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ORIGINAL ARTICLE

MicroRNA antagonist therapy during normothermic machine perfusion of donor kidneys

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Normothermic machine perfusion (NMP) is a novel clinical approach to overcome the limitations of traditional hypothermic organ preservation. NMP can be used to assess and recondition organs prior to transplant and is the subject of clinical trials in solid organ transplantation. In addition, NMP provides an opportunity to deliver therapeutic agents directly to the organ, thus avoiding many limitations associated with systemic treatment of the recipient. We report the delivery of oligonucleotide-based therapy to human kidneys during NMP, in this case to target microRNA function (antagomir). An antagomir targeting mir-24-3p localized to the endothelium and proximal tubular epithelium. Endosomal uptake during NMP conditions facilitated antagomir co-localization with proteins involved in the RNA-induced silencing complex (RISC) and demonstrated engagement of the miRNA target. This pattern of uptake was not seen during cold perfusion. Targeting mir-24-3p action increased expression of genes controlled by this microRNA, including heme oxygenase-1 and sphingosine-1-phosphate receptor 1. The expression of genes not under the control of mir-24-3p was unchanged, indicating specificity of the antagomir effect. In summary, this is the first report of ex vivo gymnotic delivery of oligonucleotide to the human kidney and demonstrates that NMP provides the platform to bind and block detrimental microRNAs in donor kidneys prior to transplantation.

KEYWORDS
basic (laboratory) research/science, gene therapy, ischemia reperfusion injury (IRI), kidney transplantation/nephrology, molecular biology: micro RNA, organ perfusion and preservation, translational research/science

Abbreviations: ASO, antisense oligonucleotide; DCD, donation after circulatory death; DGF, delayed graft function; ECD, extended criteria donor; IRI, ischemia reperfusion injury; LNA, locked nucleic acid; MAP, mean arterial pressure; mRNA, messenger RNA; NMP, normothermic machine perfusion; PTEC, proximal tubular epithelial cell; RISC, RNA-induced silencing complex.

Emily R. Thompson and Avinash Sewpaul denotes equal contribution.

1 | INTRODUCTION

Kidney transplantation remains the treatment of choice in patients with end-stage renal disease (ESRD).[1] The increasing prevalence of ESRD has led to a shortage of donor organs and the need to expand the donor pool. To bridge the gap between organ demand and supply, donation after circulatory death (DCD) donors and extended criteria donors (ECDs) are increasingly being used. DCD donors have prolonged warm ischemia times during cardiac arrest with an increased incidence of delayed graft function (DGF) compared with kidneys donated after brain death.[2]

Normothermic machine perfusion (NMP) describes a period of ex vivo re-oxygenation of the donor organ prior to implantation.[3] NMP has been postulated to reduce the severity of ischemia reperfusion injury (IRI) and lower the rate of DGF in kidney transplant recipients.[4,5] DGF is associated with inferior long-term graft outcomes[6] and, therefore, NMP could lead to improvements in graft longevity. NMP also allows the assessment and reconditioning of marginal organs that would otherwise not be used for transplantation, thereby increasing the supply of donor organs.[7,8] NMP may also provide an opportunity to deliver therapeutic agents, including drugs and cellular therapies,[9] directly to the isolated organ, reducing the dose delivered to the recipient.

MicroRNAs are short, non-coding oligonucleotides. They bind to the 3' UTR of messenger RNA (mRNA), either blocking translation or leading to mRNA degradation. Each microRNA has the potential to bind different mRNAs and therefore regulate translation of multiple proteins involved in diverse cellular functions.[10] As a consequence, microRNAs have key regulatory functions in both physiological and pathological conditions. Changes in the expression of microRNA have been implicated in a range of pathological scenarios including cancer, autoimmune and degenerative diseases. In addition, microRNAs have been extensively investigated as potential biomarkers of disease, their short length conferring stability in biological fluids and tissues.[11-13] This makes them attractive therapeutic targets, but as with other oligonucleotide-based therapies, delivering antagomirs or mimics to their desired target cell represents a significant obstacle. Previously, oligonucleotide therapies have required coupling with either a viral vector or cationic lipid/polymer vehicle for effective intracellular delivery. These methods of delivery are not ideal in immunosuppressed transplant recipients due to concerns regarding the oncogenic potential of viral vectors and pro-inflammatory immune response seen with other delivery methods.[14] However, the normal metabolic and physiological conditions possible during kidney NMP result in a perfect platform to facilitate gymnosis or "naked" delivery of the oligonucleotide without the need for encapsulation or a vector. Gymnotic delivery is also considered to have fewer potential side effects and it is cheaper to manufacture and deliver naked oligonucleotides.

We aimed to investigate the possibility of gymnotic delivery of an antagomir antisense oligonucleotide (ASO) therapy to human kidneys during NMP.

2 | MATERIALS AND METHODS

2.1 | Normothermic machine perfusion

Sixteen human kidneys deemed unsuitable for transplantation were included in this study, Table S1 (Research Ethic Committee approval 15/SC/0161). Kidneys were prepared by cannulation of the renal artery, vein, and ureter. The NMP protocol was as previously described,[3,4] detailed in Supplementary Methods. A preliminary cohort of kidneys underwent microRNA profiling studies, $n = 6$. A second cohort of kidneys were treated for 6 h during NMP with 1mg of either miRNA-24-3p antagomir ($n = 5$) or scramble sequence oligonucleotide ($n = 5$; Table 1). The oligonucleotide was added directly to the perfusate reservoir at time-zero. Oligonucleotides were purchased from Exiqon™, antagomir against miRNA-24-3p (C*T*G*C*T*G*A*A*C*T*G*A*G*C*C) and "scramble" control sequence oligonucleotide (A*C*G*T*C*T*A*T*A*C*G*C*C*C*A). Both oligonucleotides had a modified phosphorothioate backbone with a locked nucleic acid design. To allow localization, the oligonucleotides were modified with the addition of a red fluorescent label (TYE™563) to the 5' end. Treatment allocation was sequential, the first five kidneys were treated with antagomir and then the subsequent five kidneys were treated with scramble sequence. The number of biopsies taken during NMP was rationalized to minimize injury to the perfusing kidney; three core biopsies were taken at 0-, 2-, and 4-h time-points. All sites were suture-ligated. At the end of NMP, 1 cm^2 wedge biopsies were taken.

2.2 | Immunohistochemistry and imaging

Localization of labeled oligonucleotide was performed on 4 μm formalin-fixed, paraffin-embedded sections. The antigen retrieval methodology and staining protocol was optimized as outlined in Table S2. Initial localization studies were performed using a Zeiss AxioImager and the intensity of staining quantified using ImageJ™ software, as previously described.[15] Briefly, the mean fluorescent intensity of the red fluorescent antagomir (TYE™563) within the tissues section was calculated for each biopsy time-point and compared relative to time-zero. For intracellular compartment tracking of the oligonucleotide, sections were stained with the following commercially available antibodies to anti-RAB5 (early endosome), anti-RNA7 (late endosome), anti-LAMP2 (lysosome), and anti-GW182 (P bodies). Counter nuclear staining was with DAPI and a secondary antibody conjugated with AlexaFluor488™ was used. Sections were visualized using a Leica SP8 Confocal microscope. A cross section of tubule was identified and a 2.5 μM z-stack of this tubule acquired at the optical sampling interval (0.13 μM) and optimal pixel size in accordance with the Nyquist criterion. All images were captured in a standardized manner for comparison, detailed in Supplementary Methods. Colocalization was quantified using Hugyens™ software following batch deconvolution.

TABLE 1 Donor characteristics and normothermic machine perfusion parameters

	Antagomir $n = 5$	Control sequence oligonucleotide $n = 5$	p value
Age of donor years, mean (range)	56.6 (44–68)	69.8 (63–73)	<.05
Sex	M = 5 F = 0	M = 3 F = 2	.44
CIT h, mean (SEM)	20.73 (4.163)	24.15 (0.994)	.45
Kidney weight g, mean (SEM)	273.4 (28.78)	231.2 (21.88)	.27
eGFR at procurement ml/min/1.73 m^2, mean (SEM)	77.8 (15.78)	75.8 (8.351)	.91
Remuzzi score median (range)	1 (0–4)	4 (1–4)	.14
Perfusion characteristics			
Blood flowml/min/100 g, mean (SEM)	87.98 (21.86)	108.2 (31.38)	.61
Filtrate volume ml/h, mean (SEM)	267 (63.51)	150 (53.27)	.20
Quality assessment score, median (range)	1.4 (1–2)	2.2 (1–4)	.37

2.3 | In situ hybridization

Double-DIG-labeled miRCURY LNA detection probes (Exiqon™) were used to localize miRNA-24-3p. Sections were deparaffinized with xylene, rehydrated and treated with proteinase K prior to hybridization with probe at 54°c. Probe was detected with anti-digoxigenin Fab fragments conjugated to alkaline phosphatase (Enzo LifeSciences) and the substrate NBT/BCIP (Enzo LifeSciences). Slides were counterstained with nuclear fast red (Enzo LifeSciences).

2.4 | MicroRNA profiling

Total RNA was extracted from biopsies using the miRCURY™ RNA Isolation Kit (Exiqon™). After quality control (Agilent 2100 Bioanalyzer System), microRNA profiling was performed using the Exiqon microRNA microarray platform. This microarray uses high-throughput PCR to measure the expression of 752 miRNAs.

2.5 | Real-time PCR quantification of gene expression

For quantification of gene expression, following isolation, the RNA was reverse transcribed and cDNA synthesis was carried out using the Tetro cDNA synthesis kit. RNA sequence quantification was performed using TaqMan RT-qPCR on a StepOnePlus™ Real-Time PCR System. The following ThermoFisher™ TaqMan primers were used; HMOX1 hs01110250_m1, S1PR1 hs00173499_m1, TGFβ1 hs00998133_m1, TWIST1 hs01675818_s1 and HPRT1 hs02800695_m1 as a housekeeping gene.

2.6 | Statistical analysis

Data were analyzed using GraphPad PRISM (Version 8.0). Mann–Whitney U tests were applied to compare nonparametric ordinal data, and chi-squared tests or Fisher's exact tests for the analysis of categorical variables. Continuous data were analyzed using *t*-tests or by one-way analysis of variance (ANOVA with a Tukey posthoc test) when more than two groups were compared. A repeated measure-two-way ANOVA was used when the same variable was measured repeatedly during NMP. *p*-values of <.05 were considered significant.

3 | RESULTS

3.1 | The effect of normothermic machine perfusion on renal microRNA expression

MicroRNA expression was profiled in human kidneys prior to and after 1 h of NMP, $n = 6$ kidneys, Figure 1. This identified 15 candidate miRNAs that were highly expressed throughout NMP that had a fold change of <2 indicating they might be suitable targets for therapeutic manipulation. A literature review was performed and identified that six of these miRNAs have been implicated in IRI (Table 2). We subsequently focused on miR-24-3p to explore whether blockade of this detrimental miRNA could be targeted during NMP.

3.2 | Renal delivery of antagomir by normothermic machine perfusion

Sections of kidney biopsies (0, 2, 4, and 6 h) were visualized by fluorescence microscopy to identify the distribution of oligonucleotide.

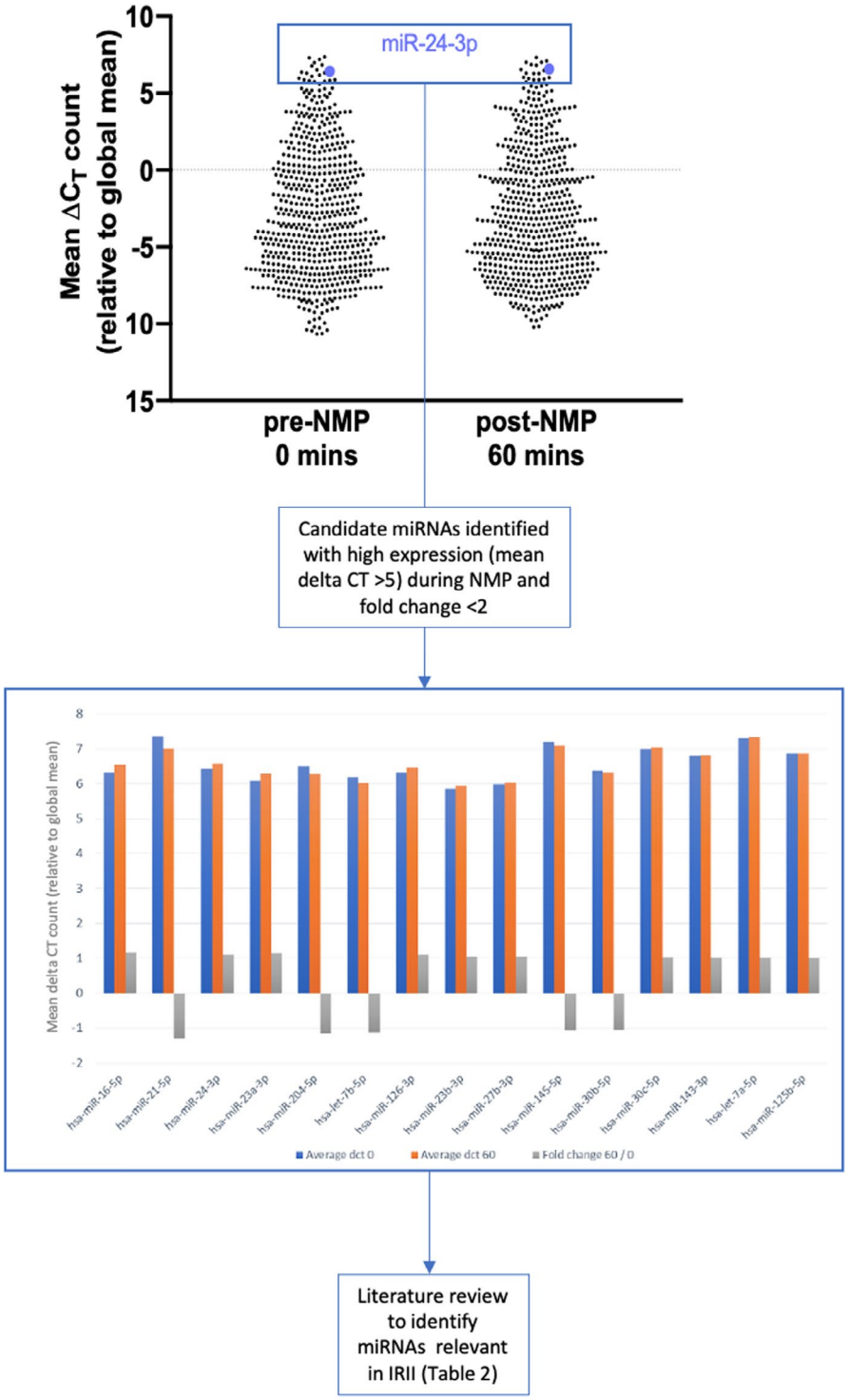


FIGURE 1 MicroRNA profiling in kidneys undergoing ex vivo normothermic machine perfusion (NMP). MicroRNA profiling was performed on kidney biopsies prior to and after 1 h of ex vivo NMP using a Nanostring microarray that included 752 microRNAs (MiR-24-3p is highlighted in blue). Mean cycle threshold values for each microRNA relative to the global mean of all microRNAs are displayed here. The bottom graph depicts the candidate microRNAs mean cycle threshold values before and after 1 h of NMP and the associated fold change. MicroRNAs that were highly expressed prior to NMP that maintained stable expression throughout were considered candidates for therapeutic blockade [Color figure can be viewed at wileyonlinelibrary.com]

After 2 h of NMP, there was evidence of antagomir retention within the kidney (Figure 2A–D). Quantification of fluorescence intensity was consistent with a time-dependent uptake of antagomir during NMP (Figure 2E). There was evidence of uptake by vascular endothelial cells (Figure 2D,F,G) and tubular epithelial cells, with a vesicular pattern of antagomir distribution in the latter (Figure 2G,H). The distribution of uptake of antagomir and scramble sequence oligonucleotides was identical. Sections were stained with aquaporin-1 to identify proximal tubular epithelial cells (PTECs) (Figure 2I). When antagomir was delivered during hypothermic machine perfusion, it could be found coating the vascular endothelium (Figure 2J,K), but there was no intracellular uptake of antagomir in PTECs, suggesting that uptake is an active process, only occurring in normothermic conditions.

TABLE 2 MicroRNAs implicated in ischemia reperfusion injury (IRI)

MiRNA	Role in IRI	Proposed mode of action	References
Let-7 family	Let-7a knockout protects against cerebral ischemia reperfusion injury	Inhibition of MAPK and JNK signaling pathways	31
	Inhibition of Let-7 family members reduces rat cardiac IRI	Altered the expression of a range of genes implicated in cardiac protection	32
Mir-21-5p	Reduced injury in Mir-21 -/- mice after experimental IRI	Generation of free radicals is reduced due to an increase in mitochondrial Mpv17l in the absence of Mir-21	13
	Higher serum Cr after bilateral renal IRI in Mir-21 -/- mice		33
Mir-24-3p	Increased in ischemic kidney and Mir-24 inhibition reduces injury in preclinical fibrotic disease	Increases hemoxygenase expression and reduces tubular cell apoptosis	11
	Protect from cardiac IRI in pre-clinical models	Down regulation of pro-apoptotic genes in cardiomyocytes	34
Mir-27	Higher levels inhibit angiogenesis and increases vascular permeability	Reduces the expression of the endothelial junctional protein VE-cadherin	35
Mir-126	Overexpression reduces IRI in mice	Increases the number of circulating hematopoietic stem cells and maintains microvascular integrity	36
Mir-145	Protects cardiomyocytes from free radical injury	Inhibits mitochondrial apoptotic pathways	37

3.3 | Intracellular trafficking of antagomir during normothermic machine perfusion

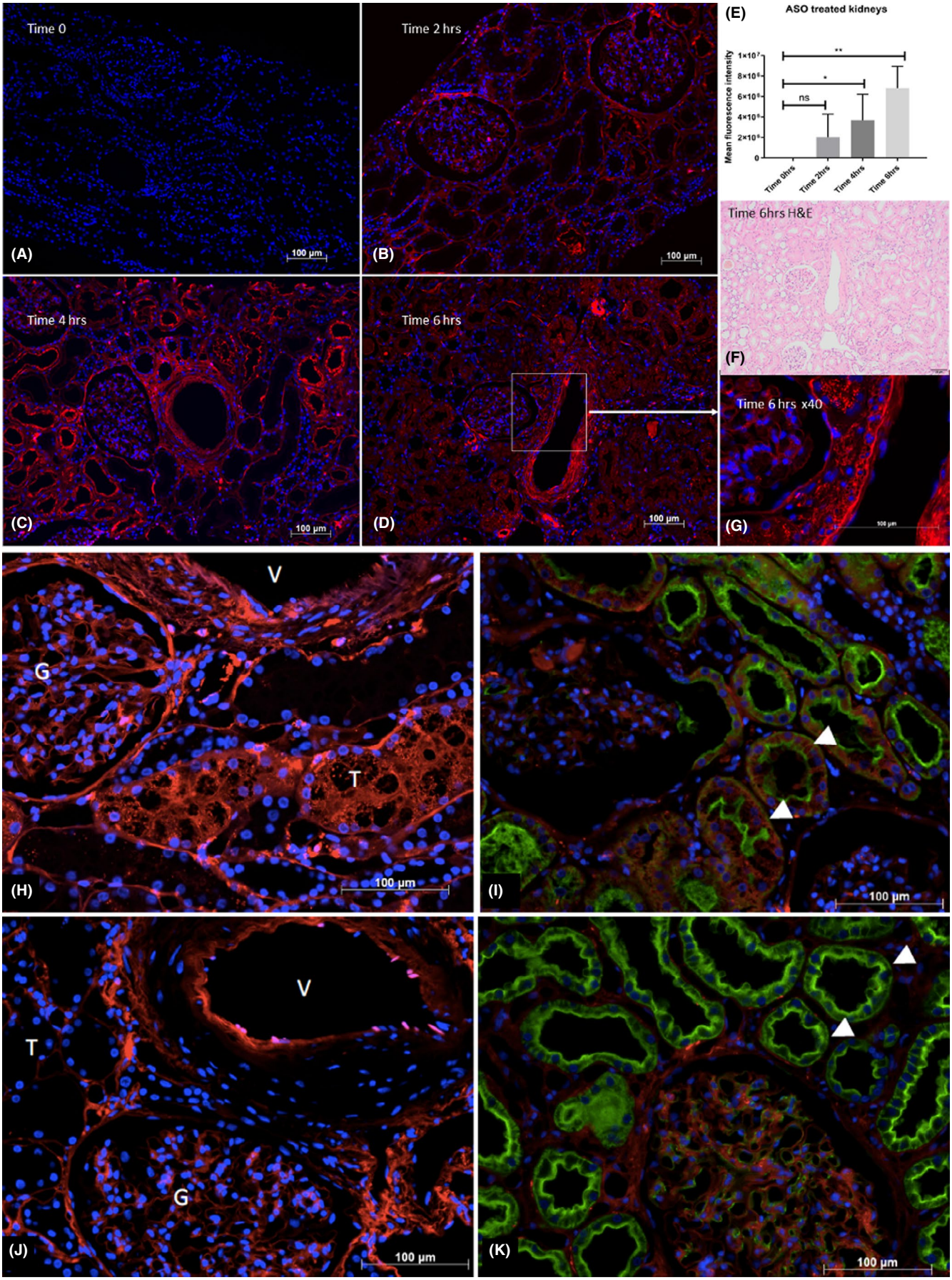
Recently, it was established that intracellular trafficking plays an important role in regulating ASO pharmacological activity; therefore, understanding this process is key to confirming desired downstream miRNA targeting.[16,17] To investigate intracellular trafficking of the antagomir, sections were examined using confocal microscopy from 0-, 2-, 4-, and 6-h biopsies. Sections were stained with antibodies against with Rab5 (early endosome), Rab7 (late endosome), LAMP2 (lysosome), and GW182 (RNA-induced silencing complex [RISC]). The kidneys treated with the antagomir during NMP demonstrated co-occurrence of antagomir with Rab5 in the early endosome. This co-occurrence increased steadily, peaking after 4 h of NMP and then declined (Figure 3, Row A). A large proportion of antagomir was no longer co-localizing with Rab5 by the end of NMP, indicating progression into other intracellular compartments. There was also moderate co-occurrence of antagomir with Rab7 at a steady rate throughout NMP (Figure 3, Row B). The antagomir used in this study is designed to bind to miRNA, which will be concentrated within RISC, GW182 is an important protein within this complex. We observed co-occurrence of the antagomir with GW182 throughout NMP (Figure 3, Row C), indicating that the antagomir is escaping the endosomal compartment and localizing to regions where there will be high concentrations of miRNA. GW182 co-localization was not seen in the scramble sequence–treated kidneys despite a similar pattern of endosomal uptake (Figure 3, Row E). Both antagomir and scramble-treated kidneys also demonstrated co-occurrence with lysosomal marker, LAMP2 (Figure 3, Row D). This gradually increased during NMP, suggesting there is also accumulation of oligonucleotide in lysosomes. During HMP, there was no evidence of co-localization of the antagomir with any intracellular compartments, (Figure 3, Row F), and the antagomir was generally found to be entirely extracellular.

3.4 | Engagement of antagomir target

In situ hybridization (ISH) was used to identify the where miR-24-3p was predominantly concentrated within the kidney and whether the antagomir was able to bind to its target. Target binding by the antagomir does not result in miRNA degradation; therefore, PCR quantification is not informative. However, when the antagomir binds it prevents other complementary nucleotide sequences binding, including the antisense ISH probe. MiR-24-3p staining was evident in both endothelial cells and the tubular epithelium in untreated NMP kidneys (Figure 4A,B). ISH for miR-24-3p was unaffected by scramble sequence treatment during NMP (Figure 4C). However, antagomir treatment during NMP resulted in reduced staining for miR-24-3p (Figure 4D), suggesting that during NMP the antagomir successfully engages its target.

3.5 | Downstream effects of antagomir treatment

An antagomir, by blocking microRNA function, will lead to an increase in target mRNA levels. Two mRNA targets of miR-24-3p blockade were investigated in more detail, heme oxygenase-1 (HMOX1) and sphingosine-1-phosphate receptor-1 (S1PR1), as they have a proposed protective role in renal IRI and other forms of acute tissue injury.[18,19] RT-pRCR demonstrated, that kidneys perfused with antagomir had a significant increase in HMOX1 and S1PR1 gene expression, $p < .005$ and $p < .05$ respectively (Figure 5A–D,). The same effect was not seen in scramble sequence–treated kidneys.


Time 0
(A)
100 µm
Time 2 hrs
(B)
100 µm
(E)
ASO treated kidneys
Mean fluorescence intensity
1×10⁷
8×10⁶
6×10⁶
4×10⁶
2×10⁶
0
**
*
ns
Time 0hrs
Time 2hrs
Time 4hrs
Time 6hrs
Time 6hrs H&E
(F)
Time 4 hrs
(C)
100 µm
Time 6 hrs
(D)
100 µm
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(G)
V
G
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100 µm
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100 µm
(K)
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FIGURE 2 Uptake of antagomir during ex vivo normothermic machine perfusion (NMP). Kidney biopsies were taken prior to (time 0 [A]) and 2 (B), 4 (C), and 6 (D) h after the introduction of labeled oligonucleotide (red) into the NMP circuit. Formalin fixed sections were deparaffinized and nuclei counter stained with DAPI. The quantity of antagomir was assessed by measuring fluorescence intensity using ImageJ software (E, $n = 10$, $^{*}p < .05$, $^{**}p < .005$, ANOVA). Hemoxylin and eosin–stained kidney section (F) to identify a blood vessel to assess endothelial cell and vessel wall uptake of antagomir (G). At 6 h after the introduction of labeled antagomir, formalin-fixed sections were deparaffinized and counterstained with DAPI. Images H and I depict sections of kidneys after ex vivo NMP demonstrating antagomir uptake was evident in the glomerulus (G), blood vessels walls (V), and in a vesicular pattern in tubular epithelial cells (T). Staining with aquaporin 1 (green) was used to identify proximal tubular epithelial cells (arrowheads) in images I and K. After cold perfusion (images J and K) labeled antagomir was seen in blood vessel walls and the glomerulus; however, staining was not seen in aquaporin 1 positive tubular cell [Color figure can be viewed at wileyonlinelibrary.com]

In addition, we assessed the expression of two genes that do not contain a miR-24-3p-binding sequence in their 3'UTR, TGFβ1, and TWIST1. No change in the expression of these genes was seen following perfusion of kidneys with either MiR-24-3p antagomir or scramble sequence oligonucleotide (Figure 5E–H). This is consistent with the hypothesis that antagonism of microRNA function will allow manipulation of specific mRNAs, predictable from in silico analysis.

3.6 | Impact of antagomir therapy on renal physiology and inflammation

There was no significant difference in NMP parameters or cytokine concentrations in the perfusate between kidneys perfused with antagomir or scramble sequence oligonucleotide (Figure S1).

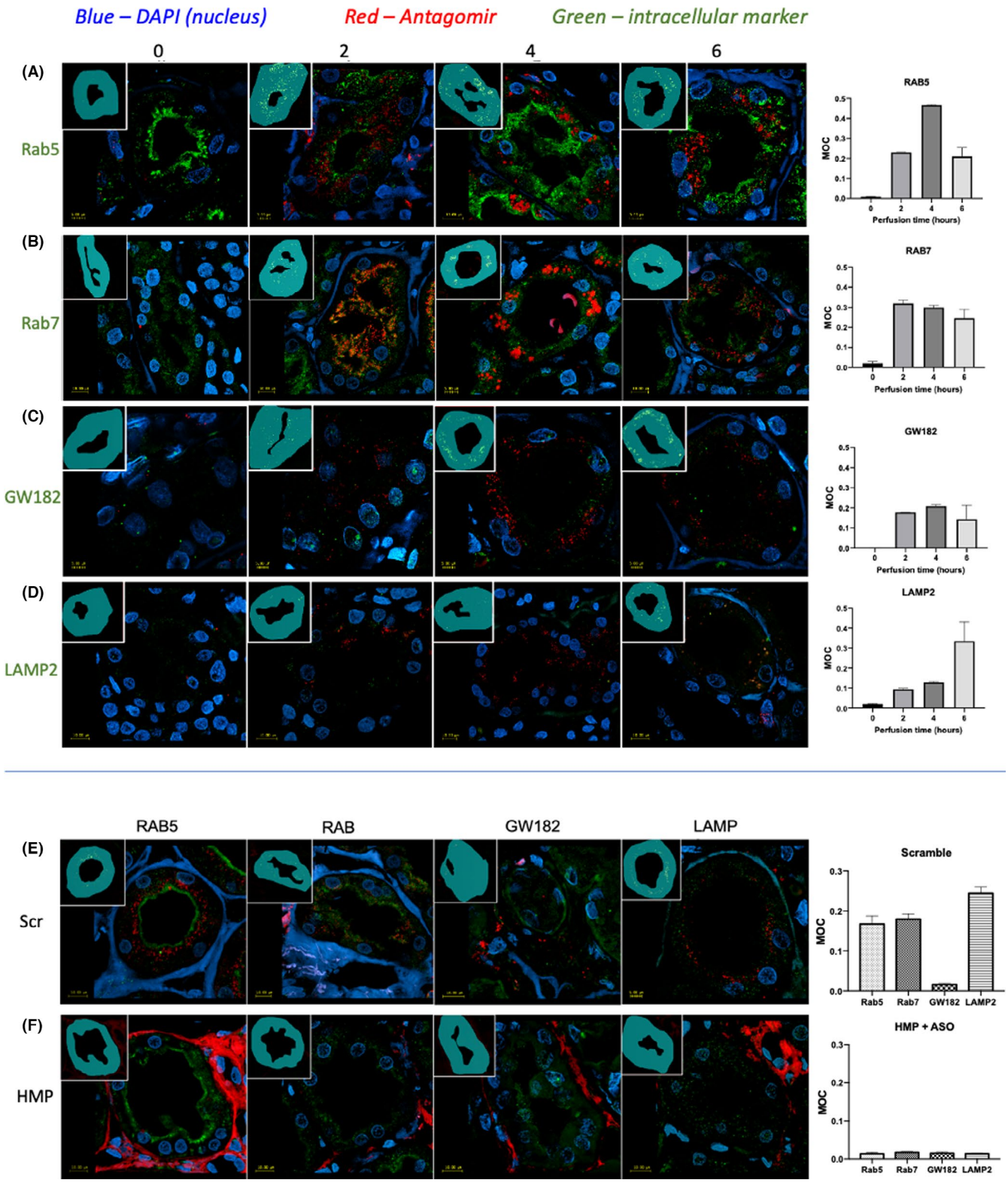
4 | DISCUSSION

This is the first study to demonstrate the potential of antagomir therapy targeting miR24-3p, delivered via NMP to an isolated kidney graft. The antagomir was taken up by tubular epithelial cells and successfully bound the target miRNA. This has the potential capacity to modify the expression of targeted genes, which may have a putative protective function within the transplant organ. NMP allows this to be done with limited systemic exposure to antagomir in the potential recipient. Many drugs have an intracellular mode of action and their effects require active cellular uptake. Although drugs that do not require cellular uptake can be administered during hypothermic machine perfusion,[20] NMP increases the range of therapies that can be administered to the isolated organ.

Profiling microRNA expression in kidneys prior to and after 1 h of normothermic perfusion identified candidate microRNAs that were highly expressed in the kidney and the expression of which was not altered by normothermic perfusion. The highly expressed microRNAs included miRNA-24-3p, which has been proposed to have a role in kidney damage, both in the pathogenesis of cellular injury and as potential biomarker of disease severity.[11] Multiple miRNAs have also previously been identified as regulating transcription during IRI, rejection and interstitial fibrosis.[21] MiR-21, miR-146, and miR-182 have been found to play important roles in IRI. MiR-146 was also found to be elevated in the urine samples of recipients of DCD kidneys compared with live donor recipients, which will have experienced a significantly less ischemic insult.[22] Lorenzen et al. demonstrated that miR-24-3p was upregulated in the kidney after transplantation, specifically in endothelial and tubular epithelial cells.[11] Overexpression of miR-24-3p resulted in increased tubular cell apoptosis and decreased cell function. In contrast, an antagomir to block miR-24-3p preserved cellular function and prevented apoptosis. This was found to be mediated through the miR-24-3p mRNA targets; heme-oxygenase 1 (HMOX1) and H2A histone family member X. In their mouse model of ischemia reperfusion injury, anti-miR-24 therapy was associated with increased survival, better kidney function and less epithelial injury. MiR-24-3p is upregulated in human kidneys that suffer prolonged cold ischemic times.[11] As such, we chose to focus our antagomir on miR-24-3p blockade and investigate its impact on downstream mRNA target, HMOX-1.

MiRNA are synthesized from the transcription of miRNA genes into a pri-miRNA to form a hairpin structure. This hairpin is cleaved by the RNase Drosha to form a pre-miRNA of approximately 70 nucleotides in length which is transported into the cytoplasm. Once in the cytoplasm the pre-miRNA is further cleaved to form mature miRNA by the enzyme dicer.[23] This mature miRNA will engage with argonaute and GW182 proteins to create the RISC.[24] The RISC will target the specific mRNA based on the complementary sequence of the miRNA (the guide strand), and the other miRNA strand will be degraded (passenger strand).[25] Once the miRNA binds the target mRNA this leads to translational repression or degradation, and as such, regulation of protein expression.

In this study, the miRNA-24-3p antagomir administered during NMP localized to the endothelium and tubular cells. Antagomirs have an intracellular mechanism of action and, therefore, will require active cellular uptake. The mechanism by which an antagomir enters the cell has important ramifications on its ability to mediate pharmacological activity.[17] An oligonucleotide must be able to escape the endosomal compartment into the cell cytoplasm to have effect.[26] Here, we demonstrate that NMP facilitates the process of active endocytic uptake particularly delivering antagomir into tubular epithelial cells. During hypothermic machine perfusion the antagomir was not taken into epithelial cells and there was no colocalization with endosomal intracellular compartments indicating NMP is actively facilitating this process. It is likely that NMP enables glomerular filtration and reabsorption of the oligonucleotide into the PTECs of the kidney. Investigating this gymnotic mechanism of uptake further revealed the ASO was trafficking through the endocytic pathway (Rab5 & Rab7) within the cell. Rab5 is a protein associated with the early endosomal compartment and begins to determine the fate of internalized molecules.[27]



Rab7 is a protein commonly associated with the late endosomal compartment; it also has a functional role in sorting of selective cargos in the early endosome in combination with Rab5.[28] It was also apparent that a proportion of ASO was escaping from the endosomal compartment into the cytoplasm to localize with specific miRNA cytoplasmic machinery (GW182). GW182 is a cytoplasmic protein crucially involved in the miRNA mediated mRNA silencing that occurs in the RISC complex.[29] GW182 directly interacts with Argonaute proteins to provide a docking platform for miRNA resulting in translational repression or mRNA degradation.[30] Interestingly, the scramble sequence oligonucleotide followed a similar pathway of endocytic uptake and trafficking through endosomal compartments, however, it did not colocalize with the RISC complex. This likely reflects the redundancy of its scramble

FIGURE 3 Tracking antagomir uptake through intracellular compartments during ex vivo normothermic machine perfusion (NMP). Upper panel—images depict typical confocal microscopy cross-sectional images of kidney tubules (multiple intensity projection, z-stacks), which were used in colocalization analysis from each time-point (0, 2, 4, and 6 h of NMP). The antagomir (red)-treated kidney sections were co-stained with specific intracellular compartment markers (green) and DAPI used as counter nuclear stain (blue). The smaller image inlaid into the top left-hand corner of each tubule cross section depicts typical rendering of red and green colocalization that was used in the quantification. The graph demonstrates the quantification of co-occurrence using Manders coefficient during the NMP timeline. Row A—colocalization of antisense oligonucleotide (ASO) with early endosome marker Rab5 in a timeline of deconvolved images of tubules taken throughout NMP (0, 2, 4, and 6 h). Row B—colocalization of ASO with late endosome marker Rab7 throughout the perfusion timeline. Row C—colocalization of ASO with RISC marker GW182 throughout the perfusion timeline. Row D—colocalization of ASO with lysosome marker LAMP2 throughout the perfusion timeline. Lower panel: Colocalization of scramble sequence oligonucleotide with intracellular compartments during ex vivo NMP and antagomir ASO during hypothermic machine perfusion. Images depict confocal microscopy of human kidney tissue sections following 6 h of treatment. Oligonucleotide (red) and co-stained for each intracellular marker (green) and DAPI as counter nuclear stain (blue). The rendered colocalization analysis of the selected region of interest (the kidney tubule cross section) is inlaid in the top left-hand corner. Row E—representative images of the scramble sequence oligonucleotide and its degree of colocalization with the different intracellular compartments following 6 h of NMP treatment. The graph depicts quantification of co-occurrence of scramble sequence oligonucleotide with intracellular compartments. Row F—representative images from the hypothermic machine perfused kidneys that were treated for 6 h with the ASO and the degree of colocalization with the intracellular compartments. The graph quantifies this and demonstrates there was very minimal colocalization of the ASO with any of intracellular compartments during HMP [Color figure can be viewed at wileyonlinelibrary.com]

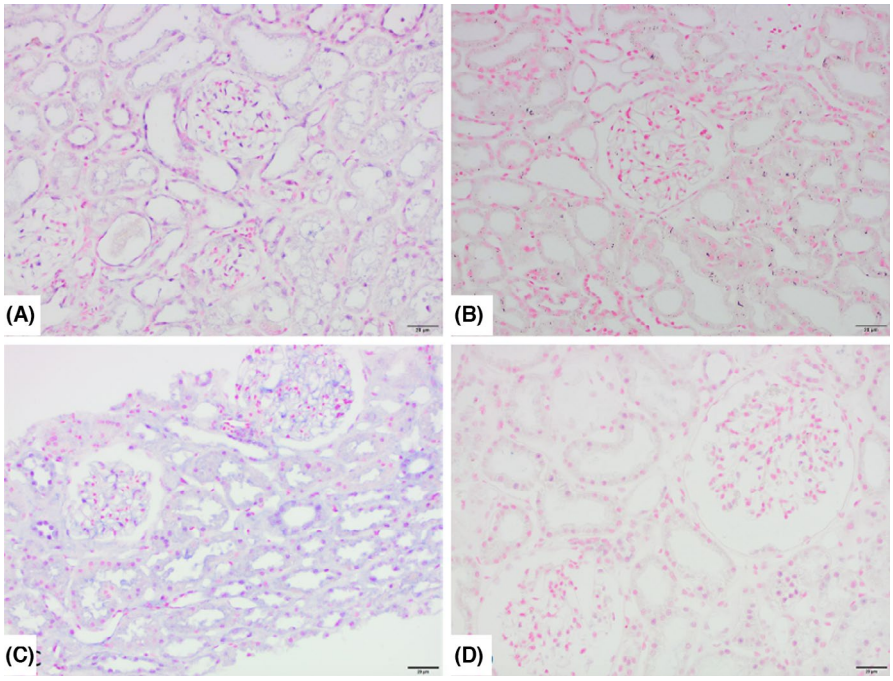


FIGURE 4 Antagomir-binding miR-24-3p target during normothermic machine perfusion (NMP): in situ hybridization with Dig-labeled antisense probe for miR-24-3p was performed on proteinase K–treated sections. Binding of probe was detected with anti-Dig, alkaline phosphatase conjugated antibody (purple). Sections were counterstained with nuclear fast red. The distribution of miR-24-3p using antisense probe in control tissue is shown (A) and control sense probe on the same tissue is shown in (B). Staining using antisense probe after 6 h ex vivo NMP with random sequence oligonucleotide (C) and with miR-24-3p antagomir (D) [Color figure can be viewed at wileyonlinelibrary.com]

sequence, which lacks any specific targets. Some of the antagomir was also within lysosomes where it may undergo degradation. It is not clear from these experiments what factors influence the proportion of antagomir that escapes into the cytoplasm versus that which is processed for degradation. The antagomir was not uniformly seen throughout the kidney, which may be an effect of dosing or localized microvascular perfusion over-concentrating the antagomir in specific regions resulting in increased lysosomal processing, this will require further investigation in future studies.

This is the first time a study has successfully demonstrated the mechanics behind gymnotic oligonucleotide therapy in NMP, ratified by the ISH studies, demonstrating that ASO delivery during NMP can bind and block a detrimental microRNA prior to transplantation. It may be that the physiologically normal environment provided by NMP coupled with the chemically modified structure of the ASO results in a synergistic combination optimizing delivery. The unique characteristics of the kidney nephron make it a highly desirable target for oligonucleotide therapies. The small oligonucleotides will be freely filtered by the glomerulus during NMP and then be reabsorbed by PTEC. The phosphorothoriate backbone of the ASO provides an electrostatic mechanism to associate with cell membrane receptor proteins, which may result in internalization.[31] In PTECs the responsible receptors are likely to be cubulin and megalin.[32,33] Using this physiology is particularly desirable in transplantation as the PTECs are the most damaged cells during IRI resulting in acute tubular necrosis and DGF.[34] Harnessing this potential of a therapy and delivery platform that naturally localizes in these damaged cells is attractive. The proposed mechanism for this is summarized in Figure 6.

One of the major hurdles facing oligonucleotide therapeutics is targeting delivery to the required site.[35] A number of different

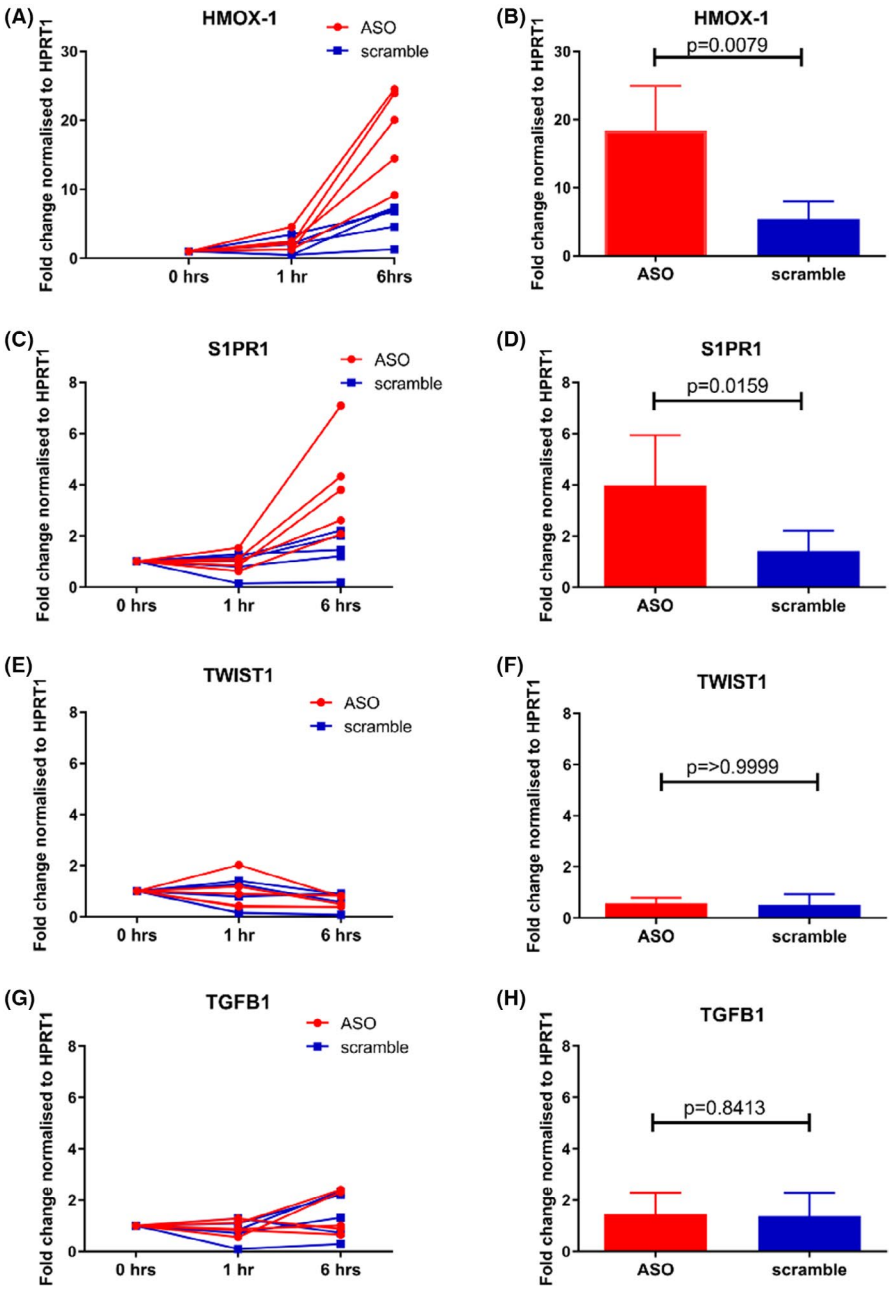


FIGURE 5 Evaluation of the expression of mir-24-3p targets. Gene expression was assessed in kidneys prior to (0 h) and at 1 and 6 h of perfusion with miR-24-3p antagomir (ASO, red) or scramble sequence oligonucleotide (scramble, blue) ($n = 5$ per group) by RT-PCR (A,C,E,G). Comparison of the expression after 6 h perfusion is shown in panels B, D, F, and H. $^{*}p > .05$, $^{**}p < .005$, unpaired *t*-test [Color figure can be viewed at wileyonlinelibrary.com]

methods have been investigated to overcome this including using a viral vector[36] or lipid nanoparticle encapsulation.[37] These methods of delivery can improve targeting, especially when coupled with the specific ligand for a receptor. However, viral vectors have a low specificity and can activate host oncogenes.[38] Lipid nanoparticles are also associated with severe infusion related reactions.[39] Gymnotic delivery of oligonucleotide therapeutics has previously been investigated but due to the inherent instability of the unmodified short oligonucleotide sequence this can be very unreliable.[40] Chemical modifications to oligonucleotide compounds have helped improve their efficiency. This most commonly includes the phosphorothiate backbone and a locked nucleic acids design, which has demonstrated increased efficiency of gymnotic uptake.[41] However, lack of targeting to the required cell type still persists, and this results in large, expensive doses of therapeutics being necessary to achieve the desired outcomes. In this study, we have demonstrated that a chemically modified oligonucleotide design coupled with organ-specific delivery during NMP can bypass many of the previous challenges associated with oligonucleotide therapy.

We were able to demonstrate the effects of antagomir treatment on gene expression, with an increase in both HMOX1 and S1PR1, two genes predicted to be regulated by mir-24-3p. HMOX1 is important to limit free radical–induced damage during reperfusion, and there is evidence that treatment with hemoxygenase or strategies to increase the expression of heme oxygenase-1 protect from IRI, including early-phase clinical studies.[42] We observed a 20-fold increase in HMOX1 expression after 6 h of NMP antagomir treatment, significantly greater than the increase seen with NMP and control

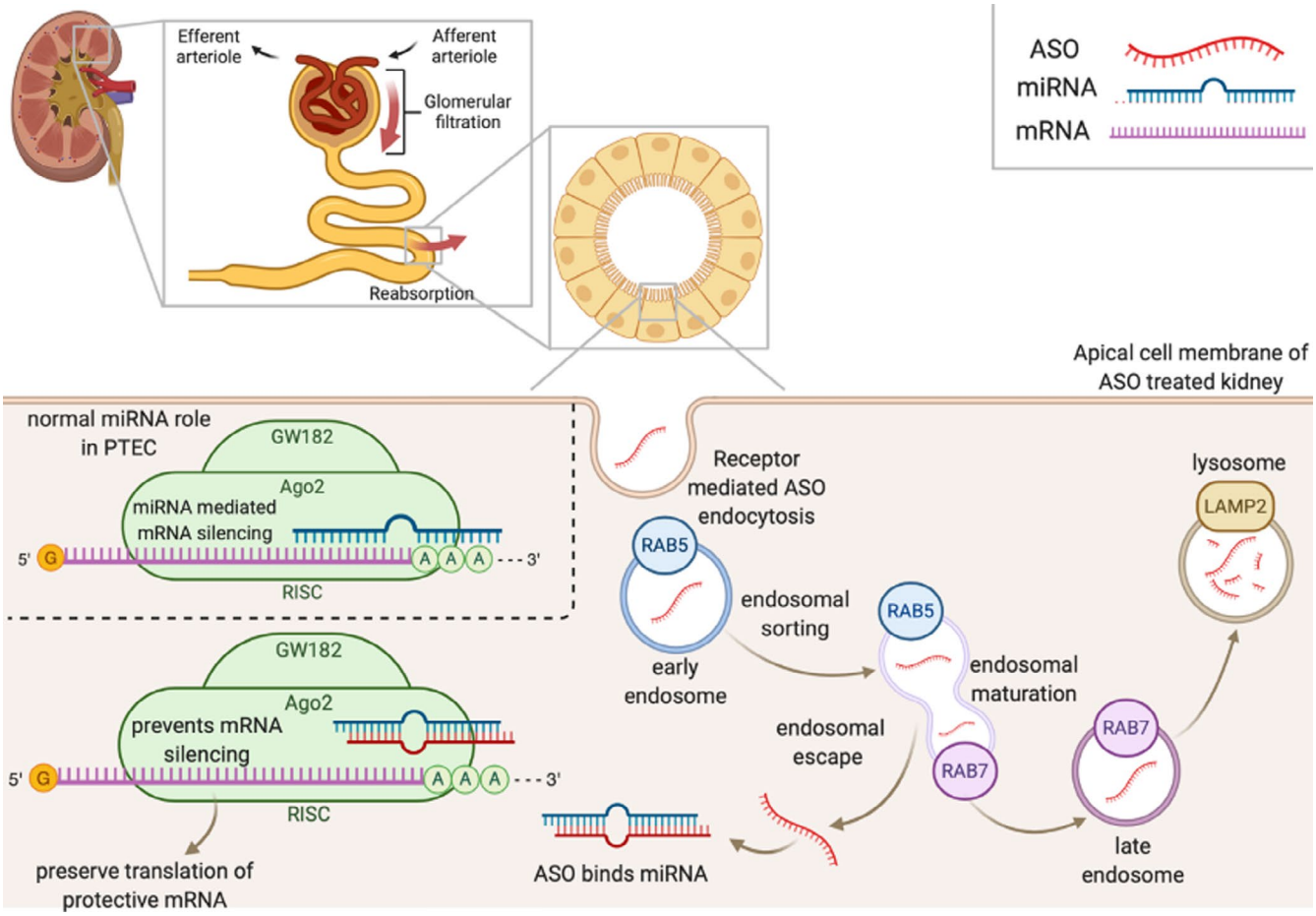


FIGURE 6 Diagram depicting proposed mechanism for antagomir uptake and trafficking interaction with miR-24 during ex vivo normothermic machine perfusion resulting in the preservation of mRNA targets [Color figure can be viewed at wileyonlinelibrary.com]

sequence oligonucleotide. The increase in HMOX1 with antagomir is greater than that seen with other interventions that have shown promise in clinical studies.[42] Similarly, expression of SIPR1, which has a role in maintaining endothelial integrity, was also significantly increased by antagomir treatment, whereas genes not targeted by antagomir were unaffected.

MicroRNAs elicit tissue and cell specific responses; therefore, delivering an antagomir systemically may result in unintended adverse effects in one organ countered by beneficial effects in the other.[43] For example, miR-24, the target in our study, has previously been demonstrated to play a critical role in modulating IRI responses in human kidney tissues and blockade results in the preservation of renal function in a mouse model.[11] However, in the heart, the overall effect of blockade is less clear—miR-24 is protective in cardiac myocytes and blockade results in increased apoptosis,[44] but, in cardiac endothelial cells, blockade results in decreased apoptosis.[45] In vitro studies performed in our group also reveal a similar cell-specific response to miR-24 blockade in the kidney; endothelial cell blockade resulted in the upregulation of HMOX1 and SOD2 coupled with a reduction in reactive oxygen species production.[46] It is likely that in vitro models are unable to recapitulate the in vivo response of a whole organ and NMP models helps us to delineate this overall response better, thereby supporting clinical translation of microRNA therapeutics.

The findings of this study have a number of limitations including the size and heterogeneity within the sample cohort. The kidneys investigated were not pairs from the same donor and were from a heterogeneous donor population meaning that our scramble and antagomir treatment groups were not perfectly matched. Future transcriptomic studies should focus on using samples taken from a paired model of therapeutic investigation in kidney NMP. The more interesting control cohort for comparison would be a paired untreated control as this would facilitate investigation of potential off-target and toxic effects associated with antagomir therapy. Translation of antagomir therapy into a clinical reality faces several challenges. The ability of one microRNA to affect multiple mRNA targets may be an advantage but may also be a disadvantage if "protective genes" are downregulated. Synthetic oligonucleotides may also have off target effects that could be detrimental to the donor organ; however, this was not evident in our study. MicroRNAs may have different effects in different organs and, a major problem facing all oligonucleotide-based therapies, is how to deliver them to their target cell. For example, miRNA-24-3p attenuates IRI in cardiac endothelial cells[45] but perpetuates IRI in kidneys. NMP is able

to overcome these obstacles; by reducing systemic exposure and delivering directly to the desired organ. The potential for this mode of delivery was also demonstrated in a recent paper using a porcine model of liver transplantation. Goldracena and colleagues demonstrated that administration of a LNA inhibitor of mir-122 during NMP enhanced functional miR-122 sequestration prior to transplantation potentially suppressing hepatitis C virus replication.[47]

In summary, this is the first description of the use of NMP to deliver antagomir to a human organ. We are able to demonstrate gymnotic cellular uptake, trafficking, and binding of the target microRNA with resultant upregulation of target mRNA. This, therefore, represents a way to alter gene expression in an organ prior to transplant. Although we have focused on miR-24-3p, many other microRNAs could be targeted and combination of treatments is feasible. Work in preclinical models will identify optimal targets and inform future clinical studies.

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DISCLOSURE

The authors of this manuscript have no conflicts of interest to disclose as described by the *American Journal of Transplantation*.

DATA AVAILABILITY STATEMENT

All data related to this study are present in the article or the Supplementary Figures found at the end of this article. The data that support the findings of this study are available from the corresponding author upon request. All reagents and perfusion consumables were commercially available.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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