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miR-217 Regulates Heme Oxygenase-1 Expression in 'Humanized' Renal Proximal Tubular Cells

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miR-217 Regulates Heme Oxygenase-1 Expression in 'Humanized' Renal Proximal Tubular Cells

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Abstract

Background: Heme oxygenase-1 (HO-1) is an inducible enzyme that affords cytoprotection in reaction to cellular stress and tissue damage. To study the *in vivo* regulation of human HO-1 gene expression at the molecular level, our laboratory previously generated HO-1 transgenic (hBAC mice) mice that lack murine HO-1 gene and overexpress a human HO-1 transgene on a BAC or bacterial artificial chromosome. The human HO-1 levels in the basal state in various tissues including the kidneys were significantly elevated in hBAC mice compared to HO-1 wild type (WT) mice. Previously, microRNAs or miRNAs have been reported to be involved in the regulation of HO-1 by binding to the 3' untranslated region or UTR of the gene. In our current study, we sought to examine the role of miRNAs in the regulation HO-1 expression in primary proximal tubule cells (PTCs) isolated from the kidneys of hBAC mice. **Methods:** Primary PTCs were isolated from the kidneys of hBAC mice and treated with four different miRNAs predicted by online web algorithms to bind to the 3' UTR of HO-1. We used bioinformatics analyses, real time polymerase chain reaction (PCR) and western blot protein analyses to examine the regulation of HO-1 expression by miRNAs in hBAC PTCs. **Results:** miRNAs 217, 377, 485, and 873 were predicted to bind to the 3' UTR of the human HO-1 gene. No significant differences were found in HO-1 mRNA levels in PTCs that were isolated from hBAC mice and treated with these miRNA mimics. This indicates that these miRNAs do not affect the stability of mRNA transcripts of HO-1. miR-217 significantly reduced HO-1 protein expression levels in PTCs isolated from hBAC mice. **Conclusion:** These studies indicate that miRNAs regulate the expression of HO-1 in PTCs and suggest that these mice represent an important tool for elucidating the mechanisms that control HO-1 expression in pathological conditions including acute kidney injury (AKI) to potentiate its protective effects.

Key words: microRNA, heme oxygenase, proximal tubule cells

Introduction

Heme oxygenase-1 (HO-1) is a microsomal inducible enzyme that is upregulated in cellular stress, tissue injury and pathophysiological states like acute kidney injury (AKI), lung injury, atherosclerosis, hypertension and cancers^{1,2}. HO-1 is cytoprotective and is induced by various stimuli like heme, certain growth factors, nitric oxide (NO), hyperoxia, as well as others. HO-1 catabolizes pro-oxidant iron-

containing heme, which is released by injured cells, resulting in the production of (i) iron (Fe^{2+}), (ii) biliverdin and (iii) carbon monoxide (CO). These byproducts of HO activity, including bilirubin generated from biliverdin reductase, exhibit powerful (i) antioxidant, (ii) anti-apoptotic and (iii) anti-inflammatory properties³. HO-1 is also involved in maintaining homeostasis of iron through the production of Fe^{2+} and co-induction with ferritin,

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an antioxidant pathway that sequesters iron. Our laboratory previously generated 'humanized HO-1 transgenic mice', which harbor an 87kb copy of the full-length human HO-1 gene segment and its upstream regulatory sequences on a bacterial artificial chromosome (BAC). These mice were bred with HO-1 knockout (KO) mice to create 'humanized HO-1 transgenic mice' (hBAC)⁴. hBAC mice lack the murine HO-1 gene, but globally overexpress a human HO-1 transgene and rescue the phenotype of the HO-1 KO mice. Interestingly, despite higher basal levels, human HO-1 expression is still inducible in hBAC mice subjected to AKI⁴. In addition, within specific tissues, the human HO-1 transgene is encoded at the highest levels in the specific cellular compartments that normally have elevated levels of HO-1 in wild type (WT) mice following injury, such as the proximal tubular cells (PTCs) of the kidney⁴. Unlike HO-1, heme oxygenase-2 (HO-2) is constitutively present at tissue-specific levels in mice and humans⁵ and its level of expression is unchanged in hBAC mice⁴.

The regulation of HO-1 expression involves an intricate regulatory network of *cis* and *trans* elements as well as signaling pathways and transcription factors⁶. MicroRNAs (miRNAs) have previously been reported to regulate the levels of HO-1 in human cells by binding to the 3' UTR of the gene⁷. miRNAs are approximately 22 nucleotides of non-coding RNA sequences that regulate gene expression after mRNA transcription⁸. miRNAs are generated by RNA polymerase II from their respective genes into pri-miRNA forms (primary transcripts) consisting of several hairpin structures. These pri-miRNAs are transformed into pre-microRNAs (seventy to a hundred nucleotides long) in the nucleus by a nuclear enzyme- Drosha (RNase III), which are then shuttled out to the cytoplasm by exportin-5 mediated export processes and converted to mature functional miRNAs by the cytosolic enzyme, Dicer. miRNAs base-pair with the 3' UTR of their target mRNA transcripts and form a complex known as microRNA containing ribonucleoprotein complex (miRNP). This complex binds with several other proteins including Argonaute to create the RNA-induced silencing complex that facilitates gene

silencing either by suppressing translation or facilitating mRNA degradation⁹.

Several studies indicate that altered or abnormal expression / levels of miRNAs can lead to several pathological disorders and cancers⁸. In addition, miRNAs have been described to play a vital role in kidney development, maintaining normal kidney function and homeostasis¹⁰. Since miRNAs regulate HO-1 expression levels⁷, the development of therapeutic strategies based on miRNAs is a promising target for intervention, given that HO-1 induction is a protective response to injury^{11, 12}. miRNA mimics are synthetic double stranded RNAs that behave like mature endogenous miRNAs after administration *in vitro* and *in vivo*. In this study, we isolated PTCs from hBAC mice as a tool to study the role of miRNA mimics in regulating expression of the human HO-1 gene in primary cells. We report that synthetic mature microRNA mimic miR-217 significantly reduced human HO-1 protein in hBAC PTCs, signifying a role for miRNAs in the regulation of HO-1 expression.

Materials and Methods

Bioinformatics analyses

The bioinformatics platforms Target Scan 6.2 (<http://www.targetscan.org>), miRGator 3.0 (<http://mirgator.kobic.re.kr/>), miRDB (<http://mirdb.org/miRDB/>), miRBase (<http://www.mirbase.org/>), and microrna.org (<http://www.microrna.org/microrna/home.do>) were used to predict miRNA target sequences in the 3' UTR of the human HO-1 gene. The transcript sequence ID *HMOX1-001* ENST00000216117 was used for the human UTR.

Isolation and culture of mouse proximal tubule cells

PTCs were isolated from the kidneys of HO-1 WT and hBAC mice (mixed FVB and C57BL/6 background) between 8-12 weeks of age by dissecting and separating the renal cortex from medulla as described previously^{13, 14}. The renal cortices were then chopped into tiny pieces, minced using single edged razor blade and filtered through a 70-µm cell sieve (FisherSci) into a 50mL conical tube containing renal epithelial cell growth medium

2 with supplement pack (#C39605 Promo Cell). The flow through medium containing the cells was centrifuged and the cells were cultured overnight in dishes layered with rat-tail type1 collagen (BD Biosciences) at 37°C in 5% CO₂. Next day, the media from these culture dishes were centrifuged and the pellet of cells (tubules) from the media was then cultured in dishes coated with rat-tail type1 collagen at 37°C in 5% CO₂. In addition, WT PTCs were treated with 10µM hemin for one hour and were grown in complete media for an additional 24 hours before protein isolation. HEK 293 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Cellgro) with 10% fetal bovine serum at 37°C in 5% CO₂. The University of Alabama at Birmingham Animal Care and Use Committee sanctioned all protocols involving animal use.

miRNA mimic treatment

Initial experiments were performed using HEK293 cells transfected with 20nM and 40nM (nanomolar concentration) of miR-217 and miR-377, respectively and cultured for 24 hours. The cells were then treated with 10µM hemin for one hour and were grown in complete media for an additional 24 hours before protein isolation⁷.

miRNA mimics were transfected as per recommendations and instructions of the manufacturer (miScript miRNA Mimics, Qiagen). Briefly, miRNA mimics were mixed with HiPerfect transfection reagent (Qiagen) for ten minutes at room temperature in 100µL of serum free media so that the transfection complexes can be formed. The transfection complexes were then dispensed drop by drop onto a 12-well plate containing approximately 16 x10⁴ PTCs/well and cultured for 72 hours. hBAC PTCs were transfected with 40nM of miRNA mimics. All Stars Negative Control siRNA (scrambled / Scr) having no homology to any known mammalian gene (#1027280; miScript miRNA Mimics, Qiagen) was used as a negative experimental control.

For RNA and miRNA quantification by real-time PCR, approximately 32 x10⁴ PTCs/well were cultured in a 6-well plate in 2 ml of complete media with 40nM miRNA mimics for 24 hours. After 24 hours of culture, RNA was extracted.

Real-time PCR

Quantitative real-time Polymerase Chain Reaction (qPCR) was done as reported previously¹⁴. Briefly, Trizol Reagent (Invitrogen) was used for isolating total cellular RNA. cDNA was synthesized using QuantiTect Reverse Transcription Kit (Qiagen). Real-time PCR using SYBR Green was done (Lifetech) and the $\Delta\Delta Ct$ method was utilized to calculate and quantitate the relative mRNA levels / expression using *GAPDH* as a loading control. Each reaction was performed in triplicate. For miRNA quantification to assess transfection efficiency, RNA was subjected to reverse transcription using miScript II RT Kit (with the Qiagen HiSpec buffer protocol) according to Qiagen company's recommendations. Real-time PCR was done with the Qiagen miRNA-specific miScript Primer Assay (forward primer) and miScript SYBR green PCR kit (Qiagen) containing the miScript Universal Primer (reverse primer). To normalize the amount of target miRNA, the endogenous reference small nuclear ribonucleic acid (snRNA) *RNU6B* gene (*RNU6-2*; Qiagen miScript Primer Assay; # MS00033740) was used. **Table 1** shows the primer sets that were used to quantify the transfection efficiency of the specific miRNA mimics used in this study and real time PCR of HO-1 gene expression. The following cycling conditions were used for miRNA quantification by real-time PCR (40 cycles): 95°C for fifteen minutes; 94°C for fifteen seconds; 55°C for thirty seconds and 70°C for thirty seconds.

Western blot analysis

Immunoblotting in this study was performed as described here¹⁴. Total protein lysates were collected using Radio immuno precipitation assay lysis buffer (RIPA lysis buffer recipe: 0.25% deoxycholic acid, 50 mM Tris/HCl, 150 mM NaCl, 1% NP-40, 1 mM sodium orthovanadate, 1 mM EGTA, 1 mM NaF) with a cocktail of protease inhibitors (Sigma). After measuring protein concentration by BCA protein assay (Thermo Scientific), 25µg of protein was electrophoretically resolved on SDS-PAGE gel. The protein from the gel was transferred to a nitrocellulose membrane. Rabbit polyclonal anti-HO-1 (ADI-SPA-894, Enzo Life Sciences), mouse monoclonal anti-GAPDH primary antibodies

Table 1. Primers for determining transfection efficiency of miRNA mimics and real time PCR analysis of HO-1 expression levels.

Assay	Target	Primer sequence
Transfection efficiency*	Hs_miR-217_1	UACUGCAUCAGGAACUGAUUGGA
	Hs_miR-377_1	AUCACACAAAGGCAACUUUUGU
	Hs_miR-873_1	GCAGGAACUUGUGAGUCUCCU
	Hs_miR-485-5p_1	AGAGGCUGGCCGUGAUGAAUUC
Real time PCR	human HO-1 F	5'-CATGACACCAAGGACCAGAG-3'
	human HO-1 R	5'-AGTGTAAAGGACCCATCGGAG-3
	human GAPDH F	5'-TCCCACTCTTCCACCTTGA-3'
	human GAPDH R	5'-AGTTGGGATAGGCCTCTCTTG-3'

F denotes forward primer; R denotes reverse primer.

*Real-time PCR for determining the miRNA mimic transfection efficiency was performed using the Qiagen miRNA-specific miScript Primer Assay (forward primer sequences listed in the table) and miScript SYBR green PCR kit (Qiagen) containing the miScript Universal Primer (reverse primer, Qiagen company's proprietary sequence).

(1:5000 dilutions), and anti-rabbit or anti-mouse immunoglobulin-G (IgG) secondary antibodies conjugated to horseradish peroxidase (HRP) (1:5000 dilutions) were used. The blots were developed and visualized using a chemiluminescence (ECL) detection substrate. Densitometric analyses were performed using the open source ImageJ software suite (<http://imagej.nih.gov/ij/>).

Statistical Analysis

The Student t-test was used for comparing data from two groups. For comparing data from more than two groups, Analysis of variance (ANOVA) with the Tukey's multiple comparisons post-test were utilized. Statistical significance was considered at $P < 0.05$ for all data sets. Graphpad Prism 6 software suite was used for performing all the statistical analyses in this study (GraphPad Software, Inc.).

Results and Discussion

hBAC renal proximal tubule cells overexpress human HO-1 protein

In vivo studies have demonstrated that the levels of HO-1 expression in basal state are globally elevated in hBAC kidneys compared to WT controls⁴. We confirmed this finding *in vitro* using western blot of protein lysates generated from PTCs isolated and cultured from WT and hBAC mice. Compared to WT PTCs, HO-1 protein expression is significantly higher in hBAC PTCs (Figure 1A and 1B).

In addition, WT PTCs were treated with 10µM hemin to induce the expression of endogenous HO-1, because the basal level of HO-1 is relatively low (Figure 1A and 1B). These data indicate that

upregulation of HO-1 by hemin in WT PTCs results in levels of HO-1 expression that are comparable to basal levels in hBAC PTCs.

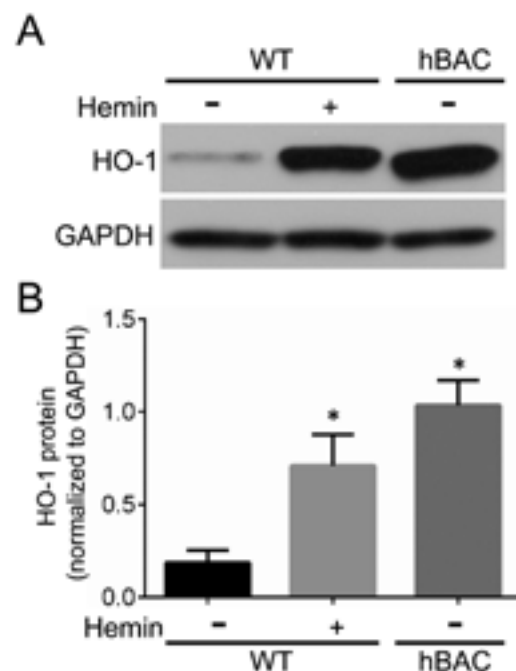


Figure 1: HO-1 expression in WT and hBAC proximal tubule cells. A) Western blot results for HO-1 protein expression in WT (treated with or without 10µM hemin for 1 hour) and hBAC PTCs are shown. GAPDH (Glyceraldehyde phosphate dehydrogenase) was used as a western blot loading control. B) Quantitation of HO-1 protein expression (from Figure 1A) normalized to GAPDH is shown. Data are represented as mean ± SEM values. * $P < 0.05$, relative to WT (n=4).

Prediction of miRNA binding in the 3' UTR of the HO-1 gene

The Target Scan 6.2, miRGator 3.0, microRNA.org, and mirDB bioinformatics algorithms predicted the presence of more than 129 different miRNA target sequences / sites in the 3' UTR of the human HO-1

gene. Table 2 lists the miRNAs predicted to bind to 3' UTR of human HO- gene (microRNA.org). miR-217, 485, 873 and 377 were identified as the most favorable candidates for further *in vitro* testing based on their context score of binding within the 3' UTR of the human HO-1 gene (Table 3), as determined by our *in silico* analyses (Figure 2).

Table 2: miRNAs predicted to bind to 3' UTR of human HO-1 gene.

miRNAs predicted to bind to Human HO-1 3'UTR
hsa-miR-377
hsa-miR-217
hsa-miR-485-5p
hsa-miR-200b
hsa-miR-200c
hsa-miR-429
hsa-miR-505
hsa-miR-22
hsa-miR-218
hsa-miR-873
hsa-miR-128

miRNAs that are predicted to bind to 3' UTR of human HO-1 gene as predicted by microRNA.org (<http://www.microRNA.org/microRNA/home.do>) are listed.

Table 3: miRNAs predicted to bind to the 3' UTR of the human HO-1 gene.

HO-1 miRNA	Total Context Score
miR-377	-0.41
miR-485-5p	-0.39
miR-217	-0.38
miR-873	-0.30

miRNAs predicted to have the lowest (favorable) total context score for binding to the 3' UTR of the human HO-1 gene are listed. Target Scan 6.2 (http://www.targetscan.org/vert_61/)

In summary, we selected miR-217, 485, 873 and 377 for experimental validation because these miRNAs had the lowest total context score and at least three of the four bioinformatics platforms predicted their binding to the human HO-1 3' UTR. In addition, miR-217 and 377 were previously demonstrated to regulate HO-1 expression⁷.

miRNA mimics do not affect HO-1 mRNA expression in hBAC proximal tubule cells

Quantitative real-time PCR was used to determine and quantify the transfection efficiency of mimics in hBAC PTCs, relative to the endogenous reference small nuclear ribonucleic acid (snRNA) RNU6B gene. Our data indicate that relative to control PTCs from hBAC kidneys treated with transfection reagent alone, miRNA mimics were transfected effectively into hBAC PTCs (Figure 3A). There were no significant differences in human HO-1 mRNA levels in hBAC PTCs after transfection with miRNA mimics, indicating that these miRNAs do not affect the stability of the HO-1 mRNA transcript (Figure 3B).

miRNA mimics reduce HO-1 protein expression in hBAC proximal tubule cells

The dose of miRNA mimics needed to achieve an observable reduction in endogenous human HO-1 expression was first determined in HEK293 cells. As reported previously⁷, two different concentrations (20 and 40nM) of miR-217 and miR-377 were tested (data not shown). Next, hBAC PTCs were used to test the effect on miR-217, 485, 873, and

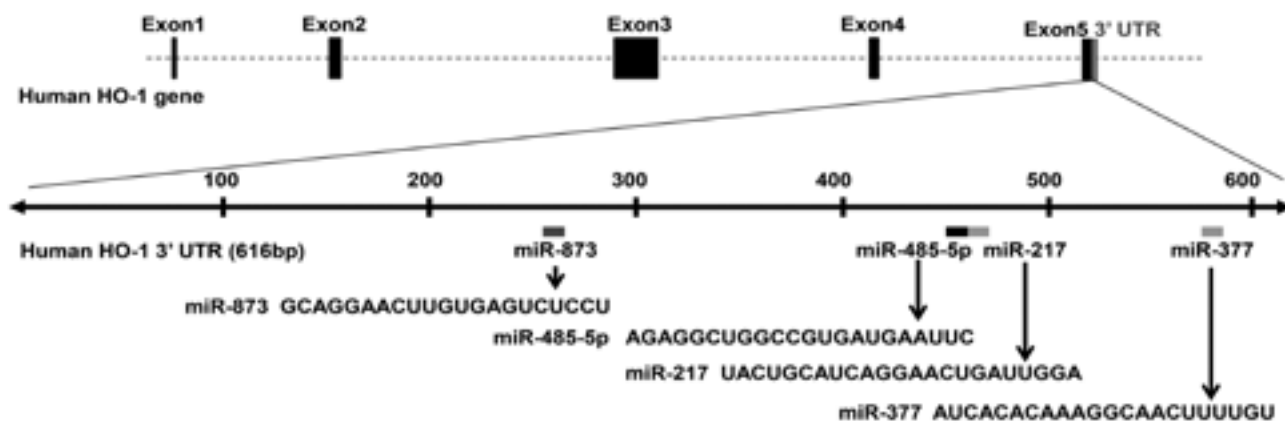


Figure 2. miRNAs predicted to bind to the 3' UTR of human HO-1 gene. Binding sites of the four miRNAs used in this study with their respective locations and sequences are shown.

377 on human HO-1 protein levels. Western blot done using an antibody cross-reactive to human HO-1 protein showed that miR-217 significantly reduced HO-1 protein expression in hBAC PTCs (Figure 3C and D). miR-377 and 485 also reduced HO-1 expression, but did not attain statistical significance. These data suggest that miR-217 suppresses basal HO-1 expression levels in PTCs isolated from hBAC mice.

Discussion

In the current study, PTCs from hBAC mice were transfected with the mature miRNA mimics, miR-217, 485, 873, and 377. These miRNA species were selected based on their favorable score (lowest total context score), which predicted their binding to the human HO-1 3' UTR (Figure 2 and Table 3). miR-217 significantly reduced HO-1 protein expression

in hBAC PTCs. Interestingly, of the four miRNA mimics predicted to bind to the human HO-1 3' UTR that were tested in this study, only miR-217 significantly reduced HO-1 protein expression in hBAC PTCs. However, we demonstrated an obvious decrease in HO-1 expression levels after miR-377, 485, and 873 treatments (Figure 3C). The capability of these miRNAs to reduce HO-1 expression, perhaps suggests that additional regulatory mechanisms (along with miRNAs) may contribute to the regulation of the human HO-1 gene in hBAC mice. It has been previously reported that miRNAs and transcriptional factors co-operatively control gene expression to regulate cellular functions in health and disease conditions^{15, 16}. However, other possible reasons for the lack of a statistically significant suppression of HO-1 expression by miR-377, 485, and 873 in hBAC PTCs could include

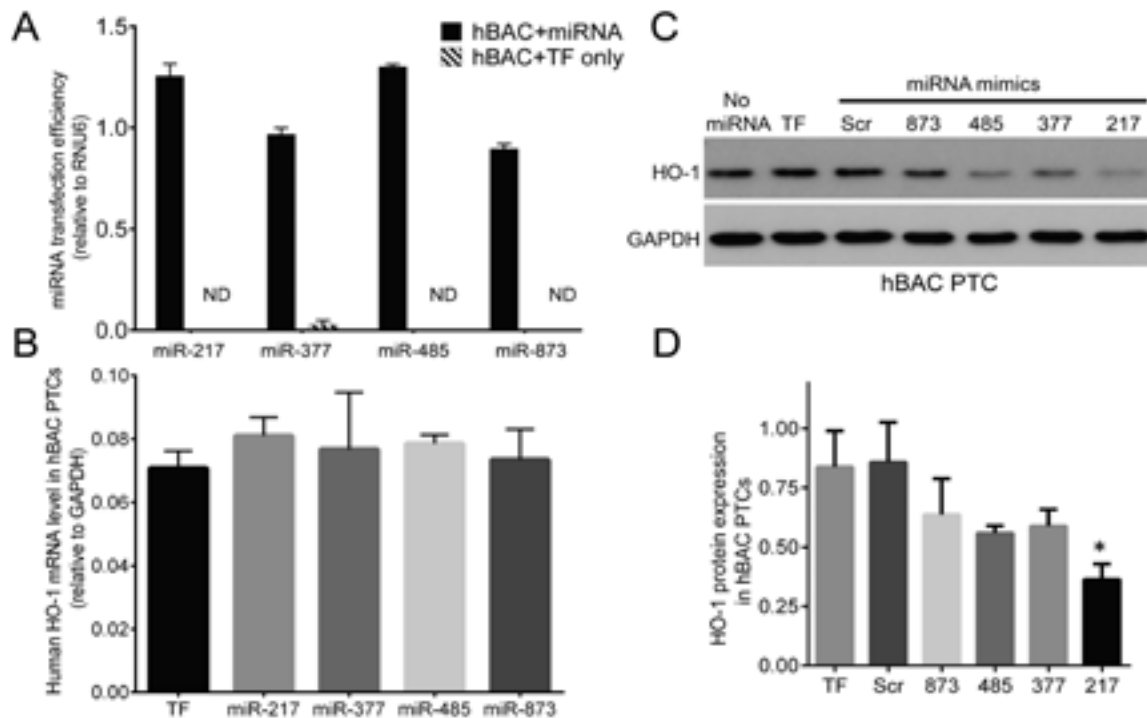


Figure 3: Effect of miRNA mimics on human HO-1 mRNA and protein levels in hBAC proximal tubule cells. A) miRNA quantification by real time PCR to assess the transfection efficiency of hBAC PTCs treated with the indicated miRNA mimic or transfection reagent only (TF) as a control is shown. Real-time PCR was done with the Qiagen miRNA-specific miScript Primer Assay (forward primer) and miScript SYBR green PCR kit (Qiagen) containing the miScript Universal Primer (reverse primer). Data are represented as mean \pm SEM values and normalized to small nuclear RNA (snRNA) RNU6B (RNU6). All these experiments were done in duplicates and repeated three times (n=3). ND- not detected. B) Real time PCR analysis of human HO-1 mRNA expression in hBAC PTCs after administration of indicated miRNAs or transfection reagent only (TF) normalized to GAPDH is shown. Data presented as mean \pm SEM values. C) hBAC PTCs were transfected with miR-873, 485, 377, 217 and All Stars Negative Control siRNA (scrambled-Scr) for 72 hours. HO-1 protein expression was assessed by western blot. D) Quantitation of HO-1 protein expression in hBAC PTCs normalized to GAPDH and shown as a fold change relative to no miRNA. Data presented as mean \pm SEM values. *P<0.05 compared to no miRNA lane. All these experiments were performed in duplicates three times (n=3). TF: transfection reagent only.

the requirement for higher doses or different combinations of these miRNAs, with or without miR-217, for achieving a significant reduction of HO-1 expression. Collectively, these data suggest that the miRNAs used in the current study regulate HO-1 protein expression in hBAC PTCs.

HO-1 is induced in numerous disease states and such induction of HO-1 is protective in AKI, ischemia-reperfusion injury in the heart and liver, as well as organ transplantation^{1-3, 17}. In fact, our laboratory has demonstrated that higher basal levels of human HO-1 in hBAC mice is protective in AKI caused by cisplatin-induced nephrotoxicity, heme-mediated rhabdomyolysis, and renal ischemia reperfusion injury^{4, 14, 18}. Many miRNAs (including miR-217 and miR-377) have been reported to be involved in the development of renal fibrosis¹⁹. Studies done *in vivo* and *in vitro* systems indicate that increased levels of transforming growth factor- β (TGF- β) can induce miR-217 and miR-377, resulting in the down regulation of PTEN (Phosphatase and Tensin Homolog) and increased production of collagen and fibronectin thus promoting a pro-fibrotic response^{20, 21}. Therefore, future studies that explore approaches to suppress the expression or stability of miRNAs such as miR-217 (and / or 377), which suppress HO-1 expression, may possibly be considered a promising novel therapeutic target.

miRNA profiling is clinically relevant as well as for investigating therapeutic targets in kidney transplantation, rejection, and experimental ischemia-reperfusion injury^{22, 23}. Several tools to enhance or reduce miRNA function or abundance are under investigation (reviewed in²⁴). For example, the delivery of exogenous small RNAs in the form of precursor miRNA or mature miRNAs diminishes the rate of tumor growth in mice. Alternatively, introduction of antagonists of specific miRNAs (like antagomiRs) can be used to inhibit or block specific miRNAs. Therefore, this study demonstrates that hBAC mice form a powerful tool that can be employed in future studies that focus on modulating miRNA levels and other regulatory elements that negatively control HO-1 expression *in vivo* and in disease states such as AKI.

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Conflict of Interest

None

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