Gene Silencing With Small Interfering RNA

Thijssen, Max F.; Bruggenwirth, Isabel M. A.; Gillooly, Andrew; Khvorova, Anastasia; Kowalik, Timothy F.; Martins, Paulo N.

Published in:
Liver Transplantation

DOI:
10.1002/lt.25383

Publication date:
2019

Gene Silencing With siRNA (RNA Interference): A New Therapeutic Option During Ex Vivo Machine Liver Perfusion Preservation

Max F. Thijssen,[1] Isabel M. A. Brüggenwirth ,[2] Andrew Gillooly,[1] Anastasia Khvorova,[3] Timothy F. Kowalik,[4] and Paulo N. Martins[1]

[1]Department of Surgery, Division of Organ Transplantation, UMass Memorial Medical Center, University of Massachusetts, Worcester, MA; [2]Department of Surgery, Section of Hepato-Pancreato-Biliary Surgery and Liver Transplantation, University Medical Center Groningen, Groningen, the Netherlands; [3]RNA Institute, University of Massachusetts Medical School, Worcester, MA; and [4]Department of Microbiology and Physiological Systems, University of Massachusetts Medical School, Worcester, MA

RNA interference (RNAi) is a natural process of posttranscriptional gene regulation that has raised a lot of attention culminating with the Nobel Prize in Medicine in 2006. RNAi-based therapeutics have been tested in experimental transplantation to reduce ischemia/reperfusion injury (IRI) with success. Modulation of genes of the innate immune system, as well as apoptotic genes, and those involved in the nuclear factor kappa B pathways can reduce liver injury in rodent liver pedicle clamping and transplantation models of IRI. However, in vivo use of RNAi faces limitations regarding the method of administration, uptake, selectivity, and stability. Machine perfusion preservation, a more recent alternative approach for liver preservation showing superior results to static cold preservation, could be used as a platform for gene interference therapeutics. Our group was the first to demonstrate uptake of small interfering RNA (siRNA) during liver machine preservation under both normothermic and hypothermic perfusion. Administering siRNA in the perfusion solution during ex vivo machine preservation has several advantages, including more efficient delivery, lower doses and cost-saving, and none/fewer side effects to other organs. Recently, the first RNAi drug was approved by the US Food and Drug Administration for clinical use, opening a new avenue for new drugs with different clinical applications. RNAi has the potential to have transformational therapeutic applications in several areas of medicine including transplantation. We believe that machine preservation offers great potential to be the ideal delivery method of siRNA to the liver graft, and future studies should be initiated to improve the clinical applicability of RNAi in solid organ transplantation.

Liver Transplantation 25 140–151 2019 AASLD.
Received September 26, 2018; accepted October 11, 2018.

Ischemia/reperfusion injury (IRI), especially in extended criteria donors (ECDs), has been associated with a high discard rate and more complications after liver transplant.[1,2] One of the pathophysiological mechanisms leading to the increased risk for developing complications after transplantation is susceptibility for IRI. IRI is a dynamic process that amplifies cellular damage in the newly transplanted organ by recruitment of inflammatory cytokines, production of reactive oxygen species, neutrophil accumulation, and complement activation.[3] The cascade of processes that occurs in IRI can result in inflammation, apoptosis, and necrosis.[4] The concept of interrupting these IRI cascades has been proposed as a way to minimize organ injury during liver transplantation. Several approaches have been described to minimize IRI, such as preventing the activation of neutrophils or Kupffer cells (KCs), reducing the generation of reactive oxygen species, and minimizing programmed cell death.[5]

Abbreviations: AGO2, argonaute 2 protein; ALT, alanine aminotransferase; BUN, blood urea nitrogen; c3, Complement 3; Chol., cholesterol; Cy3, Cy3 dye; cas3, caspase 3; cas8, caspase 8; DAPI, 4′,6-diamidino-2-phenylindole; DCA, docosanoic acid; DHA, docosahexaenoic acid; ECD, extended criteria donor; EPA, eicosapentaenoic acid; GalNAc, N-acetylgalactosamine; HGMB1, high mobility group box 1; HMP, hypothermic machine perfusion; HTK, histidine tryptophan ketoglutarate; IFIT, interferon-induced proteins with tetratriopeptide; IFN, interferon; IL, interleukin; IRF, interferon regulatory factor; IRI, ischemia/reperfusion injury; IV, intravenous; KC, Kupffer cell; LA, lithocholic acid; mRNA, messenger RNA; miRNA, microRNA; MIP2, macrophage inflammatory protein 2; MDA, malondialdehyde; MPO,

More recently, research has been carried out to study the potential effects of RNA interference (RNAi) on IRI during liver transplantation. Discovered in 1998, RNAi is a natural occurring mechanism to down-regulate gene expression by specifically targeting messenger RNA (mRNA) transcripts. RNAi was transformational and opened a new avenue for targeted therapeutic approaches culminating with the Nobel Prize in Medicine in 2006 for Andrew Fire and Craig Mello.[6] Consequently, several genes that have been identified as mediators in the pathophysiological pathway of IRI have been silenced by this technique.[7-13]

Genetic ex vivo modification of the liver is an underused option. RNAi appears to be promising in alleviating IRI and has the potential to improve postoperative graft survival. Developments in the chemistry of oligonucleotides offer more selective therapeutics for targeting the liver.[14]

myeloperoxidase; NALP3, NACHT domain, leucine-rich repeat domain and pyrin domain containing protein 3; NF-κB, nuclear factor kappa B; NGAL, neutrophil gelatinase-associated lipocalin; NMP, normothermic machine perfusion; PC, phosphocholine head group; PC-DCA, phosphocholine+docosahexaenoic acid; PC-EPA, posphocholine+eicosapentaenoic acid; PC-Chol; phosphocholine+cholesterol; PCSK9, proprotein convertase subtilisin-kexin type 9; PC-LA, phosphocholine+lithocholic acid; PC-RA, phosphocholine+retinoic acid; PC-TS, phosphocholine+α-tocopheryl succinate; PMNs, polymorphonuclear neutrophils; pre-miRNA, precursor microRNA; pri-miRNA, primary microRNA; RA, retinoic acid; RBC, red blood cell; RISC, RNA-induced silencing complex; RNAi, RNA interference; RNase, ribonuclease; shRNA, short-hairpin RNA; SCr, serum creatinine; siRNA, small interfering RNA; TID, renal tubulointerstitial damage; TLR4, toll-like receptor 4; TTR, transthyretin; TNF-α, tumor necrosis factor α; TNFR1, tumor necrosis factor receptor; TS, α-tocopheryl succinate; UW, University of Wisconsin.

Address reprint requests to Paulo N. Martins, M.D., Ph.D., F.A.S.T., F.A.C.S., F.E.B.S., Department of Surgery, Division of Organ Transplantation, UMass Memorial Medical Center, University of Massachusetts, University Campus, 55 Lake Avenue North, Worcester, MA 01655. Telephone: 508-334-2023; FAX: 508-856-1102; E-mail: paulo.martins@umassmemorial.org

Paulo N. Martins is supported by a UMass Faculty Development Grant and American Association for the Study of Liver Diseases Career in Transplantation Grant.

Anastasia Khvorova consults for Advinc and has intellectual property rights with UMass. Timothy F. Kowalik has stock ownership or equity of more than $10,000 with TATT, LLC, and has intellectual property rights with Moderna and AbViro.

View this article online at wileyonlinelibrary.com.

DOI 10.1002/lt.25383

In previous studies, IRI was attenuated by systemically injecting (donor) animals up to 48 hours prior to transplantation or clamping experiments.[9-11,13] For obvious reasons, this method of administration, in which human donors would be injected with RNAi therapeutics several hours before procurement would be clinically inapplicable. Therefore, administration of RNAi therapeutics with machine preservation can offer its beneficial effects by silencing IRI-associated pathways and prove to develop into a clinical applicable platform for administration of RNAi therapeutics in transplantation. Also, costs, which limit the applicability of RNAi, may be reduced because the treatment dosage used in machine preservation administration is based on the weight of the liver instead of the weight of the whole donor.

In this review, we examine the current status of RNAi in alleviating IRI to improve liver graft quality. We review RNAi gene targeting strategies of experimental studies in the liver and kidney. Finally, we discuss future challenges of RNAi and propose a novel technique using ex vivo machine preservation to improve the clinical applicability of RNAi in liver transplantation.

Gene Silencing With RNAi

RNAi is a mechanism of gene regulation mediated by a family of ribonucleoprotein complexes called RNA-induced silencing complex (RISC), which can be programmed to target any specific gene. Small regulatory RNA molecules assemble into RISC and guide the complex to complimentary bind to target mRNA transcripts.[15] Once the RISC is loaded with the small regulatory RNA, it can silence the target gene by several distinct mechanisms, including translational repression and degradation of mRNA with imperfect complementarity and sequence specific cleavage of perfectly complementarily mRNA.[16]

The small regulatory genes that guide RISC have similar sounding names and are categorized based on the biosynthetic pathway of the small RNA or the type of RISC in which the small RNA is found. Even though the exact molecular composition of RISC is not defined, proteins of the Argonaute family are required within the complex to bind and cleave target mRNA.[15]

Small interfering RNA (siRNA), microRNA (miRNA), and short-hairpin RNA (shRNA) are all small regulatory molecules that act within the system of RNAi. The biosynthetic pathway and the types of

TABLE 1. Overview of Differences in miRNA, shRNA, and siRNA

Small RNA molecule	Origin		Type of Binding to Target mRNA	Duration of Silencing	Type of Gene Silencing
	Location	Source			
siRNA (Synthetic)	Cytoplasm	Exogenous	Perfect/near perfect Watson-Crick base pairing	Transient	Sequence specific cleavage (AGO2)
shRNA	Nucleus	Exogenous (expression vector)	Perfect/near perfect Watson-Crick base pairing	Long term	Sequence specific cleavage (AGO2)
siRNA (originating from shRNA)	Cytoplasm	Endogenous			
miRNA	Nucleus	Endogenous	Limited complementarity	Not available (natural mechanism of gene regulation)	Translational repression and/or degradation

RISC they use differ. Differences between small regulatory molecules (siRNA, miRNA, and shRNA) and silencing mechanisms are outlined in Table 1. The mechanism of RNAi by siRNA, miRNA, and shRNA is shown pictorially in Fig. 1.

For siRNAs, the initiation event of the RNAi pathway is cleavage of double-stranded RNA into siRNA by an enzyme called dicer.[17] The siRNAs are double-stranded, 20-25–nucleotide-long RNA molecules with 5'-phosphorylation and 2-base 3' overhangs. The guide strand of the siRNA can directly incorporate in RISC and complementarily bind and cleave target mRNA with a perfect match.[18] Naturally, the siRNA originates from double-stranded RNA. However, siRNA can also be chemically synthesized and administered to the cell or organism to induce gene silencing, through the RISC machinery, after entering the cytoplasm.[16]

The shRNAs are synthesized within the nucleus by DNA vector-mediated production.[19] In the nucleus, polymerase II or III transcribes the shRNA from an external expression vector bearing a double-stranded DNA sequence with a hairpin loop.[18] The shRNA is then processed by Drosha, a ribonuclease (RNase) III endonuclease, and transported to the cytoplasm. Here, in the cytoplasm, the resulting shRNA is processed to siRNA by dicer. Subsequently, the siRNA integrates within RISC and complementarily binds target mRNA. Silencing with siRNA is transient, whereas silencing with shRNA that is constitutively expressed by promotors can result in longterm gene silencing.[16]

The miRNAs are a class of single-stranded RNAs of approximately 22 nucleotides (ranging 19~25 nucleotides) and are generated in the nucleus from endogenous transcripts. The miRNAs act as guide molecules within RISC and can lead to posttranscriptional repression and/or mRNA cleavage to down-regulate gene expression.[20] The endogenous transcripts from which the miRNA originate is known as primary microRNA (pri-miRNA). In the nucleus, the pri-miRNA is processed into a 60~70-nucleotide-long precursor microRNA (pre-miRNA) by a complex consisting of Drosha, a RNase III family enzyme, and DiGeorge syndrome chromosomal region 8, a protein that assists in positioning of Drosha.[21,22] The pre-miRNA is transported to the cytoplasm by the double-stranded RNA-binding protein exportin 5. In the cytoplasm, the pre-miRNA is processed into 22-nucleotide-long miRNA duplexes by dicer, which trims down the double-stranded RNA molecule to allow RISC loading of the guide strand. Inside RISC, the miRNA may guide binding to target mRNA by imperfect complementarity and induce translational repression or mRNA degradation, thus silencing the gene.[16]

Advances in oligonucleotide chemistry and formulation resulted in recent US Food and Drug Administration approval of the first siRNA drug for treatment of liver diseases. Patisiran is a lipid nanoparticle containing partially modified siRNAs targeting transthyretin (TTR), and it is now approved for the treatment of TTR amyloidosis, a genetic disease commonly requiring liver transplantation. The clinical schedule requires administration every 3 weeks and has been proven to be safe and efficacious.[23] Further chemical advances resulted in the development of next-generation liver targeting siRNAs, like inclisiran, a low-density lipoprotein cholesterol-lowering drug, whose duration of effect exceeds 6-12 months following a single administration.[24] This siRNA, which targets the proprotein convertase subtilisin-kexin type 9 (PCSK9), provided a reduction of PCSK9 by 74.5% after a single 300-mg dose and up to 83.8% after multiple 300-mg doses, with limited side effects.[25]

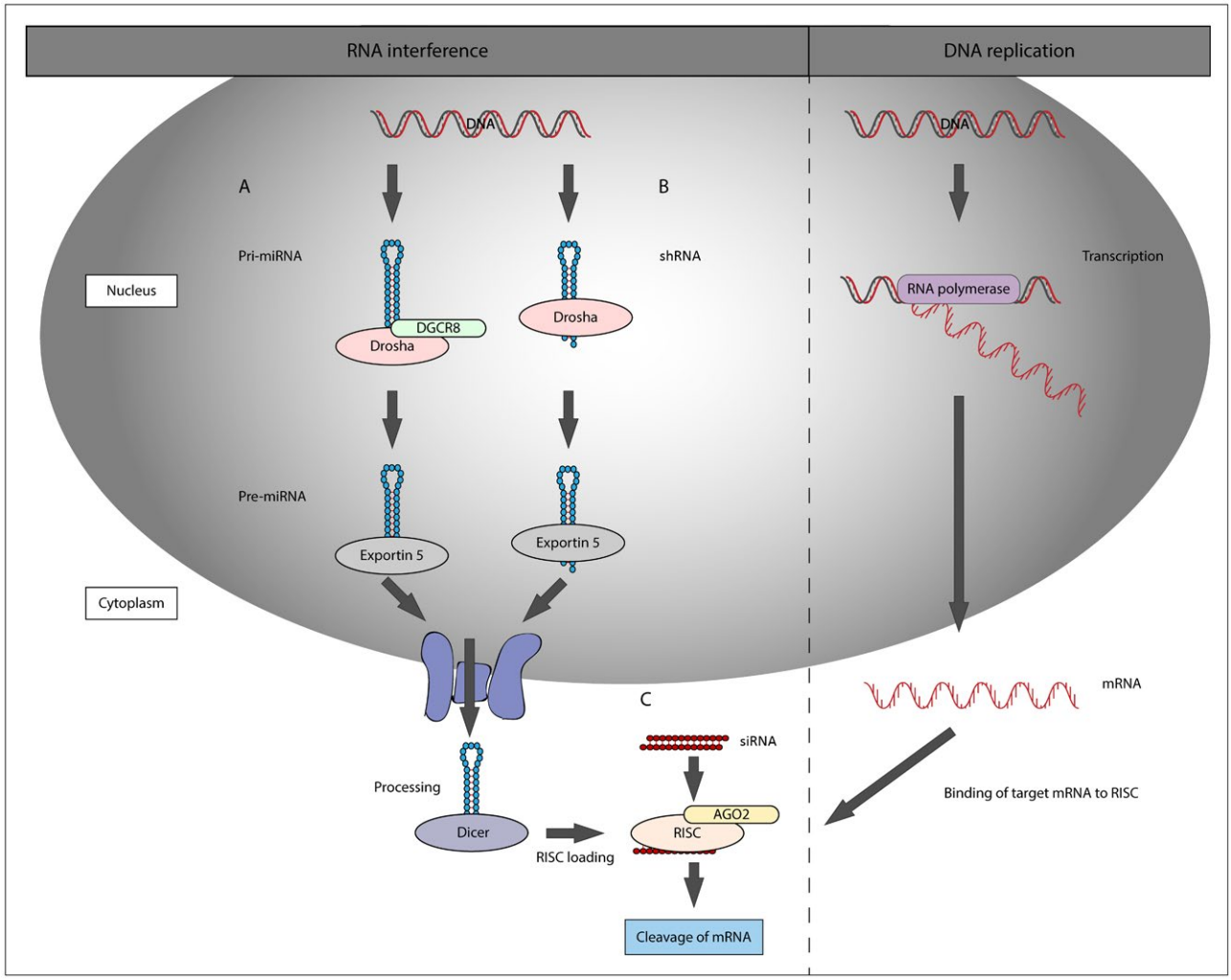

FIG. 1. Simplified mechanism of gene silencing by RNAi. (A) The pri-miRNA is transcribed from the DNA and processed by a complex containing the Drosha RNase III and DGCR8 into pre-miRNA. The complex is transported to the cytoplasm by the transport protein exportin 5; once inside the cytoplasm the pre-miRNA is processed by dicer into smaller fragments. The smaller fragments incorporate RISC and can partly bind to the target mRNA, leading to translational repression or degradation of the mRNA, silencing the gene. This pathway of RNAi occurs naturally in mammalian cells and regulates gene expression. (B) The shRNA undergoes similar processing as the miRNA in the nucleus but is artificially introduced to the cell or organism as plasmid or viral vector. The shRNA is transported to the cytoplasm by exportin 5. After processing by dicer, the steps of gene silencing are similar to silencing with siRNA, which includes RISC loading with AGO2 in the complex and perfectly matched complementary binding to the target mRNA and cleavage of the mRNA. (C) The exogenous (synthetic) siRNA can enter the cytoplasm and incorporate the RISC machinery to guide the complex through perfect matched complementary binding to the target mRNA. After binding, the mRNA is cleaved, leading to gene silencing. On the right side of the image, DNA replication with the production of target mRNA is shown.

Silencing Apoptotic Genes

Silencing apoptotic genes with RNAi can alleviate IRI in the liver. The beneficial effect of silencing apoptotic genes has been shown in liver pedicle clamping and transplantation models of both the liver and kidneys.[7,10,11] Several genes and receptors involved in the activation of apoptosis, including Fas, tumor necrosis factor receptor 1 (TNFR1), and caspases were tested in this context, with the studies and results outlined in Tables 2 and 3.

Fas (CD95) is a relevant cell-surface receptor that transduces death signals into the cell to induce apoptosis.[39] The Fas-mediated pathway of apoptosis can

TABLE 2. Overview of Translational Studies in the Liver Focusing on Alleviating IRI With RNAi

Species	Model	Type	Target	Time of Administration Before IRI and Route		Dose	Delivery Method and/or Chemical Modification	Outcome	Reference
Mice	Clamping	shRNA	TLR4	48 hours	Tail vein	50 μg	Expression vector, liposome nanoparticles	Best delivery to the liver, TLR4 expression↓, ALT↓, neutrophil infiltration and necrosis↓, MDA↓, ROS production↓, IL1 mRNA↓, TNF-α mRNA↓	Jiang et al.[9] (2011)
Mice	Clamping	shrna	NALP3	48 hours	HDI tail vein	50, 75, 100, and 150 μg	Expression vector	NALP3 mRNA↓, NALP3 protein↓, ALT↓, AST↓, apoptotic cells↓, IL1B↓, IL18↓, HMGB1 expression↓, TNF-α↓, IL6↓	Zhu et al.[13] (2011)
Mice	Clamping	shRNA	RelB	24 hours	HDI tail vein	50 μg	Expression vector	MPO↓, MDA↓, Inhibition in the decrease of superoxide dismutase, congestion↓, vacuolisation↓, massive necrosis↓, reduced expression of TNF-α	Luo et al.[8] (2012)
Rats	Transplant	shRNA	IRAK-4	6 hours storage/after anastomosis	Perfusion/ storage/ injection	n/a	UW solution	ALT↓, AST↓, attenuation histological damage, IRAK4 mRNA↓, IRAK4 protein↓, Nf-kB activity↓, TNF-α↓, IL6↓, IL1B↓	Wu et al.[12] (2016)
Mice	Clamping	siRNA	Cas3, cas8	1 hour	Portal vein	0.5 nmol	Lipiodol	Cas8 and cas3 expression 60%↓, cas8 and cas3 activity↓, apoptosis↓, PNM infiltration↓, cas3: ALT↓ and survival 50%↑, cas8: ALT↓ and survival 30%↑	Contreras et al.[7] (2004)
Rats	Transplant	siRNA	Fas	48 hours	HDI penile vein	200 μg	n/a	ALT↓, apoptotic index↓, Fas mRNA↓, Fas expression↓	Li et al.[11] (2007)
Mice	Clamping	shRNA	TNF-α	48 hours	HDI tail vein	50 μg	Expression vector	TNF-α expression↓, ALT↓, AST↓, congestion↓, vacuolisation↓, MDA↓, PMNs infiltration↓	Hernandez-Alejandro et al.[10] (2012)

TABLE 3. Overview of Translational Studies in the Kidney Focusing on Alleviating IRI With RNAi

Species	Model	Type	Target	Time of Administration Before IRI and Route		Dose	Delivery Method and/or Chemical Modification	Outcome	References
Pig	Auto transplant	Modified siRNA	Cas3	24 hours	Renal artery flushed, I.V. systemic injection	0.3 mg flushed, 0.9 mg injected	Locked nucleic acid	Cas3 mRNA and protein↓, apoptotic cells↓, MPO+↓, IL1B↓, IL6↓, NF-κB↓, IFNα↓, INFγ↓, IFNβ↓, IRF3↓, IRF7↓, IFIT1 mRNA↓, SCR↓, urea nitrogen↓, TID score↓, HMGB1 expression↓	Yang et al.[26] (2013)
Mice	Clamping	shRNA	Cas3, cas8	48 hours	HDI tail vein	50 μg	Expression vector	Cas3 and cas8 mRNA↓, cas3 and cas8 protein↓, BUN↓, SCr↓, survival↑ (cas3 and cas8 combined), necrosis↓, vacuolization↓, neutrophil infiltration↓	Zhang et al.[27] (2006)
Mice	Clamping	shRNA	C3, cas3	48 hours	HDI tail vein	50 μg	Expression vector	Cas3 and C3 mRNA↓, BUN↓, SCr↓, survival↑, tubular infarction↓, vacuolization↓, cast formation↓, neutrophil infiltration↓	Zheng et al.[28] (2006)
Mice	Clamping	shRNA	Fas, cas8	48 hours	HDI IVC	150 μg	Expression vector	BUN↓, SCr↓, survival↑, apoptotic cells↓, tubular cast formation↓, tubular necrosis↓	Du et al.[29] (2006)
Mice	Transplant	siRNA	C3, RelB, Fas	Directly and 4, 6, 12 hours	Renal artery and storage	100 μg each	Storage in HTK solution	BUN↓, SCr↓, survival↑, apoptosis↓, C3, Fas and RelB protein↓, C3, Fas and Relb mRNA↓, IL6↓, TNF-α↓, pathological damage score↓	Zheng et al.[30] (2016)
Rats	Transplant	siRNA	p53	15 minutes before/4 hours after	IV injection	12 mg/kg	2′-O methylation	SCr↓, necrotic and apoptotic cells↓, RBC flow rate↑, expression of p53 proteins↓	Imamura et al.[31] (2010)
Mice	Clamping	shRNA	p53	Directly after IRI	Left renal artery	0.33 μg, 3.3 μg	Expression vector, cationic polymer	Apoptosis↓, p53 protein↓, SCr↓, NGAL mRNA↓, improved histology, GSK-3b expression↓	Fujino et al.[32] (2013)
Mice	Clamping	shRNA	TNF-α	48 hours	HDI tail vein	50 μg	Expression vector	TNF-α mRNA↓, BUN↓, SCr↓, improved histology; infarction↓, cast formarion↓, neutrophil infiltration↓, survival↑	Hou et al.[33] (2016)
Rats	Clamping	siRNA	p53	2 hours, 30 minutes before; 4 hours, 8 hours after	Injection, femoral or juglar vein	1-4 mg/kg	2′-O methylation	SCr↓, attenuation of histological tubular and medullary injury, p53 protein↓, PUMA protein↓, MDM2 protein↓, protection against crisplatin-induced injury	Molitoris et al.[34] (2009)
Mice	Clamping	shRNA	RelB	48 hours	HDI tail vein	50 μg	Expression vector	RelB mRNA↓, BUN↓, SCr↓, attenuation pathological changes, neutrophil infiltration↓, Immunohistochemistry: TNF-α↓, IL1↓, survival↑	Feng et al.[35] (2009)
Mice	Clamping	shRNA	C3	48 hours	HDI tail vein	50 μg	Expression vector	C3 mRNA↓, C9 deposition↓, BUN↓, SCr↓, survival↑, significant attenuation of pathological changes, TNF-α↓	Zheng et al.[36] (2006)
Mice	Clamping	shRNA	C5a receptor	48 hours	HDI tail vein	50 μg	Expression vector	C5a mRNa↓, BUN↓, SCr↓, survival↑, significant attenuation of pathlogical changes, TNF-α mRNA↓, MIP2 mRNA↓, KC mRNA↓	Zheng et al.[37] (2008)
Rats	Clamping	siRNA	IKKb	48 hours	HDI left renal artery	50 μg	Naked	IKKB mRNA↓, IKKB protein↓, BUN↓, SCr↓, macrofage infiltration↓, Nf-κB activation↓, NGAL expression↓, IL18 expression ↓	Wan et al.[38] (2011)

be activated by Fas ligand. Hepatocytes express a high number of Fas receptors and are susceptible for Fas-mediated apoptosis. A study by Li et al., using a rat transplantation model, silenced Fas in the liver.[11] As a result, Fas gene expression was suppressed, and IRI was attenuated by inhibiting hepatocyte apoptosis. The effect of the therapy was assessed by a relative reduction in alanine aminotransferase (ALT), decreased apoptotic index, and a reduction in Fas mRNA levels in rat liver.[11]

Tumor necrosis factor α (TNF-α) plays a crucial role in the regulation of the inflammatory response during IRI. In addition, TNF-α can cause hepatocyte apoptosis by prolonged c-Jun N-terminal kinase activation.[40] Hernandez-Alejandro et al. used a mouse liver clamping model to test the effects of silencing TNF-α with shRNA. The authors observed lower peak ALT, less histological damage, decreased neutrophil infiltration and lower tissue malondialdehyde levels compared with mouse livers treated with scramble shRNA.[10]

Upon activation, both Fas and TNFR1 recruit a protein complex that consists of adapter proteins and procaspases. Proteases also play a role in activating apoptosis and can dismantle cells by sequential activation and cleavage of key proteins. In the downstream pathway of Fas and TNF-α, the proteases caspase 3 (cas3) and caspase 8 (cas8) can be activated, which lead to cell death.[7,41]

In a study by Contreras et al., cas3 and cas8 were silenced with siRNA in a rodent liver clamping model.[7] The cas3 and cas8 siRNA were injected through the portal vein 1 hour before clamping both the portal vein and the hepatic artery. Cas3 and cas8 gene silencing was validated in vivo by measuring mRNA using reverse-transcription polymerase chain reaction. The authors demonstrated that intraportal administration of siRNA silenced cas3 and cas8 and attenuated IRI. Damage was assessed by measuring ALT, apoptosis, histopathological features, and polymorphonuclear neutrophils (PMNs), which were all reduced in the siRNA-treated mice. In a model of prolonged ischemia, where the warm ischemia time of the liver was extended to 75 minutes, survival rates improved in the cas3-treated mice (50%) and cas8-treated mice (30%).[7] Cas3 silencing has also been achieved after infusing kidneys with University of Wisconsin solution dosed with cas3 siRNA into the renal artery. After 48 hours of cold preservation, siRNA administration aggravated renal tissue damage. However, the kidneys were not protected after transplant, which might be because of systemic complementary responses.[26]

Silencing Genes of the Nuclear Factor Kappa B Pathway and the Innate Immune System

Silencing genes of the nuclear factor kappa B (NF-κB) pathway and innate immune system attenuated IRI in the liver.[8,9,12,13] In a study by Luo et al.,[8] transcription factor RelB, a protein of the NF-κB family, was silenced. Proteins of the NF-κB family have been implicated to play a role in the development of IRI, and silencing RelB in the kidney diminished IRI.[35] Another study in kidney transplantation showed that intra-arterial perfusion of mouse donor kidneys with siRNA solution knocked down expression of complement 3, RelB, and Fas. Consequently, levels of blood urea nitrogen (BUN) and serum creatinine were reduced, fewer histopathological changes and apoptotic cells were observed, and graft survival was prolonged compared with control groups.[30] Silencing RelB in the liver mitigated IRI and led to a decrease in the generation of reactive oxygen species. In addition, histopathological liver damage and expression of TNF-α were reduced.[8] Comparable results were achieved in a study by Zhu et al. In this study, NACHT domain, leucine-rich repeat domain and pyrin domain containing protein 3 (NALP3) inflammasome-signaling pathway was blocked. A protective effect was associated with reduced production of inflammatory cytokines and reduced production of HMGB1, an important toll-like receptor ligand.[13] Inflammatory injury by NF-κB gene activation is also involved in the progression of vascular stenosis. A study in rats showed less neointimal formation and fewer proliferating cell nuclear antigen cells in vein grafts treated with NF-κB siRNA.[42]

Hurdles of RNAi Therapeutics

There are several limitations associated with the in vivo use of RNAi. The main limitations are related to the method of administration, uptake, stability, and selectivity. Naked siRNA is unstable in the blood and is degraded rapidly by serum nucleases.[43] The in vivo stability of siRNAs has been optimized by chemically modifying the RNA backbone, where advanced clinical compounds are fully modified with no RNA left.

Complex modification patterns have been evolved that contain a combination of phosphorothioates, 2′-*O*-methyl RNA and 2′-fluoro RNA.[14] In addition, the 5' phosphate of the guide strand can be stabilized with a variety of chemical entities, including 5'-(E) vinylphosphonate, which further enhance stability. These modification patterns enable multimonth activity in vivo, do not interfere with RISC complex assembly, and enhance the half-life of the loaded RISC complex.[14,44]

To effectively induce gene silencing, siRNA must enter the cytoplasm of the target cell. The negatively charged nature of the siRNA molecule is proven to be a hurdle in crossing the cellular membrane. In addition, the siRNA complex must navigate through the circulatory system and avoid kidney filtration.[45] To enhance cellular uptake and selectivity, liposomes, polymers, nanoparticles, and conjugated modifications have been used as delivery systems.[45,46]

Development of multivalent *N*-acetylgalactosamine (GalNAc) has revolutionized the field of liver siRNA delivery. Conjugation of trivalent GalNAc to fully chemically stabilized siRNAs enables efficient hepatocyte delivery both in vitro and in vivo.[47] GalNAc internalization is based on recognition by asialoglycoprotein receptors overexpressed in hepatocytes. This strategy should be amenable for modulation of gene expression in liver transplants ex vivo. In early variants of the same concept, toll-like receptor 4 (TLR4), a signaling component of the innate immune system, was silenced with galactose-modified, liposomal formulater siRNA. Silencing TLR4 resulted in attenuation of IRI.[9]

Machine Preservation: a New Ex Vivo Delivery System of RNAi Therapeutics

Machine preservation is an exciting means to improve liver graft quality prior to transplantation. Machine perfusion is currently undergoing clinical trials, with the technology offering advantages to the gold standard of simple cold storage.[48,49] Machine preservation of ECD livers has been shown to improve outcomes after liver transplantation. Specifically, hypothermic machine perfusion (HMP) reduces peribiliary gland damage in treated donation after circulatory death livers versus controls.[50] However, at this time, perfusion at cold temperatures lacks a reliable method to assess the energy (adenosine triphosphate) status of the liver,[51] and a consensus regarding a biomarker profile of useable HMP livers has not been reached.[52] At normothermic perfusion temperatures, even with extended preservation times, a marked decrease (~50%) in peak ALT was observed in treated livers compared with static cold storage grafts.[53] In addition to HMP, normothermic machine perfusion (NMP) allows for viability assessment of the organ prior to

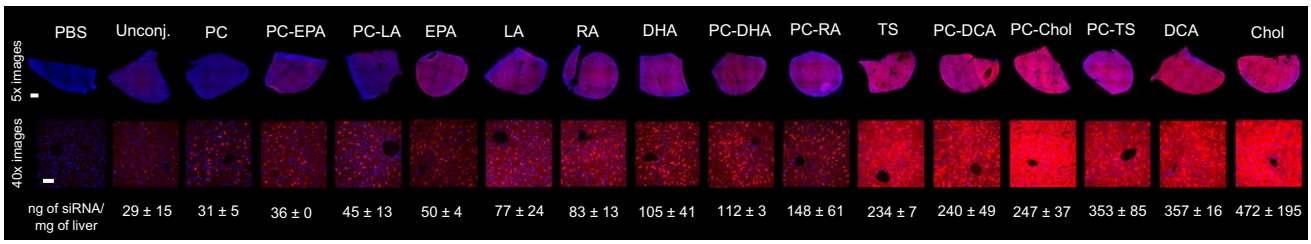

FIG. 2. Distribution of various Cy3-conjugated siRNAs in the mouse liver. Mice were injected subcutatenously with respective 3′ lipid-conjugated siRNAs at 20 mg/kg. Tissue was collected 48 hours after injection. Nuclei is visualized with DAPI in blue; siRNA is visualized in red via 5′ Cy3 fluorophore. Scale bars: 1 mm at 5×; 50 μm at 40×. Quantification of siRNA in liver tissue was measured by PNA hybridization assay. Lipid conjugate key: PC (phosphocholine head group), RA (retinoic acid), PC-RA (phosphocholine + retinoic acid), DHA (docosahexaenoic acid), PC-DCA (phosphocholine + docosahexaenoic acid), TS (α-tocopheryl succinate), PC-TS (phosphocholine + α-tocopheryl succinate), EPA (eicosapentaenoic acid), PC-EPA (posphocholine + eicosapentaenoic acid), DCA (docosanoic acid), PC-DCA (phosphocholine + docosanoic acid), LA (lithocholic acid), PC-LA (phosphocholine + lithocholic acid), Chol. (cholesterol), and PC-Chol (phosphocholine + cholesterol).

TABLE 4. Proposed Advantages and Limitations of siRNA Administration Methods in Transplantation

Administration Method	Machine Preservation	Injection	Explanation
Advantages	Organ-specific	Simpler	Perfused organ targeted only instead of systemically targeting all organs
	Cheaper		Less adverse effects to other organs
	Administration method clinically more applicable		Dose is dependent of the organ weight instead of the body weight
			Start siRNA treatment after procurement versus pretreating donors
Disadvantages	Requires machine preservation expertise	Systemically	Application requires machine preservation expertise
		More expensive	
		Off-target effects	

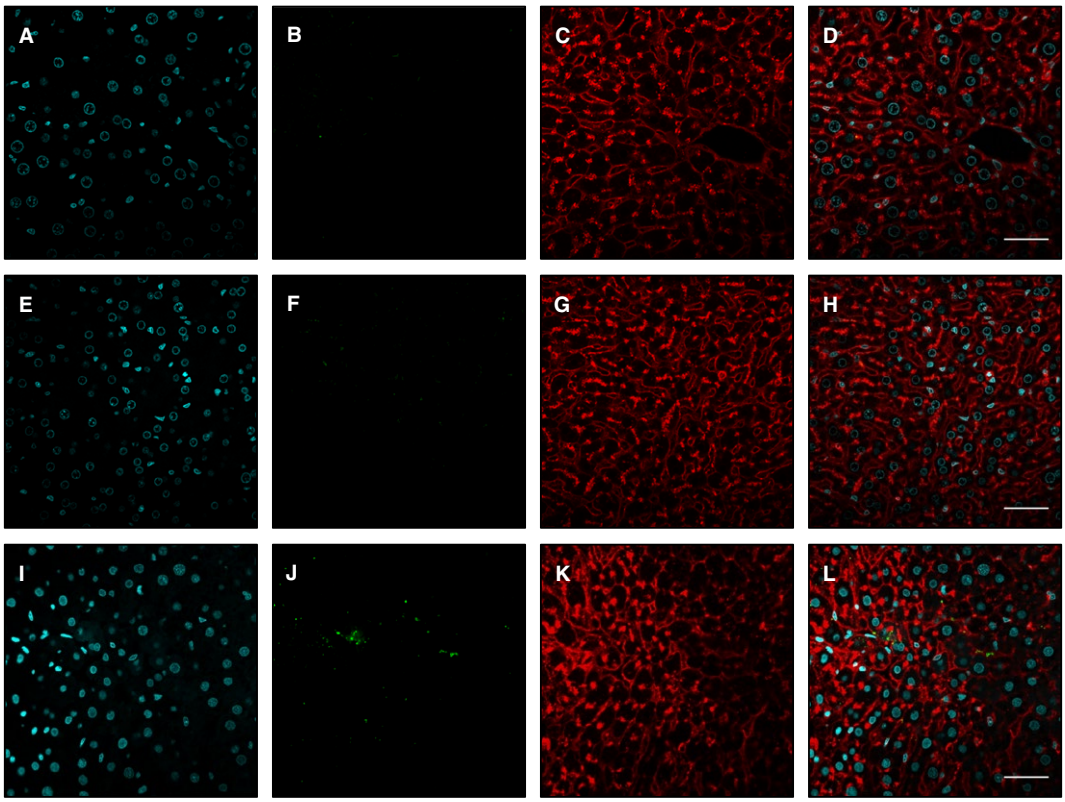

FIG. 3. Alexa Fluor–conjugated p53 siRNA (1 mg/kg of liver) is uptaken by rat hepatocytes during NMP with Williams' E medium. Scale represents 20 μm. (A)-(D) Untreated liver (no lipofectamine or siRNA) before machine perfusion. (A) Nuclei visualized in blue with DAPI, (B) siRNA in green, (C) cell membranes in red with wheat germ agglutinin conjugated to Alexa Fluor 647, and (D) with a merged image. (E)-(H) Liver treated with lipofectamine nanoparticles alone (no siRNA) and perfused for 2 hours at 37°C. (I)-(L) Liver treated with lipofectamine with p53 siRNA and perfused for 2 hours at 37°C.

transplantation. Hemodynamic, synthetic, and metabolic parameters can be used to assess the viability of the graft.[49,54] Administering chemically stabilized siRNA (2'O-methylation) leads to cellular uptake of the siRNA by the hepatocytes.[55] In vivo uptake can be enhanced even further by encapsulating siRNA in lipid-based nanoparticles or by using lipid-based delivery reagents.[9,56] GalNAc-conjugated siRNA, which drives receptor-mediated uptake by hepatocytes, is another promising candidate for delivery with machine perfusion to the liver. GalNAc-conjugated siRNAs are fully modified using an alternating 2'-fluoro, 2'-O-methylation pattern to improve stability.[47,57,58] Other 3' lipid modifications to siRNA can drastically change distribution patterns in liver uptake, with the most lipophilic capable of uniformly penetrating mouse

liver tissue without interrupting the siRNA's ability to interact with RISC machinery (Fig. 2). By selectively changing the siRNA backbone, a stable and targeted molecule can be synthesized specifically for delivery to liver grafts during the perfusion period.

Ex vivo graft therapy has the advantage of reducing adverse effects related to systemic therapy. Other major advantages of machine perfusion with siRNA are outlined in Table 4. Recently, research from our group reported for the first time the use of siRNA during machine preservation. We have shown that 21 base pair siRNAs can be successfully delivered to rat liver grafts during machine perfusion directly from the perfusate solution, both at hypothermic (4°C) and normothermic (37°C) temperatures. To accomplish this, we used siRNA together with lipid-based nanoparticles in the rat model to silence the apoptotic gene p53.[59,60] Figure 3 shows confocal microscopy images of p53 siRNA uptake by the liver during NMP. A summary of the potential advantages and disadvantages is demonstrated in Table 4.

In conclusion, increasing evidence exists that supports the proposition that RNAi-based therapies can improve transplantation success. SiRNA delivery with machine perfusion may be especially beneficial for improving the viability of ECD organs by alleviating IRI. We believe that machine preservation offers great potential for the delivery of siRNA to the liver. Not only livers, but all other organs that can be preserved by machine perfusion, eg, kidneys, pancreas, heart, lungs, could also benefit from this concept. We think that delivery by machine perfusion can improve target-cell specificity in a clinically more achievable administration platform for RNAi therapeutics. RNAi during ex vivo machine preservation would not only be important to increase the graft tolerance to IRI, but it could target genes to decrease the risk of rejection (eg, major histocompatibility complex antigens), induce tolerance, or prevent viral infections (eg, hepatitis C virus). Experimental research should provide proof whether this concept is viable, and future studies should be initiated to improve the clinical applicability of RNAi therapy in solid organ transplantation.

REFERENCES

1) Pezzati D, Ghinolfi D, De Simone P, Balzano E, Filipponi F. Strategies to optimize the use of marginal donors in liver transplantation. World J Hepatol 2015;7:2636-2647.
2) Fondevila C, Hessheimer AJ, Ruiz A, Calatayud D, Ferrer J, Charco R, et al. Liver transplant using donors after unexpected cardiac death: novel preservation protocol and acceptance criteria. Am J Transplant 2007;7:1849-1855.
3) Abu-Amara M, Yang SY, Tapuria N, Fuller B, Davidson B, Seifalian A. Liver ischemia/reperfusion injury: processes in inflammatory networks–a review. Liver Transpl 2010;16:1016-1032.
4) Lopez-Neblina F, Toledo AH, Toledo-Pereyra LH. Molecular biology of apoptosis in ischemia and reperfusion. J Invest Surg 2005;18:335-350.
5) Peralta C, Jiménez-Castro MB, Gracia-Sancho J. Hepatic ischemia and reperfusion injury: effects on the liver sinusoidal milieu. J Hepatol 2013;59:1094-1106.
6) Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC. Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. Nature 1998;391:806-811.
7) Contreras JL, Vilatoba M, Eckstein C, Bilbao G, Anthony Thompson J, Eckhoff DE. Caspase-8 and caspase-3 small interfering RNA decreases ischemia/reperfusion injury to the liver in mice. Surgery 2004;136:390-400.
8) Luo L, Lu J, Li WC, Shan J, Li FS, Long D, et al. RNA interference targeting RelB attenuates liver ischemia/reperfusion injury. J Surg Res 2012;178:898-906.
9) Jiang N, Zhang X, Zheng X, Chen D, Zhang Y, Siu LK, et al. Targeted gene silencing of TLR4 using liposomal nanoparticles for preventing liver ischemia reperfusion injury. Am J Transplant 2011;11:1835-1844.
10) Hernandez-Alejandro R, Zhang X, Croome KP, Zheng X, Parfitt J, Chen D, et al. Reduction of liver ischemia reperfusion injury by silencing of TNF-alpha gene with shRNA. J Surg Res 2012;176:614-620.
11) Li X, Zhang JF, Lu MQ, Yang Y, Xu C, Li H, et al. Alleviation of ischemia-reperfusion injury in rat liver transplantation by induction of small interference RNA targeting Fas. Langenbecks Arch Surg 2007;392:345-351.
12) Wu Y, Liu Y, Li M, Liu Z, Gong J. IRAK-4-shRNA prevents ischemia/reperfusion injury via different perfusion periods through the portal vein after liver transplantation in rat. Transplant Proc 2016;48:2803-2808.
13) Zhu P, Duan L, Chen J, Xiong A, Xu Q, Zhang H, et al. Gene silencing of NALP3 protects against liver ischemia-reperfusion injury in mice. Hum Gene Ther 2011;22:853-864.
14) Khvorova A, Watts JK. The chemical evolution of oligonucleotide therapies of clinical utility. Nat Biotechnol 2017;35:238-248.
15) Pratt AJ, MacRae IJ. The RNA-induced silencing complex: a versatile gene-silencing machine. J Biol Chem 2009;284:17897-17901.
16) Burnett JC, Rossi JJ. RNA-based therapeutics: current progress and future prospects. Chem Biol 2012;19:60-71.
17) Rand TA, Petersen S, Du F, Wang X. Argonaute2 cleaves the anti-guide strand of siRNA during RISC activation. Cell 2005;123:621-629.
18) Deng Y, Wang CC, Choy KW, Du Q, Chen J, Wang Q, et al. Therapeutic potentials of gene silencing by RNA interference: principles, challenges, and new strategies. Gene 2014;538:217-227.
19) Moore CB, Guthrie EH, Huang MT, Taxman DJ. Short hairpin RNA (shRNA): design, delivery, and assessment of gene knockdown. Methods Mol Biol 2010;629:141-158.
20) Han J, Lee Y, Yeom KH, Kim YK, Jin H, Kim VN. The Drosha-DGCR20 complex in primary microRNA processing. Genes Dev 2004;18:3016-3027.
21) Kim D, Rossi J. RNAi mechanisms and applications. Biotechniques 2008;44:613-616.

22) Yeom KH, Lee Y, Han J, Suh MR, Kim VN. Characterization of DGCR22/Pasha, the essential cofactor for Drosha in primary miRNA processing. Nucleic Acids Res 2006;34:4622-4629.
23) Adams D, Gonzalez-Duarte A, O'Riordan WD, Yang CC, Ueda M, Kristen AV, et al. Patisiran, an RNAi therapeutic, for hereditary transthyretin amyloidosis. N Engl J Med 2018;379:11-21.
24) Khvorova A. Oligonucleotide therapeutics - a new class of cholesterol-lowering drugs. N Engl J Med 2017;376:4-7.
25) Fitzgerald K, White S, Borodovsky A, Bettencourt BR, Strahs A, Clausen V, et al. A highly durable RNAi therapeutic inhibitor of PCSK9. N Engl J Med 2017;376:41-51.
26) Yang C, Jia Y, Zhao T, Xue Y, Zhao Z, Zhang J, et al. Naked caspase 3 small interfering RNA is effective in cold preservation but not in autotransplantation of porcine kidneys. J Surg Res 2013;181:342-354.
27) Zhang X, Zheng X, Sun H, Feng B, Chen G, Vladau C, et al. Prevention of renal ischemic injury by silencing the expression of renal caspase 3 and caspase 8. Transplantation 2006;82:1728-1732.
28) Zheng X, Zhang X, Sun H, Feng B, Li M, Chen G, et al. Protection of renal ischemia injury using combination gene silencing of complement 3 and caspase 3 genes. Transplantation 2006;82:1781-1786.
29) Du C, Wang S, Diao H, Guan Q, Zhong R, Jevnikar AM. Increasing resistance of tubular epithelial cells to apoptosis by shRNA therapy ameliorates renal ischemia-reperfusion injury. Am J Transplant 2006;6:2256-2267.
30) Zheng X, Zang G, Jiang J, He W, Johnston NJ, Ling H, et al. Attenuating ischemia-reperfusion injury in kidney transplantation by perfusing donor organs with siRNA cocktail solution. Transplantation 2016;100:743-752.
31) Imamura R, Isaka Y, Sandoval RM, Ori A, Adamsky S, Feinstein E, et al. Intravital two-photon microscopy assessment of renal protection efficacy of siRNA for p53 in experimental rat kidney transplantation models. Cell Transplant 2010;19:1659-1670.
32) Fujino T, Muhib S, Sato N, Hasebe N. Silencing of p53 RNA through transarterial delivery ameliorates renal tubular injury and downregulates GSK-3beta expression after ischemia-reperfusion injury. Am J Physiol Renal Physiol 2013;305:F1617-F1627.
33) Hou L, Chen G, Feng B, Zhang XS, Zheng XF, Xiang Y, et al. Small interfering RNA targeting TNF-alpha gene significantly attenuates renal ischemia-reperfusion injury in mice. J Huazhong Univ Sci Technolog Med Sci 2016;36:634-638.
34) Molitoris BA, Dagher PC, Sandoval RM, Campos SB, Ashush H, Fridman E, et al. siRNA targeted to p53 attenuates ischemic and cisplatin-induced acute kidney injury. J Am Soc Nephrol 2009;20:1754-1764.
35) Feng B, Chen G, Zheng X, Sun H, Zhang X, Zhang ZX, et al. Small interfering RNA targeting RelB protects against renal ischemia-reperfusion injury. Transplantation 2009;87:1283-1289.
36) Zheng X, Feng B, Chen G, Zhang X, Li M, Sun H, et al. Preventing renal ischemia-reperfusion injury using small interfering RNA by targeting complement 3 gene. Am J Transplant 2006;6:2099-2108.
37) Zheng X, Zhang X, Feng B, Sun H, Suzuki M, Ichim T, et al. Gene silencing of complement C5a receptor using siRNA for preventing ischemia/reperfusion injury. Am J Pathol 2008;173:973-980.
38) Wan X, Fan L, Hu B, Yang J, Li X, Chen X, Cao C. Small interfering RNA targeting IKKbeta prevents renal ischemia-reperfusion injury in rats. Am J Physiol Renal Physiol 2011;300:F857-F863.
39) Cursio R, Filippa N, Miele C, Colosetti P, Auberger P, Van Obberghen E, Gugenheim J. Fas ligand expression following normothermic liver ischemia-reperfusion. J Surg Res 2005;125:30-36.
40) Schlayer HJ, Laaff H, Peters T, Woort-Menker M, Estler HC, Karck U, et al. Involvement of tumor necrosis factor in endotoxin-triggered neutrophil adherence to sinusoidal endothelial cells of mouse liver and its modulation in acute phase. J Hepatol 1988;7:239-249.
41) Elmore S. Apoptosis: a review of programmed cell death. Toxicol Pathol 2007;35:495-516.
42) Meng XB, Bi XL, Zhao HL, Feng JB, Zhang JP, Song GM, et al. Small interfering RNA targeting nuclear factor kappa B to prevent vein graft stenosis in rat models. Transplant Proc 2013;45:2553-2558.
43) Layzer JM, McCaffrey AP, Tanner AK, Huang Z, Kay MA, Sullenger BA. In vivo activity of nuclease-resistant siRNAs. RNA 2004;10:766-771.
44) Aagaard L, Rossi JJ. RNAi therapeutics: principles, prospects and challenges. Adv Drug Deliv Rev 2007;59:75-86.
45) Whitehead KA, Langer R, Anderson DG. Knocking down barriers: advances in siRNA delivery. Nat Rev Drug Discov 2009;8:129-138.
46) Wang J, Lu Z, Wientjes MG, Au JL. Delivery of siRNA therapeutics: barriers and carriers. AAPS J 2010;12:492-503.
47) Nair JK, Willoughby JL, Chan A, Charisse K, Alam MR, Wang Q, et al. Multivalent *N*-acetylgalactosamine-conjugated siRNA localizes in hepatocytes and elicits robust RNAi-mediated gene silencing. J Am Chem Soc 2014;136:16958-16961.
48) Selzner M, Goldaracena N, Echeverri J, Kaths JM, Linares I, Selzner N, et al. Normothermic ex vivo liver perfusion using steen solution as perfusate for human liver transplantation: First North American results. Liver Transpl 2016;22:1501-1508.
49) Ravikumar R, Jassem W, Mergental H, Heaton N, Mirza D, Perera MT, et al. Liver transplantation after ex vivo normothermic machine preservation: a phase 1 (first-in-man) clinical trial. Am J Transplant 2016;16:1779-1787.
50) van Rijn R, van Leeuwen OB, Matton APM, Burlage LC, Wiersema-Buist J, van den Heuvel MC, et al. Hypothermic oxygenated machine perfusion reduces bile duct reperfusion injury after transplantation of donation after circulatory death livers. Liver Transpl 2018;24:655-664.
51) Schlegel A, Muller X, Dutkowski P. Hypothermic machine preservation of the liver: state of the art. Curr Transplant Rep 2018;5:93-102.
52) Quillin RC 3rd, Guarrera JV. Hypothermic machine perfusion in liver transplantation. Liver Transpl 2018;24:276-281.
53) Nasralla D, Coussios CC, Mergental H, Akhtar MZ, Butler AJ, Ceresa CDL, et al. for Consortium for Organ Preservation in Europe. A randomized trial of normothermic preservation in liver transplantation. Nature 2018;557:50-56.
54) Marecki H, Bozorgzadeh A, Porte RJ, Leuvenink HG, Uygun K, Martins PN. Liver ex situ machine perfusion preservation: a review of the methodology and results of large animal studies and clinical trials. Liver Transpl 2017;23:679-695.
55) Czauderna F, Fechtner M, Dames S, Aygün H, Klippel A, Pronk GJ, et al. Structural variations and stabilising modifications of synthetic siRNAs in mammalian cells. Nucleic Acids Res 2003;31:2705-2716.
56) Eguchi A, De Mollerat Du Jeu X, Johnson CD, Nektaria A, Feldstein AE. Liver bid suppression for treatment of fibrosis associated with non-alcoholic steatohepatitis. J Hepatol 2016;64:699-707.
57) Hassler MR, Turanov AA, Alterman JF, Haraszti RA, Coles AH, Osborn MF, et al. Comparison of partially and fully

chemically-modified siRNA in conjugate-mediated delivery in vivo. Nucleic Acids Res 2018;46:2185-2196.
58) Stavchansky S, Tung IL. Effects of hypothermia on drug absorption. Pharm Res 1987;4:248-250.
59) Thijssen MF, Moore CG, Xiaofei E, Wang X, Mandrekar P, Bozorgzadeh A, et al. Silencing p53 pathway of apoptosis alleviates ischemia-reperfusion injury (IRI) in the liver. J Am Coll Surg 2017;225(suppl 2):E167.
60) Moore C, Thijssen M, Wang X, Mandrekar P, Xiaofei E, Abdi R, et al. Gene silencing with p53 si-RNA downregulates inflammatory markers in the liver: potential utilization during normothermic machine preservation. Am J Transplant 2017;17(suppl 3).