MicroRNA antagonist therapy during normothermic machine perfusion of donor kidneys

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Abstract

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 localised to the endothelium and proximal tubular epithelium. Endosomal uptake during NMP conditions facilitated antagomir co-localisation 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.

Abbreviations: ASO – antisense oligonucleotide NMP-Normothermic Machine Perfusion, PTEC-Proximal Tubular Epithelial Cell, DCD-Donation after circulatory death, ECD-Extended criteria donor, DGF- delayed graft function, IRI – Ischemia Reperfusion injury, MAP-Mean Arterial Pressure, mRNA-messenger RNA, LNA- Locked Nucleic Acid, RISC – RNA-induced silencing complex

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 (ECD) are increasingly being used. DCD donors have prolonged warm ischemia times during cardiac arrest with an an increased incidence of delayed graft function (DGF) compared to kidneys donated after brain death (DBD)[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 oliginucleotides.

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

Materials and Methods

Normothermic Machine Perfusion

Sixteen human kidneys deemed unsuitable for transplantation were included in this study, Supplementaty Table 1 (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 hours 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 localisation, the oligonucleotides were modified with the addition of a red fluorescent label (TYE™563) to the 5' end. Treatment allocation was sequential, the first 5 kidneys were treated with antagomir and then the subsequent 5 kidneys were treated with scramble sequence. The number of biopsies taken during NMP was rationalised to minimise injury to the perfusing kidney; 3 core biopsies were taken at 0, 2 and 4 hour time-points. All sites were suture ligated. At the end of NMP, 1cm^2 wedge biopsies were taken.

Immunohistochemistry and Imaging

Localisation of labelled oligonucleotide was performed on 4μm formalin fixed, paraffin embedded sections. The antigen retrieval methodology and staining protocol was optimised as outlined in Supplementary Table 2. Initial localisation 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 (MFI) 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) & anti-GW182 (P bodies). Counter nuclear staining was with DAPI and a secondary antibody conjugated with AlexaFluor488™ was used. Sections were visualised using a Leica SP8 Confocal microscope. A cross-section of tubule was identified and a 2.5μM z-stack of this tubule

acquired at the optical sampling interval (0.13µM) and optimal pixel size in accordance with the Nyquist criterion. All images were captured in a standardised manner for comparison, detailed in supplementary methods. Colocalisation was quantified using Hugyens™ software following batch deconvolution.

In situ Hybridisation

Double-DIG labelled miRCURY LNA detection probes (Exiqon™) were used to localize miRNA-24-3p. Sections were deparaffinised with xylene, rehydrated and treated with proteinase K prior to hybridization with probe at 54^{o}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).

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 utilises high throughput PCR to measure the expression of 752 miRNAs.

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, TGF$\beta 1$ hs00998133_m1, TWIST1 hs01675818_s1 and HPRT1 hs02800695_m1 as a housekeeping gene.

Statistical analysis

Data was analysed using GraphPad PRISM (Version 8.0). Mann-Whitney U tests were applied to compare non-parametric ordinal data, and chi-squared tests or Fisher's exact tests for analysis of categorical variables. Continuous data was analysed 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 <0.05 were considered significant**.**

Results

The Effect of EVNP on Renal MicroRNA Expression

MicroRNA expression was profiled in human kidneys prior to and after 1 hour 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 6 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.

Renal delivery of antagomir by NMP

Sections of kidney biopies (0, 2, 4 & 6 hours) were visualised by fluorescence microscopy to identify the distribution of oligonucleotide. After 2 hours 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 and G) and tubular epithelial cells, with a vesicular pattern of antagomir distribution in the latter (figure 2G and 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 (PTEC) (figure 2I). When antagomir was delivered during hypothermic machine perfusion it could be found coating the vascular endothelium (figure 2 J and K) but there was no intracellular uptake of antagomir in PTECs, suggesting that uptake is an active process, only occurring in normothermic conditions.

Intracellular trafficking of antagomir during NMP

Recently, it was established that intracellular trafficking plays an important role in regulating antisense oligonucleotide 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, & 6 hour biopsies. Sections were stained with antibodies against with Rab5 (early endosome), Rab7 (late endosome), LAMP2 (lysosome) and GW182 (RNA-induced silencing complex). 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 hours of NMP and then declined (Figure 3, Row A). A large proportion of antagomir was no longer co-localising 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-occurence of the antagomir with GW182 throughout NMP (Figure 3, Row C), indicating that the antagomir is escaping the endosomal compartment and localising to regions where there will be high concentrations of miRNA. GW182 co-localisation 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-occurence 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-localisation of the antagomir with any intracellular compartments, (Figure 3, Row F) and the antagomir was generally found to be entirely extracellular.

Engagement of antagomir target

In situ hybridisation (ISH) was used to identify the where miR-24-3p was predmoninatly 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 anti-sense *ISH* probe. MiR-24-3p staining was evident in both endothelial cells and the tubular epithelium in untreated NMP kidneys (Figure 4 A and B). *In situ hybridisation* 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.

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 ischemia reperfusion injury 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, $p<0.005$ and $p< 0.05$ respectively (figure 5A-D,). The same effect was not seen in scramble sequence treated kidneys. 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.

Impact of Antagomir therapy on renal physiology and inflammation

There was no significant difference in NMP parameters or cytokine concentrations in the perfusiate between kidneys perfused with antagomir or scramble sequence oligonucleotide (supplementary figure 1).

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 admistered to the isolated organ.

Profiling microRNA expression in kidneys prior to and after 1 hour 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 when compared to live donor recipients which will have experienced a significantly less ischaemic 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 ischaemia 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 ischaemic times[11]. As such, we chose to focus our antagomir on miR-24-3p blockade and investigate its impact on downstreamm mRNA target, HMOX-1.

MiRNA are synthesised 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 RNA induced silencing complex (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 bedegraded (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 localised 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[26]. An oligonucleotide must be able to escape the endosomal compartment into the cell cytoplasm to have effect[27]. 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-colocalisation 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 proximal tubular epithelial cells 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 internalised molecules[28]. 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[29]. It was also apparent that a proportion of ASO was escaping from the endosomal compartment into the cytoplasm to localise 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[30]. GW182 directly interacts with Argonaute proteins to provide a docking platform for miRNA resulting in translational repression or mRNA degradation[31]. Interestingly, the scramble sequence oligonucleotide followed a similar pathway of endocytic uptake and trafficking through endosomal compartments, however, it did not colocalise with the RISC complex. This likely reflects the redundancy of its scramble 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 localised 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 in-situ hybridisation 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 optimising 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 internalisation[32]. In PTECs the responsible receptors are likely to be cubulin and megalin[33,34]. Utilising this physiology is particularly desirable in transplantation as the PTECs are the most damaged cells during IRI resulting in acute tubular necrosis and DGF[35]. Harnessing this potential of a therapy and delivery platform that naturally localises in these damaged cells is attractive. The proposed mechanism for this is summarised in Figure 6.

One of the major hurdles facing oligonucleotide therapeutics is targeting delivery to the required site[36]. A number of different methods have been investigated to overcome this including using a viral vector[37] or lipid nanoparticle encapsulation[38]. 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[39]. Lipid nanoparticles are also associated with severe infusion related reactions[40]. 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[41]. Chemical modifications to oligonucleotide compounds have helped improve their efficiency. This most commonly includes the phosphothoroiate backbone and a locked nucleic acids design which has demonstrated increased efficiency of gymnotic uptake[42]. 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 haemoxygenase or strategies to increase the expression of heme oxygenase-1 protect from IRI, including early phase clinical studies[43]. We observed a 20-fold increase in *HMOX1* expression after 6 hours of NMP antagomir treatment, significantly greater than the increase seen with NMP and control sequence oligonucleotide. The increase in *HMOX1* with antagmoir is greater than that seen with other interventions that have shown promise in clinical studies[44]. 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[45]. 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 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[46], but, in cardiac endothelial cells blockade results in decreased apoptosis[47]. *In vitro* studies performed in our group also reveal a similar cell specificresponse to miR-24 blockade in the kidney; endothelial cell blockade resulted in upregulation of HMOX1 and SOD2 coupled with a reduction in reactive oxygen species production[48]. 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 heteroegenitiy 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 utilising 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[47] but perpetuates IRI in kidneys. NMP is able to overcome these obstacles; by reducing sytemic 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 collegues 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[50].

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 focussed on miR-24-3p many other microRNAs could be

targeted and combination of treatments is feasible. Work in pre-clinical models will identify optimal targets and inform future clinical studies.

Figure legends

Figure 1: MicroRNA profiling in kidneys undergoing ex vivo NMP.

MicroRNA profiling was performed on kidney biopsies prior to and after 1 hour 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 hour of NMP and the associated fold change. MicroRNAs which were highly expressed prior to NMP that maintained stable expression through out were considered candidates for therapeutic blockade.

Figure 2. Uptake of antagomir during ex vivo NMP

Kidney biopsies were taken prior to (time 0 (A)) and 2 (B), 4 (C) and 6 (D) hours after the introduction of labelled oligonucleotide (red) into the NMP circuit. Formalin fixed sections were de-paraffinised and nuclei counter stained with DAPI. The quantity of antagomir was assessed by measuring fluorescence intensity using ImageJ software (E, n=10, *p<0.05, **p<0.005, ANOVA). Haemaoxylin and Eosin stained kidney section (F) to identify a blood vessel to assess endothelial cell and vessel wall uptake of antagomir (G). Six hours after introduction of labelled antagomir formalin fixed sections were de-paraffanised and counterstained with DAPI. Images H & 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 Image I & K. After cold perfusion (Image J & K) labelled antagomir was seen in blood vessel walls and the glomerulus, however, staining was not seen in aquaporin 1 positive tubular cell.

Figure 3: Tracking Antagomir uptake through intracellular compartments during ex vivo NMP. Upper panel – images depict typical confocal microscopy cross-sectional images of kidney tubules (multiple intensity projection, z-stacks) which were used in colocalisation analysis from each time point (0, 2, 4, & 6 hour 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 & green colocalisation that was used in the quantification. The graph demonstrates the quantification of co-occurrence using Manders co-efficient during the NMP timeline. **Row A** – colocalisation of ASO with early endosome marker Rab5 in a timeline of deconvolved images of tubules taken throughout NMP (0, 2, 4, & 6 hour). **Row B** –colocalisation of ASO with late endosome marker Rab7 throughout the perfusion timeline. **Row C** –colocalisation of ASO with RISC marker GW182 throughout the perfusion timeline. **Row D** - depict colocalisation of ASO with lysosome marker LAMP2 throughout the perfusion timeline. **Lower Panel:** Colocalisation 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 hours 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** – depicts representative images of the scramble sequence oligonucleotide and its degree of colocalisation with the different intracellular compartments following 6 hours of NMP treatment. The graph depicts quantification of co-occurrence of scramble sequence oligonucleotide with intracellular compartments. **Row F** – depicts representative images from the hypothermic machine perfused kidneys that were treated for 6 hours with the ASO and the degree of colocalisation with the intracellular compartments. The graph quantifies this and demonstrates there was very minimal colocalisation of the ASO with any of intracellular compartments during HMP.

Figure 4. Antagomir binding miR-24-3p target during NMP - In situ hybridisation with Dig-labelled anti-sense 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 (E) and control sense probe on the same tissue is shown in (F). Staining using antisense probe after 6 hours ex vivo NMP with random sequence oligonucleotide (G) and with miR-24-3p antagomir (H).

Figure 5: Evaluation of the expression of mir-24-3p targets

Gene expression was assessed in kidneys prior to (0 hrs) and at 1 and 6 hours of perfusion with miR-24-3p antogomir (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 hours perfusion is shown in panels B,D,F and H. *P>0.05, **P<0.005, unpaired t-test)

Figure 6: Diagram depicting proposed mechanism for antagomir uptake and trafficking interaction with miR-24 during *ex vivo* NMP resulting in preservation of mRNA targets

Supplementary figure 1: Impact of antagomir and scramble therapy on renal physiology and perfusate cytokine concentrations.

Renal blood flow and mean arterial pressure where recorded at serial time points throughout NMP. antagomir (red) or scramble sequence oligonucleotide (blue); n= 5 per group, repeated measures two-way ANOVA. There was no significant difference in urine output after the first 1 hour of NMP between antagomir and scramble treated kidneys; n=5 per group, t-test Cytokine concentrations was measured in the perfusate using a MesoScale Discovery multiplex array at multiple timepoints during perfusion of kidneys with antagomir (red) or scramble sequence oligonucleotide (blue); n= 5 per group. Concentrations were normalised to the weight of the kidney. There was no significant difference in cytokine concentration between the two treatment cohorts, repeated measures two-way ANOVA.

Table captions

Table 1: Summary of clinical donor characteristics in antagomir and scramble treated kidneys.

Table 2: MicroRNAs implicated in ischaemia reperfusion injury also identified in profiling studies

Supplementary Table 1: Detailed information of all kidneys studied

Supplementary Table 2: Antibody optimization procotol

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Disclosures

The authors declare no conflicts of interest.

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