Published in final edited form as:

Transplantation. 2013 November 27; 96(10): 926–934. doi:10.1097/TP.0b013e3182a20398.

Regulation of Molecular Pathways in Ischemia Reperfusion Injury after Liver Transplantation

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Abstract

Background—Ischemia/reperfusion (I/R) injury is a multifactorial phenomenon that occurs during the transplant event and frequently compromise early graft function after liver transplantation (LT). Current comprehension of molecular mechanisms and regulation processes of I/R injury lacks clarity. MicroRNA (miRNA) regulation results critical in several biological processes. **Patients and Methods:** This study evaluated gene expression and microRNA (miRNA) expression profiles using microarrays in 34 graft biopsies collected at pre-implantation (L1) and at 90 minutes post-reperfusion (L2) from consecutives deceased donor LT recipients. MiRNA profiles were first analyzed. Data integration analysis (gene expression / microRNA expression) aimed to identify potential target genes for each identified miRNA from the L1/L2 differential gene expression profile.

Results—Pairwise comparison analyses identified 40 miRNAs and 3,168 significantly differentially expressed genes at post-reperfusion time compared with pre-reperfusion time. Pathway analysis of miRNAs associated these profiles with anti-apoptosis, inhibition of cellular proliferation, and pro-inflammatory processes. Target analysis identified a miRNA-associated molecular profile of 2,172 genes involved in cellular growth and proliferation modulation by cell

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Author Contributions

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Writing of the paper: RCG, VRM, DGM

Performance of the research: RCG, JLS, SL, DGM

Contributed new reagents or analytic tools: DEL, DGM

Data analysis: RCG, VRM, CID

Conflicts of Interest

The authors declare no conflict of interest

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cycle regulation, cell death and survival, and pro- and anti-inflammatory processes. MiRNA-independent genes involved pro-inflammatory molecules.

Conclusion—We identified a miRNA profile involved in post-transcriptional regulatory mechanisms in I/R injury post-LT. A better understanding of these molecular processes involved in I/R may contribute to develop new strategies to minimize graft injury.

Keywords

Ischemia reperfusion injury; liver transplantation; microRNA; gene expression; microarray

INTRODUCTION

Liver transplantation (LT) is a recognized lifesaving treatment for patients with end stage liver disease (1,2). As result of recent progress in immunosuppression management, the indications and need of graft for transplantation had increased (2). Besides significant advances in LT outcomes, the management of factors associated with organ preservation, and reperfusion still remain critical (3). Further understanding of molecular pathways and post-transcriptional regulators involved in ischemia/reperfusion (I/R) injury might lead to biomarkers discovery, and novel therapeutic interventions to minimize graft injury.

The multifactorial I/R injury phenomenon is known to be initiated by oxygen deprivation (ischemia) and further exacerbate after blood flow restoration (reperfusion). Severity of I/R injury is associated with graft function post-LT (3-5). Extended cold and warm ischemia times (CIT and WIT, respectively) increase the risk of severe I/R injury and subsequent graft dysfunction (6-9). Also I/R injury effects seems to be less tolerated on graft from extended criteria donors (ECD) and donor after cardiac death (DCD) (10,11). Additionally, another important donor factors associated with I/R injury severity include donor age (> 60-years-old), pressors requirements, hypernatremia, prolonged intensity care stay, African-American race, and graft steatosis (3,7,9,12).

Oxygen deprivation and metabolite reduction during CIT induce mitochondrial dysfunction and triggers a decrease in adenosine 5'-triphosphate (ATP) synthesis resulting in failure of ATP-dependent enzymes. Graft microvascular integrity is the first compromised component of the cascade. Sinusoidal endothelial cells (SEC) are primarily affected by intracellular ion Ca^{2+} accumulation associated with Ca^{2+} -ATPase failure (5,13-15). Hepatocytes are preferentially affected during WIT after $\text{Na}^{+}/\text{K}^{+}$ -ATP dependent pump failure producing cellular edema and apoptosis (5,14).

Ischemic tissue damage is further exacerbated during reperfusion in a two-phase process (4). Post-reperfusion tissue damage results from the activation of innate immune mechanisms in the graft (16). Kupffer cells are the principal tissue damage inducer within 2 hours post-reperfusion (early phase). Upon activation through toll-like receptor 4 (TLR-4) signaling and complement system activated Kupffer cells induce oxidative stress and pro-inflammatory molecules after releasing reactive oxygen species (ROS) and cytokines (TNF- α , IL-1, and IL-6), respectively. SEC cells are also a simultaneous source of TNF- α and

IL-10. This pro-inflammatory scenario provokes activated leukocyte (mainly neutrophils) infiltration and platelet adhesion post reperfusion (late phase) (4,5,14,15,17).

MicroRNAs (miRNAs) demonstrated to be key post-transcriptional regulators of several biological processes and further deregulated in diseases and malignancies (15,16). The current knowledge of miRNAs role in I/R injury has been preferentially studied in myocardial ischemia and associated with angiogenesis and cardiomyocytes apoptosis inhibition (18). Furthermore, additional studies also proposed miRNA-associated protective roles for I/R injury in small intestine epithelial cells (19,20), proximal tubular kidney cells (21), and brain neurons (22). In the recent years, miRNAs had also been investigated and found to be key regulators in human pathophysiological processes of the liver physiology and pathology (23). This characteristic along with high molecular stability and availability turns miRNAs as very valuable biomarkers in transplantation. However, the role of miRNAs and the impact in gene expression regulation occurring in liver I/R injury remain scarce (24). The present study aimed to identify molecular pathways and regulatory effectors associated with graft I/R injury early post-LT.

RESULTS

Study cohort

Demographic and clinico-pathological characteristics of the study cohort for LT recipients, donors, and graft are detailed in Table 1. Hepatitis C virus (HCV) cirrhosis, alcohol abuse, or in combination were principal LT indications (64.8%). Donor population was also mostly represented by Caucasian/male, overweight, negative viral serology, with a mean CIT < 8 hours, mean WIT < 45 minutes, and minimal (10%) graft steatosis (Table 1).

Molecular profiling post-reperfusion

Molecular profiling using microarrays was performed in paired liver biopsy at pre-implantation (L1) and at early post-reperfusion (L2) times to identify 1) miRNA and gene expression tissue profiles, and 2) Data integration to identify target genes post-transcriptionally regulated by this miRNA profile (Figure 1).

Pairwise comparisons were performed between L1 vs. L2 samples individually for miRNAs and genes (34 of each). From miRNA microarrays, 40 miRNAs (21 up-regulated and 19 down-regulated) were identified significantly differentially expressed post-LT. From the total group of LT patients, 80% (32/40) of miRNAs were identified with FDR < 10%. MiRNAs miR-4484, miR-451a, miR-1246, and miR-486-5p were identified with FDR < 1% after restricting criteria (Table 2).

Gene expression microarray analysis identified a total of 3,895 probesets, representing 3,168 mapped genes, significantly differentially expressed when comparing L1 vs. L2 biopsy samples. From the expression profile analysis only one-third (965/3,168) of genes were found up-regulated while the major percentage (69.5%; 2,203/3,168 genes) revealed negative regulation after reperfusion.

Biological characterization of identified miRNAs

Ontology and pathway analyses were performed to determine the biological relevance of the differentially expressed I/R injury-associated miRNAs post-LT using IPA tool. A set of 25 miRNAs were found to exert specific cellular and molecular functions. None clear biological roles have been established yet for the remaining 15 miRNAs further corroborated by examination of published reports (Table 2). Interestingly, two associated network functions incorporated 21 out of 25 miRNAs with proven biological function. The top scored network (score: 22), including 11 miRNAs associated molecules, related to development of malignancy processes (*HER2/ERBB2*, *TP53*), proliferative disorders (*RBI*), and tissue response to hypoxia (*HIF1A*) (Supplementary Figure S1A). The second network (score: 21), incorporating 10 miRNAs, associated with inflammatory processes (*ABCB1*) and connective tissue development (*P4HA2*) as top biological functions (Supplementary Figure S1B).

Molecular and cellular functional analyses were performed in accordance with miRNAs expression trends to propose their functional roles post-LT. The expression of 14 miRNAs is known to be regulated by oxygen level stimuli accordingly responded to hypoxia (miR-139-5p, miR-451a, miR-16-5p, miR-21-5p, miR-223-3p, miR-27a-5p), ischemia preconditioning (miR-100-5p, miR-331-3p, miR-125b-5p, miR-21-5p, miR-27b), and non-hepatic ischemia reperfusion (miR-29-b-2-5p, miR-30c-5p, miR-192-3p). Interestingly, molecular function analysis suggested a critical role of those miRNAs in cell cycle progression, cellular proliferation, and apoptosis regulation as primary homeostatic mechanisms as well as inflammation and fibrogenesis in response to I/R-associated tissue injury. Indeed, expression levels of 5 miRNAs (miR: 100-5p, 378a-5p and 3p, 486-5p, and 532-3p) were in concordance with p53 predicted activation (Figure 2). Expression levels of miR-451a, miR-125-5p, and miR-185-5p predicted proliferation inhibition through cell cycle arrest. Similarly, the expression analysis of miR-21, miR-30c, miR-16, miR-100, miR-125, and miR-331, which were associated with liver malignances and hyper-proliferative events, denoted a non-proliferative cellular profile at early post-reperfusion. In combination with anti-proliferative profiles, the expression level of 6 miRNAs (miR: 331-3p, 378a-3p, 150-5p, 16-5p, 21-5p, and 192-3p) involved in programmed cell death regulation processes suggested anti-apoptosis.

A second group of miRNAs was found to be involved in inflammatory processes and fibrogenesis development. Post graft reperfusion up-regulation of miR-638, miR-185-5p, and miR-21 together with down-regulation of miR-150-5p and miR-30c-5p are in accordance with early inflammatory processes. In addition, expression trend analysis of miRNAs miR-150-5p, miR-30c-5p, and miR-21-5p along with miR-27b-5p, miR-29b-2p, and miR-192-3p suggested initial steps of fibrosis development.

Overall, the miRNA profile with proven biological role and identified early post graft reperfusion suggests: 1) pro-inflammation (5/25 miRNAs; 20%), 2) negative regulation of apoptosis (6/25 miRNAs; 24%), 3) delay of cellular proliferation (9/25 miRNAs; 36%), and 4) potential fibrogenesis development in response to I/R injury-associated tissue damage

(6/25 miRNAs; 24%). Some miRNAs were found to be involved in more than one biological process as described above.

Identification of miRNA target genes at early graft I/R injury

Data integration analysis including miRNA and genomic profiles was aimed to determine I/R injury post-transcriptionally regulated molecular pathways early post-LT. Detection of mRNA:miRNA interactions were performed by filtering the identified genomic profile with predicted target genes for individual miRNAs. In total, 5,423 potential mRNA:miRNA interactions were identified (Table 2). This total number of mRNA:miRNA interactions represent 68.6% (2,172/3,168) of the identified differentially expressed genes, which 66.1% of those targeted of at least two miRNAs.

Molecular regulatory networks at early graft I/R injury

Gene ontology and pathway analysis was initially restricted to the miRNA-associated profile of 2,127 genes. Most significant cellular functions were related to cellular growth and proliferation (p -value range: $7.33\text{E-}36$ – $3.75\text{E-}05$), and cell death and survival (p -value range: $1.23\text{E-}34$ – $4.71\text{E-}05$), which both included the 68.4% of genes (34.2% each cellular function) of the analyzed profile. Specific cellular function and activation trend are listed in Supplementary Table S1.

Detailed analysis of most significant deregulated canonical pathways suggested cell cycle arrest and inhibition of cell proliferation. In particular, 296 genes (13.6%) were found to be involved in cell cycle regulation. Post-reperfusion molecular setting showed up-regulation for cell cycle arrest factors (p21^{CIP1}, p27^{KIP1}, FoxO1) and down-regulation of Cyclin D and Cyclin E encoding genes. Cellular proliferation regulatory factors c-Jun and c-Fos encoding genes were significantly up-regulated (Figure 2). Furthermore, encoding genes for anti-apoptotic factors Bcl-XL, BIM, and FLIP (Caspase 8 and FADD-like negative regulator) were found up-regulated early post-reperfusion. Pro-apoptotic factors PUMA and BAK encoding genes were down-regulated in contrast to up-regulation of Noxa (up-regulated by hypoxia) and Caspase-3 genes (Supplementary Figure S2). An illustration of the resulting integrative analysis is shown in the Supplementary Figure S3. The described expression profile may denote protective anti-apoptotic and cell survival mechanisms against cellular insults. In addition, the pathway activation analysis in hepatic parenchymal cells also revealed prevalent anti-apoptosis of hepatocytes (z : -2.188).

Initial inflammatory processes were indicated by deregulation of pro- and anti-inflammatory molecular profiles. In total, 12% (255/2172) of genes were involved in inflammatory processes, 5% (116/2172) of genes in cell-mediated immune reaction, and 14% (314/2172) of genes were associated with immune diseases. Activation of NF- κ B signaling downstream genes included leukocyte adhesion molecules (*ICAM-1*), prostaglandin synthases (*COX2*), and inflammatory cytokines (*IL1 β* , *CXCL2*, and *SELE*). Up-regulated c-Jun/c-Fos AP1 members were also associated with pro-inflammation (Supplementary Figure S4A). Indeed, signaling predictive analysis postulated AP1-mediated transcriptional up-regulation of IL-2, IL-6, IL8, IFN- γ , and MMP1, as well as up-regulation of several NF- κ B complex-regulated

cytokines and chemokines (Supplementary Figure S4B). Also, a group of 292 (13.4%) genes were associated with connective tissue disorders.

Biological interpretation of miRNA-independent genes

Data integration analyses determined a total of 1014 genes predicted to be not post-transcriptionally regulated by the hereby identified miRNA profile associated with I/R injury. Molecular pathway analysis demonstrated association with hepatic parenchyma pro-inflammatory induction. The list of activated molecular and cellular functions related to recruitment, migration, and trafficking of innate immune cells are shown in Supplementary Table S2. Top significant canonical pathways and signaling cascades were found to be involved in innate immune response activation. Up-regulation of NF- κ B and its downstream transcriptionally regulated molecules including several cytokines and chemokines (IL-6, IL-8, CCL2, CCL3, CCL4, and CXCL1), genes predicted inflammatory initiation through leukocyte (neutrophils) recruitment and trans-epithelial migration of macrophages (Supplementary Figure S5). Interestingly, up-regulation of most those molecules was previously identified from the miRNA-associated molecular profile (Supplementary Figure S4B).

Real time PCR confirmation

The expression of 8 miRNAs and 10 genes selected following established criteria were tested in all samples by qPCR. Statistical significance and expression trends were comparable with microarray results (Table 3).

DISCUSSION

Liver graft injury and dysfunction early post-LT is a transient pathophysiological process surrounded by multiple deregulated molecular mechanisms. Initial innate inflammation exacerbates I/R injury severity deriving in irreversible hepatocyte dysfunction, biliary tree damage and even acute rejection episodes (5,10,11). Inflammatory and molecular mechanisms of cellular detriment are triggered by increased ROS release (25,26). This phenomenon seems to be more severe when factors such as donor quality and aging, extended cold/warm ischemia times are present (5-7).

Still the regulatory molecular mechanisms involved in I/R injury-post transplantation had not been further elucidated. From the best of our knowledge, this is the first integrative study that identified a miRNA set and its potential target genes (genomic profile) at early graft I/R injury in LT recipients. Independent molecular pathway analyses demonstrated two well-differentiated groups of cellular events during I/R process. The interaction between miRNA and gene expression profiles propose, 1) cell cycle arrest and reduced cellular proliferation which promote increased survival of hepatocytes though apoptosis of epithelial cells, and 2) innate inflammatory response through up-regulation of key transcription factors involved in positive regulation of cytokines encoding genes, as principal associated pathways.

The miRNA profile identified from the present study revealed negative regulatory effects in proliferation and apoptosis while positively involved in inflammatory processes leading to graft injury. Farid *et al* (27) demonstrated that high-abundant hepatocyte miRNAs miR-122,

miR-148a, and miR-194, but not miR-192, can be differentially expressed in response to liver injury severity after 1 hour post graft reperfusion. Moreover, it suggested miR-122 expression level as biomarker for acute cellular rejection. In comparison, from our miRNA profile those miRNAs remained unmodified except miR-192, which was identified and further confirmed by qPCR in our study set. Additionally, most of liver injury profiling studies in the literature, were only performed in animal models though without in depth biological exploration (28-31).

It is important the common identification of up-regulated miR-21 among those reports mainly from hepatic regeneration studies (29,30). It has been demonstrated that miR-21 plays an essential role in proliferation and anti-apoptosis of liver cells by AP-1 transcription factor complex mediated up-regulation (24,32). From our analysis, miR-21 was found up-regulated post graft reperfusion. Similarly, miR-223 was also up-regulated in L2 biopsies. In accordance with our results, similar findings about miR-223 were also encountered in I/R injury in a rodent model (33). Interestingly, both miRNAs associated with liver injury and regeneration belongs to the miRNA group regulated by ischemia events.

Multiple overlapped I/R injury molecular mechanisms lead to specific gene pathways deregulation. Conti *et al* (34) proposed genomic profiles associated with balanced apoptosis, cell cycle regulation, and innate inflammation post-reperfusion. This molecular study was performed in graft biopsy samples collected at liver procurement time and at 2-3 hours post-reperfusion. Despite different sample collection time points in comparison with our study there are extensive similarities between genomic profiles, which may indicate that the major genomic deregulation occur early but may be better identified at post-reperfusion time. In addition, Jassem *et al* (35) and Borozan *et al* (36) obtained similar molecular profiles associated with acute graft stress from deceased and living liver donors, respectively, demonstrating the basic nature of the phenomenon in liver transplantation. Early I/R injury mechanisms include inhibition of cell proliferation, balanced pro/anti-apoptosis, and initial inflammation (34,37). In the present study, the results support that those mechanisms may be post-transcriptionally regulated by miRNAs. The identified miRNA-associated genomic profile suggests cell cycle arrest and inhibition of cell proliferation through p21^{CIP1} and p27^{KIP1} cyclin-dependent kinase inhibitors (38). Importantly, up-regulation of AP-1 molecules c-Jun, JunD, JunB, c-Fos, FosB, and c-Fos-like 1/2 encoding genes were found associated to miRNAs. Its overexpression responds to deregulated redox-activity induced by hypoxia (39). Associated miRNAs included miR-16 (c-Jun, Fos-L1), miR-139 (JunB, c-Fos), miR-30d (c-Jun, JunB), miR-149 (JunB, JunD), miR-331 and miR-532 (Fos-L2). AP-1 critically inhibits cell cycle and apoptosis at I/R injury when c-Jun or JunD are present and differentially expressed (40,41). In addition, c-Jun N terminal Kinase (JNK) activity regulates AP-1 activation while its inhibition reduce c-Jun phosphorylation blocking AP-1, BAK expression, and caspase-3 activation therefore preventing necrosis and apoptosis (42). Hereby, the identified miRNA-associated profile are in concordance with the previously described mechanisms (Supplementary Figure S3), showing a latent apoptotic mechanisms to promote tissue repair.

Up-regulated pro-inflammatory cytokines and chemokines encoding genes preferentially via NF-κB signaling were not associated with miRNA regulation. NF-κB cascade activation

depends on TNF α and ROS releasing, complement system, and TLR signaling activation during ischemia time (43). Its activation might protect or induce tissue damage depending on ROS release directly correlated with tissue hypoxia extension (44). However, exacerbated gradient increment of CC/CXC pro-inflammatory chemokines induce recruitment and infiltration of macrophages and polymorphonuclear leukocytes (PMNs), predominately neutrophils, which interact with ICAM1 receptors on hepatocytes (16,45). From the analysis of our results, CC chemokines (CCL2, CCL3, CCL4) suggest to be independent regulated by miRNAs differing from most of CXC chemokines (CXCL2, CXCL3), prostaglandins, and ICAM1 molecule. MiRNA-dependent (IL-10) and independent (IL-6) regulated anti-inflammatory cytokines concur with previous reports and mostly respond to a protective effect of NF- κ B through IL-6 upregulation and increasing hepatocyte survival (16,34,44).

In conclusion, the present study proposes miRNA-mediated post-transcriptional regulation during early phase of I/R injury post-LT. A better understanding of these molecular mechanisms associated with I/R injury might provide insights for biomarker discovery and potential novel therapeutic targets.

PATIENTS AND METHODS

Study design

The Institutional Review Board at the University of Virginia approved the study (IRB-HSR# 15986). Written informed consents were obtained from all LT patients. The study included 17 consecutive LT patients who received grafts from deceased heart beating donors, and who did not present early surgical complications post-LT. Paired biopsy samples ($n = 34$) were collected from each subject at pre-implantation after CIT (L1) and at 90 minutes post-reperfusion (L2). Biopsy samples were collected in RNeasyTM reagent (Ambion Inc., Austin, TX, USA) and stored at -80°C .

RNA isolation and microarray hybridization

Total RNA extraction and quality control analyses were previously described (46). Gene expression AffymetrixTM HG-U133A v2.0 GeneChip[®] microarrays ($n = 34$) hybridization, quality control, and normalization were performed as reported previously (47). Total RNA treated with poly (A) tailing reaction followed by ligation of a biotinylated signal molecule was used for Affymetrix GeneChip[®] miRNA v3.0 microarrays ($n = 34$) hybridization, which assesses miRNAs from 153 organisms. It includes 1,733 mature human miRNAs (Sanger miRNA database v17) and human snoRNAs and scaRNAs derived from the snoRNABase and Ensembl Archive.

Microarray analysis

After hybridization each chip was scanned on an Affymetrix GeneChip[®] Scanner 3000 G7. Raw intensities for every probe were stored in electronic files (.DAT and .CEL formats) by the GeneChip[®] Operating Software (GCOS). Gene expression microarray statistical analyses were performed over the total number of 22,277 probesets and including control probesets to rule out significant differences between groups. For miRNA microarrays,

Wilcoxon Test was used for detection of present calls above intensity background. Quality control was evaluated using five different spike oligonucleotides, which was found comparable among miRNA microarrays (Supplementary Figure S6). Dataset was restricted to human probesets (1,733 hsa-miRNAs).

Independent pairwise comparisons (L1 vs. L2) were fit using two-sample *t*-test in the R programming environment (48). *P*-values ≤ 0.01 were considered significant. False discovery rates (FDR) were estimated using Benjamini and Hochberg method (49). A controlled FDR $< 1\%$ was considered significant. Fold-change values were used for differential expression magnitudes.

Data integration analysis

MiRNAs associated cellular functions as well as predicted target genes were obtained from Ingenuity Pathway Analysis (IPA) software database (www.ingenuity.com). Entrez-Pubmed (www.ncbi.nlm.nih.gov) bibliographic research was performed to corroborate absence of associated cellular function for specific miRNAs. Identified differentially expressed target genes were included in spreadsheets work lists and uploaded to IPA tool. IPA generated activation z-score (*z*) was used for molecular pathways interpretation. Briefly, it estimates the behavior and relationship among several scores to the calculated mean. Null z-score (*z* = 0) indicates similar statistical behavior. Positive or negative z-score indicate shifted trend from mean value.

Real time PCR

Differentially expressed molecules were tested in same RNA samples by Taqman® qPCR. Eight top significant miRNAs were selected based on proven biological role. Ten most significant genes from miRNA-associated (*n* = 5) and independent (*n* = 5) genomic profiles were also tested (Supplementary Table S3). Reverse transcription reactions were performed using TaqMan® miRNA and TaqMan® gene expression reverse transcription kits (Applied Biosystems, CA, USA). Expression analyses were performed using delta-Ct model (mean *Ct*_{target} - mean *Ct*_{standard}). Statistical significances were considered using a *p*-value < 0.05 .

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding Sources

The research results included in this report were supported by a National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) grant, R01DK080074.

ABBREVIATIONS

ALT	alanine aminotransferase
AST	aspartate aminotransferase

ATP	adenosine 5'-triphosphate
CIT	cold ischemia time
FDR	false discovery rate
HBV	hepatitis B virus
HCV	hepatitis C virus
IPA	ingenuity pathway analysis
I/R	ischemia/reperfusion
LT	liver transplantation
MiRNA	microRNA
PMN	polymorphonuclear cells
QPCR	quantitative polymerase chain reaction
ROS	reactive oxygen species
SEC	sinusoidal endothelial cells
TLR-4	toll-like receptor 4
WIT	warm ischemia time

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Figure 1. Schematic representation of the study design

The diagram responds to the reasoning flow from paired sample collection until data integration analysis. The abbreviation mRNA:miRNA means microRNA transcript target.

Figure 2. Cell cycle and cellular proliferation

Molecular pathway representing downstream deregulated molecules involved in regulation of cell cycle progression and cellular proliferation associated with IR injury at early post-LT. Symbols and lines color codes are as follows. Symbols: Red (up-regulation), Green (down-regulation), Orange (predicted activation), Blue (predicted inhibition), White (not present molecules). Lines: Orange line (leads to activation), Blue (leads to inhibition), Yellow (findings inconsistent with state of downstream molecule), Gray (effect not predicted).

Table 1

Demographics and clinical characteristics

Study variable	Parameter	Observation
Number of cases		17
Graft Recipients Demographics		
Age (years)	Mean $\pm$ SD (range)	55 $\pm$ 9 (24 – 67)
Gender (%)	Male	10 (58.8)
	Female	7 (41.2)
Race (%)	White	16 (94.1)
	Black African/American	1 (5.9)
Waiting list time (days)	Mean% $\pm$ SD (range)	316 $\pm$ 743 (3 – 3237)
MELD at listing	Mean% $\pm$ SD (range)	21.8 $\pm$ 9.4 (10 – 47)
MELD at LT	Mean% $\pm$ SD (range)	18.6 $\pm$ 8.1 (7 – 36)
LT indications	Alcohol abuse (ETOH), n (%)	5 (29.6)
	Hepatitis C (HCV) cirrhosis, n (%)	3 (17.6)
	ETOH - HCV, n (%)	3 (17.6)
	Cryptogenic cirrhosis, n (%)	3 (17.6)
	Other, n (%) *	3 (17.6)
Length of stay post-LT (days)	Mean% $\pm$ SD (range)	11.5 $\pm$ 8.5 (4 – 30)
Transaminases 1-week follow-up	ALT (UI/L), mean $\pm$ SD (range)	161 $\pm$ 113 (57 – 480)
	AST (UI/L), mean $\pm$ SD (range)	68 $\pm$ 51 (19 – 198)
Donor Demographics		
Age (years)	Mean $\pm$ SD (range)	47 $\pm$ 18 (16 – 73)
Gender (%)	Male	12 (70.6)
	Female	5 (29.4)
Race (%)	White	13 (76.5)
	Black African/American	3 (17.6)
	Other	1 (5.9)
Pressors (%)	Yes	13 (76.5)
	No	4 (23.5)
BMI (kg/m2)	Mean $\pm$ SD (range)	27.5 $\pm$ 4.6 (17.9 – 34.9)
ICU stay in days (Mean $\pm$ SD (range))	Declaration of death	2.5 $\pm$ 2.6 (0.5 – 9)
	To cross clamp	3.8 $\pm$ 2.5 (2 – 11)
Ventilation (days)	Mean $\pm$ SD (range)	2.9 $\pm$ 1.9 (2 – 9)
Serum Sodium (mEq/L)	Mean $\pm$ SD (range)	152 $\pm$ 9 (135 – 168)
ALT (UI/L)	Mean $\pm$ SD (range)	88 $\pm$ 113 (12 – 434)
AST (UI/L)	Mean $\pm$ SD (range)	104 $\pm$ 126 (17 – 477)
Total bilirubin	Mean $\pm$ SD (range)	1.2 $\pm$ 0.7 (0.6 – 2.9)
Cause of Death (%)	Stroke	7 (41.2)

Study variable	Parameter	Observation
	Head trauma	5 (29.4)
	Anoxia	5 (29.4)
Graft Preservation and Histology		
CIT (hours)	Mean $\pm$ SD (range)	6.2 $\pm$ 2.0 (4.6 – 12.7)
WIT (minutes)	Mean $\pm$ SD (range)	28.9 $\pm$ 9.3 (22 – 58)
Steatosis [†]	Mean% $\pm$ SD (range)	4.1 $\pm$ 3.6 (0 – 10)

MELD: model of end stage liver disease; BMI: Body Mass Index; SD: Standard deviation.

* Other indications for LT included: chronic hepatitis B (1 case), primary sclerosis cholangitis (1 case), and fasciola hepatica (1 case).

[†] Total steatosis was calculated by fat percentage observed from both hepatic lobes by either biopsy or visual estimation.

Table 2

MicroRNAs associated with graft ischemia reperfusion injury in liver transplantation

Probe set	miRNA ID	L2 vs. L1 (Fold change)	p-value	FDR (%)	Proposed biological role	mRNA:miRNA Interactions (n) *
hsa-miR-4484_st	miR-4484	↑ (4.0)	2.51E-07	0.04	No	37
hsa-miR-1246_st	miR-1246	↑ (3.0)	8.73E-06	0.6	No	46
hsa-miR-486-5p_st	miR-486-5p	↑ (6.7)	1.95E-05	0.9	Yes	93
hsa-miR-451_st	miR-451a	↑ (3.7)	3.59E-05	0.9	Yes	35
hsa-miR-27b-star_st	miR-27b-5p	↓ (1.4)	1.24E-04	2.2	Yes	2
hsa-miR-4532_st	miR-4532	↑ (1.8)	1.60E-04	2.3	No	192
hsa-miR-99a_st	miR-100-5p	↓ (1.2)	1.76E-04	2.3	Yes	45
hsa-miR-4668-5p_st	miR-4668-5p	↑ (1.8)	2.24E-04	2.4	No	157
hsa-miR-1280_st	miR-1280	↓ (1-5)	2.28E-04	2.4	No	163
hsa-miR-885-5p_st	miR-885-5p	↓ (1.3)	4.65E-04	3.3	Yes	54
hsa-miR-192-star_st	miR-192-3p	↓ (1.3)	4.74E-04	3.3	Yes	3
hsa-miR-1281_st	miR-1281	↓ (1.7)	5.39E-04	3.3	No	111
hsa-miR-331-3p_st	miR-331-3p	↓ (1.5)	5.53E-04	3.3	Yes	300
hsa-miR-3613-3p_st	miR-3613-3p	↑ (1.9)	6.08E-04	3.5	No	17
hsa-miR-4454_st	miR-4454	↓ (1.3)	7.10E-04	3.8	No	25
hsa-miR-139-5p_st	miR-139-5p	↓ (1.2)	7.59E-04	3.9	Yes	143
hsa-miR-223_st	miR-223-3p	↑ (2.0)	1.81E-03	6.3	Yes	140
hsa-miR-149-star_st	miR-149-3p	↑ (1.4)	1.89E-03	6.3	Yes	544
hsa-miR-28-3p_st	miR-28-3p	↓ (1.1)	1.89E-03	6.3	Yes	49
hsa-miR-1825_st	miR-1825	↓ (2.1)	1.90E-03	6.3	No	134
hsa-miR-532-3p_st	miR-532-3p	↓ (1.4)	2.18E-03	6.8	Yes	224
hsa-miR-150_st	miR-150-5p	↓ (1.4)	2.69E-03	7.6	Yes	187
hsa-miR-378-star_st	miR-378a-5p	↓ (1.3)	3.61E-03	9.3	Yes	1
hsa-miR-185_st	miR-185-5p	↑ (1.3)	3.82E-03	9.5	Yes	282
hsa-miR-638_st	miR-638	↑ (1.3)	3.92E-03	9.5	Yes	51
hsa-miR-4745-5p_st	miR-4481	↑ (1.4)	3.98E-03	9.5	No	178
hsa-miR-3197_st	miR-3197	↑ (1.8)	4.07E-03	9.6	No	92
hsa-miR-29b-2-star_st	miR-29b-2-5p	↓ (1.3)	4.17E-03	9.6	Yes	4
hsa-miR-378f_st	miR-378a-3p	↓ (1.2)	4.39E-03	9.6	Yes	154
hsa-miR-27a-star_st	miR-27a-5p	↑ (2.5)	4.75E-03	9.9	Yes	2
hsa-miR-4281_st	miR-4281	↑ (1.3)	4.82E-03	9.9	No	145
hsa-miR-497_st	miR-16-5p	↓ (1.1)	4.92E-03	9.9	Yes	425
hsa-miR-4449_st	miR-4449	↑ (1.5)	5.67E-03	10.6	No	85
hsa-miR-2861_st	miR-2861	↑ (1.3)	7.46E-03	11.5	Yes	291
hsa-miR-21_st	miR-21	↑ (1.2)	7.74E-03	11.8	Yes	114

Probe set	miRNA ID	L2 vs. L1 (Fold change)	p-value	FDR (%)	Proposed biological role	mRNA:miRNA Interactions (n) *
hsa-miR-4466_st	miR-4466	↑ (1.3)	7.86E-03	11.8	Yes	100
hsa-miR-4787-5p_st	miR-4787-5p	↑ (1.2)	8.40E-03	12.0	No	133
hsa-miR-125a-5p_st	miR-125a-5p	↓ (1.2)	8.76E-03	12.2	Yes	305
hsa-miR-30d_st	miR-30d	↓ (1.1)	9.04E-03	12.4	Yes	293
hsa-miR-3621_st	miR-3621	↑ (1.4)	9.57E-03	13.0	No	68

* mRNA:miRNA includes all potential direct and indirect interactions between the identified miRNA and gene expression profiles. (↑) Up-regulation; (↓) Down-regulation.

Table 3

Real time PCR confirmation analysis

Molecule ID	Microarray		Taqman qPCR	
	L2 vs. L1 (fold change)	<i>p</i> -value ([*])	L2 vs. L1 (fold change)	<i>p</i> -value
miRNAs				
miR-486	↑ (6.7)	1.9E-05	↑ (5.7)	9.7E-09
miR-451	↑ (3.7)	3.6E-05	↑ (4.3)	8.6E-07
miR-192 [*]	↓ (1.3)	5.0E-04	↓ (1.2)	1.7E-02
miR-27b [*]	↓ (1.4)	1.0E-04	↓ (1.3)	5.9E-03
miR-223	↑ (2.0)	1.8E-03	↑ (1.7)	3.8E-03
miR-331	↓ (1.5)	6.0E-04	↓ (1.2)	2.3E-02
miR-99a	↓ (1.2)	2.0E-04	↓ (1.2)	2.9E-02
miR-885	↓ (1.3)	5.0E-04	↓ (1.8)	7.5E-03
miRNA-associated genes				
ATF3	↑ (11.7)	7.8E-10	↑ (20.6)	2.9E-13
PTGS2	↑ (7.1)	2.7E-08	↑ (8.2)	2.6E-10
JUN	↑ (3.4)	2.5E-08	↑ (5.4)	8.1E-09
FOS	↑ (6.3)	1.8E-05	↑ (10.6)	2.6E-08
CLMN	↓ (2.5)	4.6E-03	↓ (1.6)	3.8E-02
Independent genes				
MYC	↑ (3.6)	2.4E-07	↑ (4.9)	1.1E-06
GADD45A	↑ (1.7)	1.4E-06	↑ (2.0)	3.2E-06
CCL3	↑ (5.2)	1.6E-08	↑ (5.3)	3.2E-07
IL-8	↑ (8.1)	8.0E-09	↑ (12.6)	4.4E-10
IL-6	↑ (2.5)	5.3E-06	↑ (24.7)	3.1E-10

^{*} *p*-values calculated as average from two or more probesets found representing one gene. From all cases, probeset expression trends were similar within the represented gene. (↑) Up-regulation; (↓) Down-regulation.