miRNA target sites (Supplemental Table 3).

***In vitro* validation of SNP predictions:** In order to test the hypothesis that germline variations can alter mRNA-miRNA interactions, we selected a SNP (rs11574744; T>A) in the *HNF4A* 3'-UTR for further *in vitro* validation (Figure 5A). This SNP was predicted by both PolymiRTS and Patrocles programs to destroy a miRNA binding site (Supplemental Table 3). The expected result of this would be an increased luciferase activity because of a reduced negative regulation by the miRNA. In our first functional study, we compared the activity of pIS-0, pIS-HNF4A and pIS-HNF4A_SNP plasmids. The luciferase activity from the plasmid

with the variant HNF4A was two-fold higher ($p < .001$) than the wild type pIS-HNF4A plasmid (Figure 5B).

In our second functional study, we co-transfected the plasmids (pIS-0 or pIS-HNF4A or pIS-HNF4A_SNP) with hsa-miR-34a, hsa-miR-449a and negative control (cel-miR-67). When compared to the negative control miRNA (cel-miR-67), hsa-miR-34a, and hsa-miR-449a reduced the pIS-HNF4A luciferase activity by 25% and 28% respectively; while these two miRNAs reduced the pIS-HNF4A_SNP luciferase activity by only 6% and 9%, respectively (Figure 5C). The luciferase activity was significantly higher ($p < .05$) in the pIS-HNF4A_SNP plasmid transfected cells compared to the pIS-HNF4A transfected cells, when transfected with either the miR-34a or the miR-449a miRNAs (Figure 5C).

We used RNAFold (Gruber et al., 2008) to determine if this SNP changed the predicted mRNA secondary structure. We included 70 bp nucleotide flank on either side of the SNP (Kertesz et al., 2007) to assess the minimum folding energy and secondary structure. The minimum folding energy was the same for both the wildtype and SNP sequences (Supplemental Figure 3). Similarly, no differences were observed when we extended the flanking sequence to 200 bp on either side of the SNP.

In order to determine the genotype frequency of rs11574744 in different ethnicities, we designed a custom TaqMan assay (ABI, Forest City, CA) to genotype the Coriell human diversity panel comprising of 94 Caucasian, 89 African-American and 87 Asian DNA samples. The results of our genotyping suggested that the SNP is present only in African American. The minor allele frequency (MAF) was 3.4%.

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In order to determine if this *HNF4A* SNP affects CYP2D6 enzyme activity, we genotyped the rs11574744 SNP in 151 Caucasians and 243 African-Americans that were previously phenotyped for CYP2D6 with dextromethorphan (Gaedigk et al., 2008). The hypothesis was that the SNP destroys a mRNA-miRNA interaction, thereby increasing HNF4A mRNA protein expression and resulting in increased expression of downstream target mRNA (eg., CYP2D6). The SNP was present only in the African-American cohort (MAF = 4.6%). We compared the urinary dextromethorphan/dextrorphan (DM/DX) ratio in subjects with wildtype vs rs11574744 carrier (both homozygous and heterozygous) genotypes. Although not statistically significant ($p=0.10$), the DM/DX ratio was numerically lower in subjects who were carriers of the variant, which is consistent with our *in vitro* mechanistic studies. When the analysis was focused on only subjects who were genotypically extensive metabolizers (functional CYP2D6 activity scores of 1.5 or 2.0), there was also a trend in the same direction ($p= 0.16$; mean±SEM of logDM/DX of wildtype and carriers were -1.84±0.05 and -2.07±0.14, respectively).

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DISCUSSION

Interindividual variability in drug metabolism remains a significant contributor to differences in drug efficacy and toxicity. Some of this variability is due to known genetic variations and environmental factors that inhibit the enzymes or alter their expression levels. However, the mechanisms that underlie much of the variability are yet unknown. The studies presented here provide evidence for a role of miRNAs in the regulation of drug disposition through transcriptional factors that regulate drug metabolizing enzymes.

Our bioinformatic analysis predicted that *HNF4A* is targeted by many miRNAs. HNF4A is an important transcriptional 'master regulator' of phase I and II enzymes, transporters and transcriptional factors (Kamiyama et al., 2007). Thus, regulation of *HNF4A* gene expression by miRNAs would likely affect many genes involved in drug metabolism and disposition. HNF4A is also expressed in kidney, intestine and pancreas; in those tissues, it controls lipid (Hayhurst et al., 2001) and glucose metabolism (Stoffel and Duncan, 1997). Therefore, targeting of *HNF4A* by miRNAs may also affect other functions. Nine different isoforms of HNF4A have been reported (Harries et al., 2008). Since the predominant isoform of HNF4A that is expressed in the liver is isoform 2 (Ihara et al., 2005) which contains the full length 3'-UTR, it is likely to be regulated by miRNAs.

As with many bioinformatic predictions, there was substantial inter-algorithm variability in the miRNAs that were predicted to target each gene. Part of this variability may be due to the different miBase database versions that are used by each algorithm; they ranged from versions 9 to 11. The variability may also be due to differences in the algorithms; these include differences in parameters such as, degree of complementarity, differences in 3'-UTR annotations and species

conservation. The total number of miRNAs predicted by the algorithms will continue to change as more miRNAs are being discovered and as the prediction algorithms are fine-tuned. These predicted miRNAs provided a starting point for subsequent laboratory analyses.

Following our bioinformatic predictions, our *in vitro* studies also supported a role of miRNAs in the regulation of *HNF4A*. Five of the miRNAs regulated the pIS-HNF4A luciferase plasmid and at least two of those also regulated HNF4A protein expression. Since the miRNAs did not significantly reduce the HNF4A mRNA levels, we presume that they are regulating HNF4A expression by blocking the translation of HNF4A mRNA into protein, which is a common mechanism of regulation by miRNAs (Ambros, 2004; Bartel, 2004). This is supported by our results showing that the HNF4A-targeting miRNAs also suppressed the expression of a downstream *HNF4A* target, PXR. Similar to the actions of many miRNAs reported so far, the miRNAs only partially blocked *HNF4A* expression and activity. This is consistent with the role of miRNAs as a fine-tuning mechanism in the regulation of the target genes. However, it could also be that when multiple miRNAs together target a gene, there are actually very large effects. Since HNF4A mRNA is predicted to be target by many miRNAs, additional testing will be required to determine which other miRNAs may target *HNF4A* and what the collective impact is on *HNF4A* gene expression.

There are a wide variety of HNF4A-regulated genes that we could study, however, we chose to focus on *PXR* first, because it is regulated by HNF4A in HepG2 cells (Iwazaki et al., 2008). Two of the miRNAs did in fact alter PXR expression. Although the bioinformatic analysis suggested that *PXR* itself may also be a weak target of these miRNAs, they are less likely to cause the observed reduction in PXR mRNA expression because the *PXR* target sequence contained several mismatches. Similar to PXR, it is likely that several of the other

drug metabolizing enzymes that are downstream targets of HNF4A are also indirectly regulated by miRNAs that target *HNF4A*. Testing those downstream targets will be best done in future studies using primary hepatocytes, since the mRNA and protein expression of most of the drug metabolizing enzymes are either low or absent in the immortalized cell lines available. It will also be of interest to determine if any of these miRNAs are regulated by xenobiotics and drugs. One of the HNF4A regulating miRNAs that we identified in this study, has-miR-34a, was also recently shown to regulate HNF4A protein, but not mRNA levels, in a different model system, which further support our findings (Takagi et al., 2010).

MiRNA functions can be altered by genetic variants that affect the miRNA binding to the target mRNA; these variants are called miRSNPs (Mishra et al., 2007). They occur either in the miRNA or in the mRNA. MiRSNPs that create a new functional miRNA binding sites would cause additional down regulation of the target gene. In contrast, miRSNPs that destroy miRNA target sites would result in a loss of targeting and elevated expression of the target gene. MiRSNPs have been shown to alter mRNA-miRNA interactions in genes such as dihydrofolate reductase (Mishra et al., 2007) and estrogen receptor α (Adams et al., 2007). Genetic variants in the 3'-UTRs of genes have not typically been given high priority in functional studies; however, based on our analyses, they may contribute to interindividual variability in the expression of the drug metabolizing enzymes. Some studies have reported on the SNPs in the 3'-UTRs of *CYP* genes that may be associated with altered phenotypes; these include *CYP19A1* (Dunning et al., 2004) and *CYP2A6* (Wang et al., 2006). It is conceivable that these SNPs may be a target of miRNAs. We have identified SNPs in the *HNF4A* 3'-UTR that are predicted to alter the miRNA interactions (Supplemental Table 3). Our *in vitro* luciferase assay revealed that at least one of these genetic variants can alter mRNA-miRNA interactions (Figure 5). Furthermore, in our

clinical study, we observed a trend in the expected direction towards lower CYP2D6 activity in subjects with the variant *HNF4A*. Because of the low minor allele frequency of this SNP, larger African American population studies will be required to confirm these findings.

Both the algorithms, PolymiRTS and Patrocles, only predicted if the SNPs in the 'seed' region of the miRNA target sites affect the base-pairing between mRNA and miRNA. However, SNPs in 'non-seed' regions can also affect miRNAs that bind either upstream or downstream of the SNP (Mishra et al., 2007). Neither of these algorithms predict such loss or gain of such mRNA-miRNA interactions. Similarly, SNPs in the mature miRNAs and pre-miRNA may also affect the mRNA-miRNA interaction. Since very few of the miRNA genes have been resequenced in depth, the genetic variants in those genes are not well characterized. Therefore, we have not included them in this analysis.

Our findings provide evidence for the role of miRNAs in the regulation of the transcriptional factor HNF4A. Recent studies have shown that other proteins involved in drug metabolism (Pan et al., 2009; Takagi et al., 2008; To et al., 2008; Tsuchiya et al., 2006) are also subject to miRNA regulation. Our results, taken together with those findings, suggest a complex regulatory mechanism for CYPs by miRNAs. The identification of the endogenous hepatic miRNAs that regulate *CYP* genes directly or indirectly should help us to better understand the variability in therapeutic efficacy and toxicity for patients to numerous commonly used drugs. Further, identifying polymorphisms that alter the drug metabolizing mRNA-miRNA interactions would likely be a clinically important biomarker for guiding the use of CYP metabolized drugs. Ultimately, we expect that these new biomarkers would help improve the efficacy and reduce the side effects of the commonly prescribed drugs.

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AUTHORSHIP CONTRIBUTIONS:

Participated in research design: Ramamoorthy, Flockhart, Benson, and Skaar.

Conducted experiments: Ramamoorthy, Gaedigk, Benson, and Bradford.

Performed data analysis: Ramamoorthy and Li.

Wrote or contributed to the writing of the manuscript: Ramamoorthy, Skaar, Gaedigk, Li, Flockhart, Benson, and Bradford.

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FOOTNOTES:

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Todd C. Skaar

1001 W. 10th Street

WD Myers Building W7123

Indianapolis, IN. 46202.

Phone: 317-630-8795

FAX: 317-630-8185

Email: tskaar@iupui.edu

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FIGURE LEGENDS:

Figure 1: Regulation of pIS-HNF4A by miRNAs. HeLa cells were co-transfected with: (A) pIS-0 or pIS-HNF4A luciferase plasmids, along with the *Renilla* reporter plasmid for normalization. These cells were also transfected with miRNA Mimics. The data are expressed as the pIS-HNF4A luciferase activity corrected for *Renilla* luciferase and normalized to negative control (cel-miR-67) within each experiment (mean ± SEM; n=3 independent experiments); (B) pIS-HNF4A luciferase plasmid along with the *Renilla* reporter plasmid for normalization. These cells were also transfected with miRNA Mimics individually (cel-miR-67, hsa-miR-34a, hsa-miR-34b*, or hsa-miR-765; 30 nM) or together (hsa-miR-34b* + hsa-miR-34c-5p, or hsa-miR-34c-5p + hsa-miR-765; 15 nM each). All data are expressed as the pIS-HNF4A luciferase activity corrected for *Renilla* luciferase and normalized to the negative control (cel-miR-67) within each experiment (mean ± SEM; n=3 independent experiments). * indicates $p < .05$ and ** indicates $p < .001$ compared to the negative control.

Figure 2: Mature miRNAs expression in human cell lines and hepatocytes. Total RNA, including the small RNAs, was isolated from three different human hepatocytes preparations and from the HeLa and HepG2 cell lines. To show the variability between hepatocyte preparations, the results from each hepatocyte preparation are graphed separately. RT-PCR assays were performed with U6 snRNA as the internal control. The relative miRNA expression was calculated as $2^{-(\Delta Ct)}$. Values were multiplied by 10^3 to simplify data presentation. The values shown in the graph were obtained from triplicate assays in a single PCR experiment. Error bars are not shown since true biological replicates (plated on different days) cannot be obtained from human hepatocyte isolations. Therefore, separate bars are shown for each hepatocyte preparation.

Figure 3: Regulation of HNF4A protein by miRNAs. Representative Western blot using anti-HNF4A and anti-β-actin (internal control) antibodies. HepG2 cells were transfected with miRNAs (hsa-miR-34a, hsa-miR-449a, hsa-miR-493*, or cel-miR-67; 100nM) or siRNAs (HNF4A siRNA, or negative control siRNA; 100 nM). At 72 hours post-transfection, nuclear protein was isolated and western blot assays were performed.

Figure 4: Regulation of HNF4A and PXR mRNA by miRNAs. HepG2 cells were transfected with miRNAs (hsa-miR-34a, hsa-miR-449a, hsa-miR-493*, or cel-miR-67; 100 nM) or siRNAs (HNF4A siRNA, or negative control siRNA; 100 nM). At 72 hours post-transfection, RNA was isolated and RT-PCR assays were performed. GAPDH was used as the internal control. The miRNA transfections were normalized to cel-miR-67 and the siRNA transfections were normalized to negative control siRNA. The relative mRNA expression was calculated as $2^{-(\Delta\Delta Ct)}$ (mean ± SEM; n=3 independent experiments performed in triplicates). (A) Expression of HNF4A mRNA and (B) Expression of PXR mRNA. ** indicates $p < .001$.

Figure 5: *In vitro* validation of SNP predictions. (A) Predicted miRNA interaction with wildtype and variant (rs11574744) *HNF4A* 3'-UTR. (B) Transfection of HeLa cell line with 200 ng of control pIS-0 plasmid or pIS-HNF4A or pIS-HNF4A_SNP constructs with the *Renilla* luciferase plasmid as internal control. Dual luciferase assays were performed at 24 h. Data are expressed as the pIS-HNF4A and pIS-HNF4A_SNP luciferase activity corrected for *Renilla* luciferase and normalized to pIS-0 within each experiment (mean ± SEM). (C) HeLa cells were co-transfected with 4 μg of pIS-HNF4A, or pIS-HNF4A_SNP luciferase constructs, along with *Renilla* reporter plasmid for normalization. The cells were also transfected with miRNA Mimics (hsa-miR-34a or hsa-miR449a; 30 nM). All data are expressed as the pIS-HNF4A or pIS-HNF4A_SNP luciferase activity corrected for *Renilla* luciferase and normalized

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to negative control (cel-miR-67) within each experiment (mean ± SEM; n=3 independent experiments). The assays were done in triplicates on three different days. * indicates $p < .05$.

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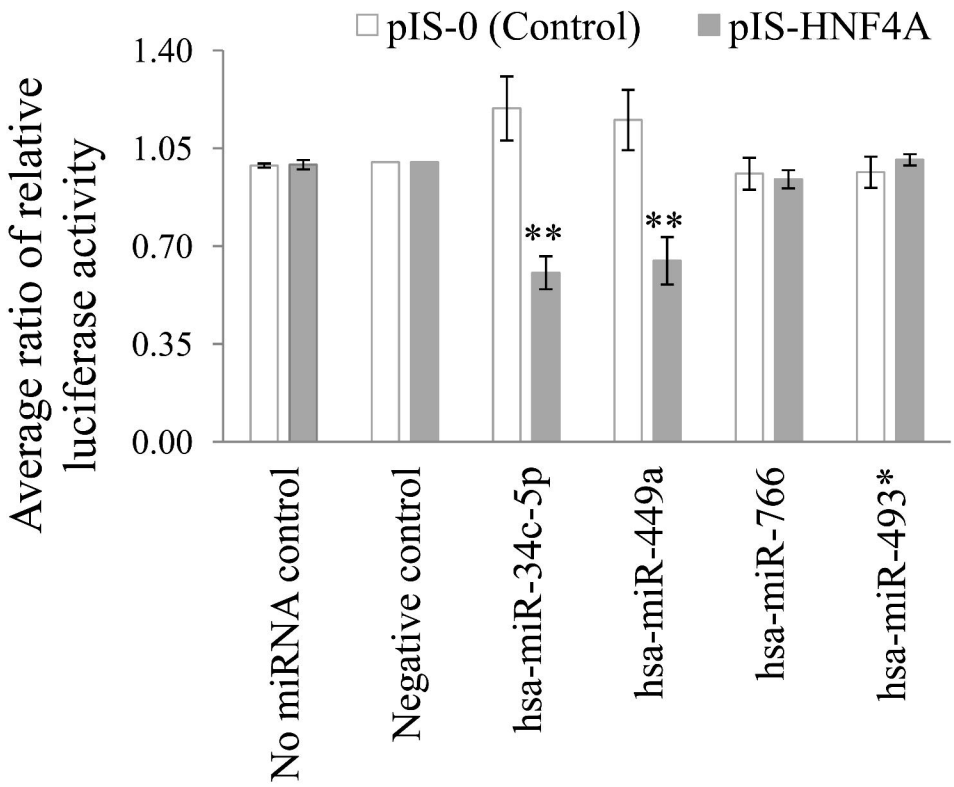
Figure 1

(A)



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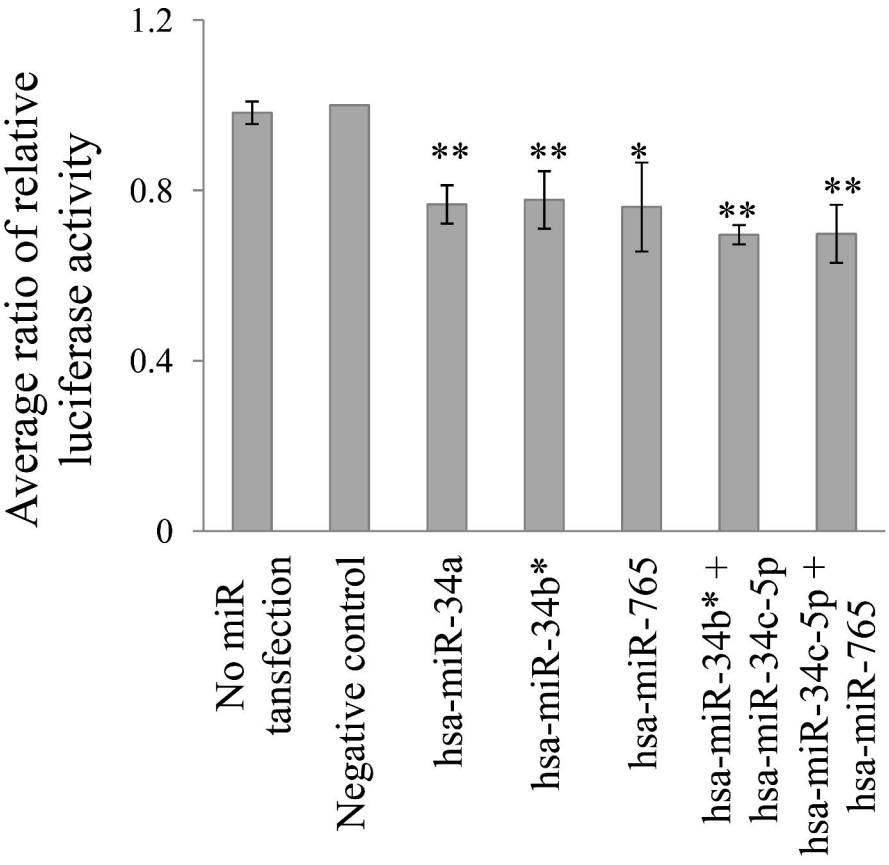

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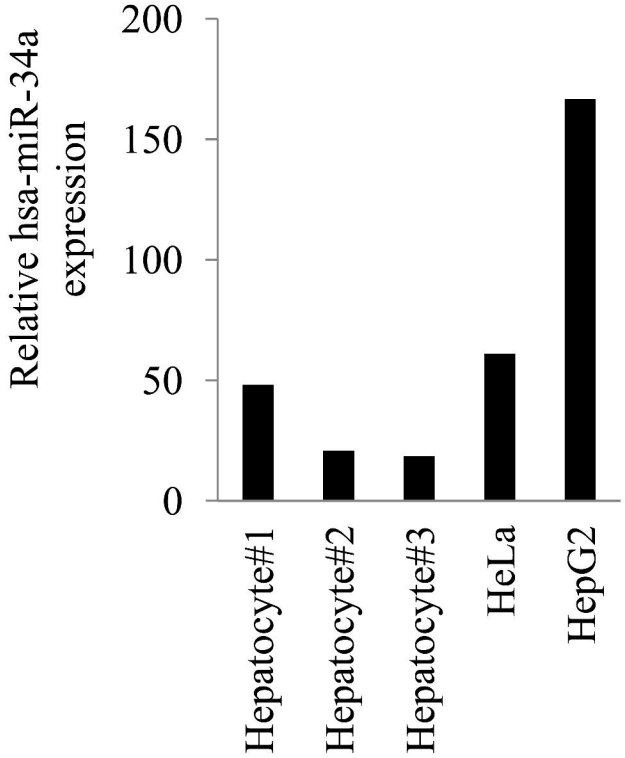
Figure 2

(A)



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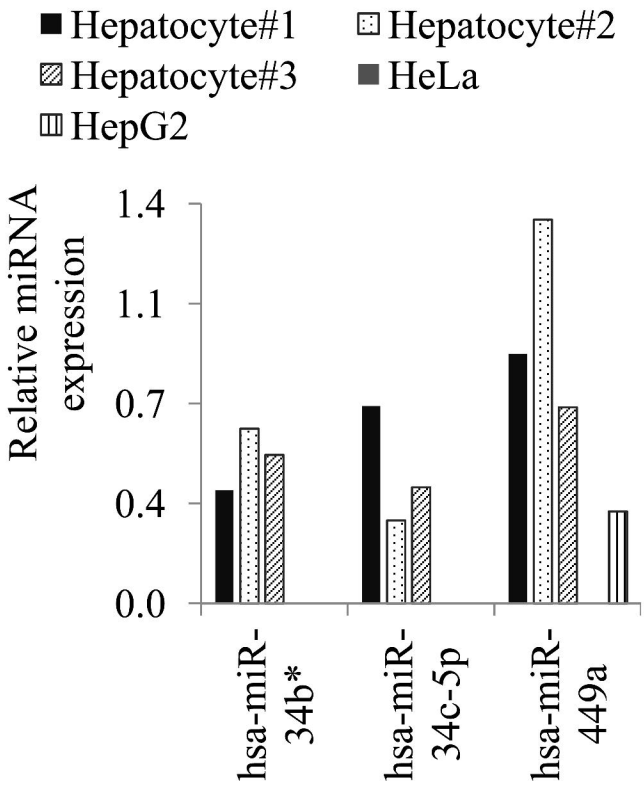

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Figure 3

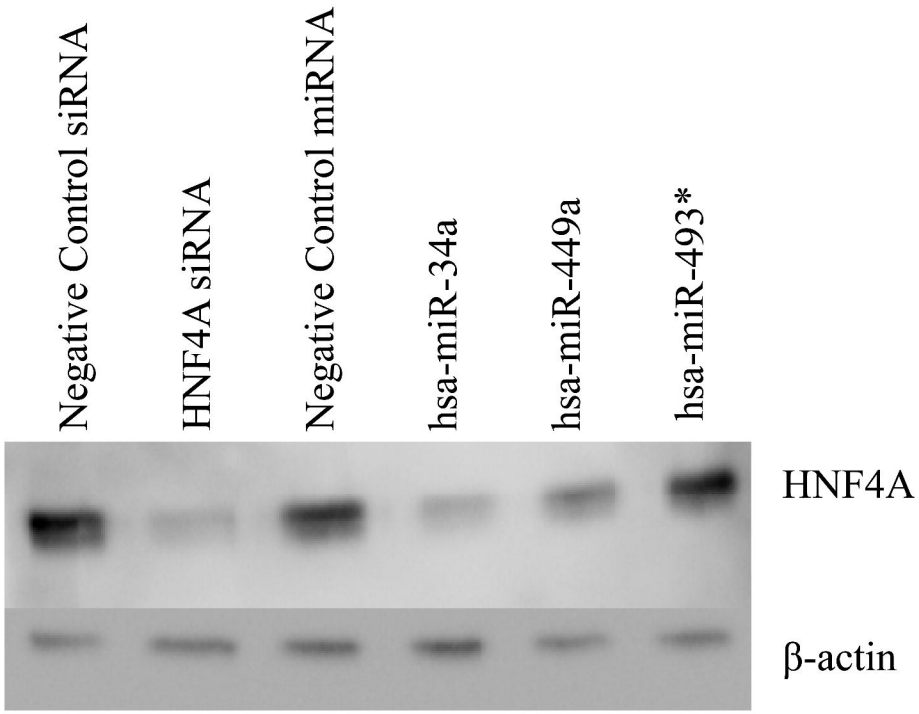

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Figure 4

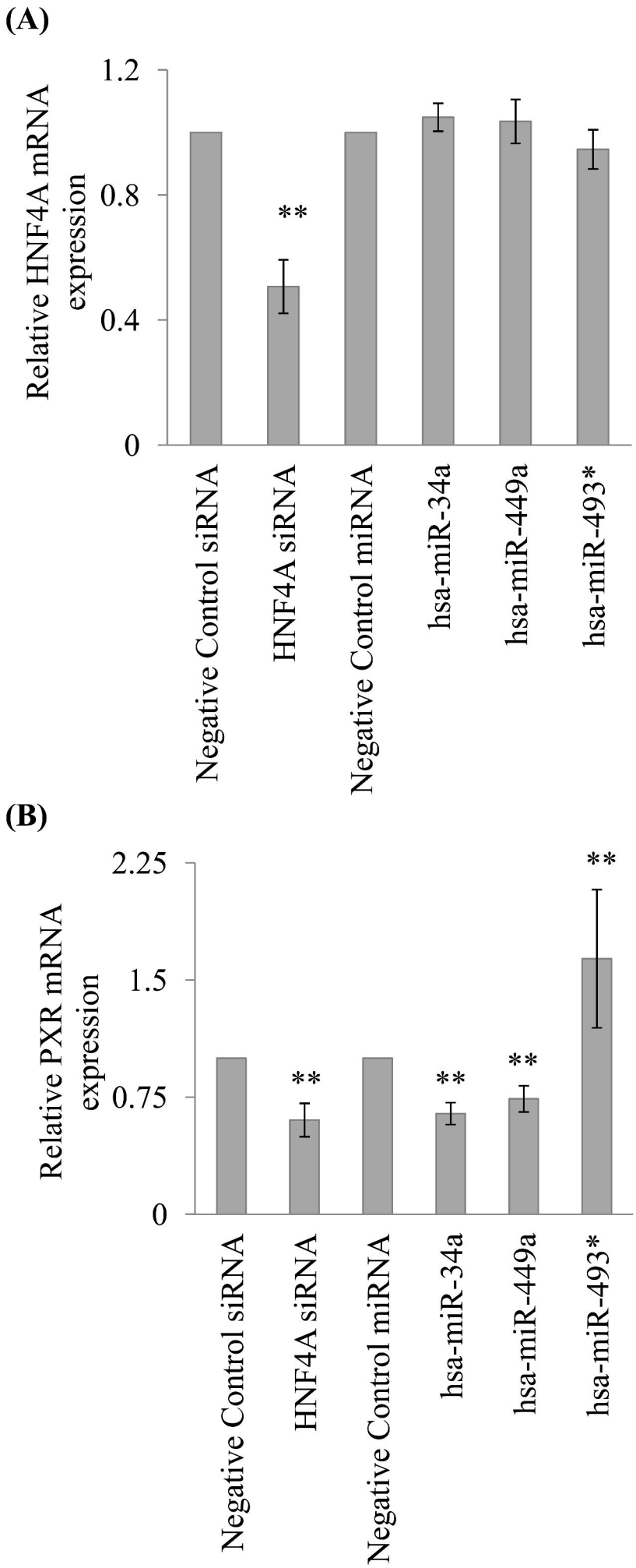

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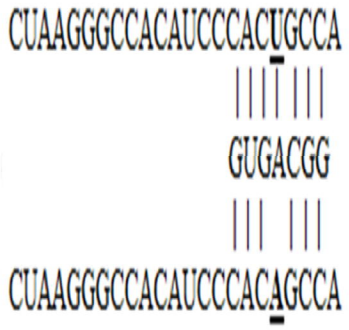
Figure 5

(A)

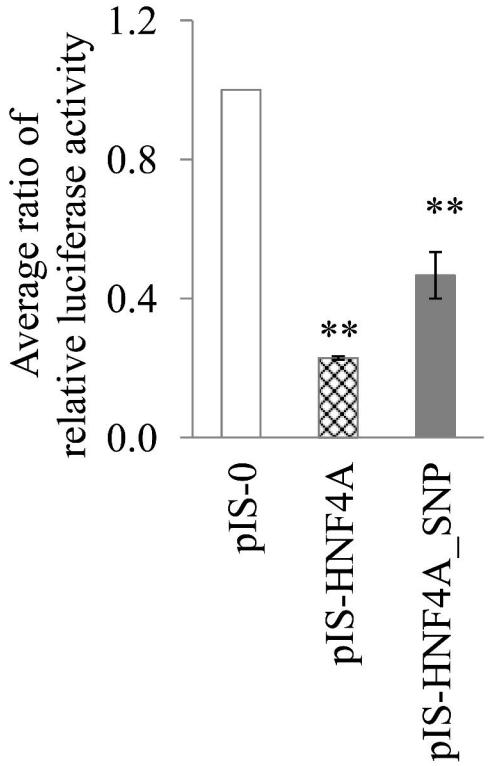


Wildtype

'Seed' sequence (2-7 bp) of hsa-miR-34a/-34b*/-34c-5p/-449a/-449b

Variant

(B)



(C)