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Moesin Functionality in Hypothermic Liver Preservation Injury

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Abstract

The objective of this study was to determine how expression and functionality of the cytoskeletal linker protein moesin is involved in hepatic hypothermic preservation injury. Mouse livers were cold stored in University of Wisconsin (UW) solution and reperused on an Isolated Perfused Liver (IPL) device for one hour. Human hepatocytes (HepG2) and human or murine Sinusoidal Endothelial Cells (SECs) were cold stored and rewarmed to induce hypothermic preservation injury. The cells were transfected with: wild type moesin, an siRNA duplex specific for moesin, and the moesin mutants T558D and T558A. Tissue and cell moesin expression and its binding to actin were determined by western blot. Liver IPL functional outcomes deteriorated proportional to the length of cold storage, which correlated with moesin disassociation from the actin cytoskeleton. Cell viability (LDH and WST-8) in the cell models progressively declined with increasing preservation time, which also correlated with moesin disassociation. Transfection of a moesin containing plasmid or an siRNA duplex specific for moesin into HepG2 cells resulted in increased and decreased moesin expression, respectively. Overexpression of moesin protected while moesin knock-down potentiated preservation injury in the HepG2 cell model. Hepatocytes expressing the T558A (inactive) and T558D (active) moesin binding mutants demonstrated significantly more and less preservation injury, respectively. Cold storage time dependently caused hepatocyte detachment from the matrix and cell death, which was prevented by the T558D active moesin mutation. In conclusion, moesin is causally involved in hypothermic liver cell preservation injury through control of its active binding molecular functionality.

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Keywords

Cytoskeletal System; Plasma Membrane; Reperfusion Injury; Organ Donor; ERM; Liver Transplantation

Introduction

Liver transplantation continues to be the treatment of choice for patients with end stage liver disease. The limiting factor is the availability of suitable liver grafts. In 2012, 6,256 livers were transplanted in the US out of a pool of 16,446 (38%). Conversely, 62% or over 10,000 patients were left on the waiting list for a graft [9]. It has recently been shown that using liver grafts from extended criteria organ donors decreases the wait time without adversely affecting post-transplant survival [23]. However, as the quality of donor livers continues to decrease because we are selecting donors that were once considered unusable, significant improvements in organ preservation will be required to maintain adequate function in these grafts.

The amount of cold exposure during hypothermic liver preservation is directly related to the amount of preservation injury experienced by the graft after transplantation [7]. In fact, cold ischemia time is one of the single most important risk factor for liver preservation injury [22] and it causes later graft injury even when normal function returns shortly after liver transplantation [1]. Cold ischemia, which is proportional to graft preservation injury after transplantation [20;26], significantly sensitizes the hepatocyte cell membrane to rewarming injury. Furthermore, cold exposure during hypothermic preservation of livers causes sinusoidal endothelial cells (SEC) to die and detach from the peri-sinusoidal matrix plate. This exposes underlying tissue factor, which activates coagulation and promotes thrombosis leading to low flow and further ischemia at reperfusion [2;17]. Detachment and subsequent apoptotic cell death in hepatocytes is also dependent on $\beta 1$ integrin failure with the cell membrane [19]. Therefore, a reasonable strategy to protect liver grafts from cold ischemic injury during organ preservation is to understand the fundamental mechanisms of how the cell membrane and underlying structures are injured during hypothermic preservation in order to identify rational therapeutic targets.

Cell membrane ultrastructure (microvilli), cell membrane organization, and cell polarity are developed and maintained, in large part, by the sub-lamellar cytoskeletal system [15]. This system consists mostly of fodrin and the band 4.1 superfamily of proteins, which consists of protein 4.1, ezrin, radixin, and moesin (FERM). These proteins all have a FERM domain at the amino terminus that binds a wide variety of membrane associated target proteins and an actin binding domain (ABD) at the carboxy terminus that links to actin [4]. These linker proteins help support ultrastructural components of epithelial cells, which are important to the function of highly epithelialized organs like the liver. While most cells produce all of these proteins, the cells of the liver expresses moesin and radixin but not ezrin [5].

The functional significance of ERM proteins in hypothermic preservation injury is suggested by a series of studies of ezrin expression and functionality in renal tubular epithelium [16;24;25]. Ezrin disassociates from the actin cytoskeleton proportional to the degree of

preservation injury in renal tubules [16]. Ezrin is protective in preservation injury by virtue of its degree of expression and its binding configuration. This implicates a role of the threonine-567 (T567) specific phosphorylation site of ezrin [24;25] since this site controls ezrin binding. Liver tissue doesn't readily express ezrin but it does display membrane injury with cold ischemia that is similar in kidneys. Therefore, an analogous hepatic ERM mechanism of cell membrane injury during cold storage preservation likely exists involving moesin and radixin in the liver. The role of moesin in hypothermic liver preservation injury was the specific focus of this study. Our hypothesis is that moesin serves to protect hepatocytes during hypothermic liver preservation injury by its membrane linker functionality, which facilitates cell adhesion and viability. The following experiments were designed to test this hypothesis.

Materials and Methods

Mouse Liver Preservation Model

All studies involving animals were first approved by the Virginia Commonwealth University (VCU) Institutional Animal Care and Use Committee (IACUC). VCU is an AAALAC accredited institution. Mice were anesthetized with isoflurane and the liver was quickly removed after in-situ flushing with cold UW solution through the portal vein. Livers were cold stored in UW solution at 2-4° C on ice in a refrigerator for 0, 24, or 48 hours. After cold storage, the livers were reperused with Krebs buffer on an IPL for an additional 60 min with oxygenation to simulate reperfusion at transplantation. This was done as previously described in detail [14]. The purpose was to demonstrate a correlation between cold storage time and preservation injury outcomes in the liver after reperfusion for later comparisons with tissue moesin binding. The measured endpoints to assess liver preservation injury were bile production, vascular resistance, and lactate dehydrogenase (LDH) release into the IPL buffer. In 3 additional mice, sinusoidal endothelial cell attachment was determined by the in-situ trypan blue test as previously described [14]. In a separate group of mouse livers, tissue samples were obtained before cold storage, after 6 or 24 hours of cold storage, and after 24 hours of cold storage and 1 hour of normothermic oxygenated reperfusion on the IPL. The tissue was extracted and assayed for moesin in its unbound and its cytoskeletal bound state by western blot.

Cell Preservation Model

A cell model of hypothermic liver preservation injury was developed using both sinusoidal endothelial cells and hepatocytes. Mouse Sinusoidal Endothelial Cells (mSEC, a gift from Dr. Vijay Shah, Mayo Clinic), Human Sinusoidal Endothelial Cells (hSEC, ScienCell, Carlesbad, CA), and human hepatocytes (HepG2 cells, ATCC, Manassas, VA) were grown separately to 80% confluence in 96-well plates in DMEM with 5% fetal calf serum. To induce hypothermic preservation injury, the plates were placed in an air tight chamber (Tupperware), which was purged with nitrogen for 5 min and placed in a refrigerator on ice for 4-48 hours. Following cold storage ischemia, the cells were rewarmed and reoxygenated (to simulate reperfusion at transplantation) by placing the plates back into the 37° C CO2 incubator for 1 hour. During the rewarming period, some wells contained 10 µl of WST-8 for the detection of cell viability and mitochondrial metabolism. After 60 min, the plates

were read (450 nM) to assess WST-8 oxidation. LDH release was determined with a CytoTox 96 assay kit to assess membrane integrity. Some cells that were plated in 6 well plates were extracted with a differential moesin extraction buffer after reperfusion to measure moesin disassociation as previously described in detail for ezrin [16].

Materials

Prk7 Moesin-YFP, an expression plasmid containing mouse wild-type Moesin cDNA, was a gift from Dr. Kodi S. Ravichandran, University of Virginia School of Medicine, Charlottesville, VA. The human sinusoidal endothelial cell line hSEC was purchased from American Type Culture Collection (ATCC). CytoTox 96 non-radioactive cytotoxicity assay kits (LDH based) were ordered from Promega Corp. (Madison, WI). Cell proliferation reagent WST-8 was ordered from Cayman Chemical (Ann Arbor, Michigan).

Site-Directed Mutagenesis

To substitute the COOH-terminal threonine 558 with an alanine (T558A) or an aspartate (T558D), site-directed mutation was done with the QuikChange®II site-directed mutagenesis kit (Agilent technologies, Santa Clara CA). The oligonucleotides used to introduce the T558A and T558D mutations in Moesin were: Mutation T to A, forward primer: CCGAGACAAATACAAGGCCCTGCGCCAGATCCGGC, reverse primer: GCCGGATCTGGCGCAGGGCCTTGTATTTGTCTCGG. Mutation T to D, forward primer: CCGAGACAAATACAAGGACCTGCGCCAGATCCGG, reverse primer: CCGGATCTGGCGCAGGTCCTTGTATTTGTCTCGG. Samples were denatured at 95°C for 30 s and then subjected to 12 cycles of amplification (95°C, 30 s; 55°C, 1 min; 68°C, 12 min) for the T558D and T558A mutations, respectively. The mutations were confirmed by DNA sequencing.

Stably Transfected Cell Lines

Stably transfected HepG2- Moesin T558, HepG2-Moesin T558A, and HepG2-Moesin T558D cells were created by transfecting HepG2 cells with the Prk7 Moesin-YFP, Prk7 Moesin-T558A-YFP, or the Prk7 Moesin-T558D-YFP plasmid. Cells were purified by flow cytometry to create stable cell lines.

Moesin Knockdown

Cells at 80% confluence were transfected using Lipofectamine 2000 Reagent with a Stealth RNAi (Invitrogen) specific for mouse moesin for 48 hrs and validated by western blot.

Moesin Disassociation Assays

Free moesin was assessed using a differential, extraction buffer to separate the bound and unbound components followed by western blot to quantitate the unbound signals. Liver tissue biopsies or cells were extracted in 2-ml Tenbroek ground glass homogenizers using 300 µl of a buffer consisting of 0.5% Triton X-100. The extract was analysis by western blot using goat anti-mouse moesin primary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) with ECL detection.

Data Analysis

All data were expressed as mean $\pm$ SD. Differences between the means were determined by one-way parametric ANOVA with Bonferroni's multiple comparison correction. P values less than or equal to 0.05 were considered statistically significant. Cell culture experiments were replicated 4-6 times and liver studies were replicated 6 times.

Results

Liver preservation injury was found to be directly proportional to the amount of cold storage ischemia time in mouse livers following IPL reperfusion (Figure 1A-1D). This model induces similar preservation injury seen with liver transplantation [3;11;12;22] so it serves as a basis for studying the moesin sub-lamellar cytoskeletal system in preservation injury.

Liver moesin disassociation before and after reperfusion on the IPL clearly show a significant increase in moesin breakdown from the actin cytoskeleton (Figure 2), which is proportional to both the time of cold storage and the degree of preservation injury (Figure 1). Moesin disassociation occurred after cold ischemic exposure and before rewarming (Figure 2A) but was not reversed after rewarming (Figure 2B).

Figure 3 shows that cold ischemia exposure time in SEC cells is inversely proportional to cell survival at reperfusion. Specifically, LDH release increased (Panel 3A) and WST-8 activity decreased (Panel 3B) as cold storage time increased. Moesin extracted from these cells after reperfusion show that the free unbound moesin signal strengthens as the cold storage time increases. This indicates that moesin becomes increasingly unbound to actin as cold storage injury increases. The correlation between moesin breakdown, cold ischemia exposure time, and preservation injury (Figures 1- 3) strongly implies a causal link between moesin breakdown in the cold and hypothermia dependent preservation injury. The next experiments were designed to test this causation.

More definitive evidence for a causal role of moesin in hypothermic preservation injury in the liver was developed from cell lines that over-express or under-express native moesin. HepG2 cells transfected with a plasmid containing the native moesin gene or cells transfected with a moesin specific siRNA duplex were cold stored and rewarmed. Post-reperfusion hepatocyte viability (WST-8 activity), which is an index of preservation injury [24], progressively declined as the cold storage time increased (Figure 4A). This response was significantly exacerbated with moesin knock-down (siRNA) and significantly attenuated with moesin over-expression (Figure 4A). The desired effect of both moesin transfection (overexpression) and moesin knock-down (under expression) on actual cell moesin protein levels was verified by moesin western blot (Figure 4B).

The role of the molecular binding configuration of moesin on preservation injury was evaluated in the HepG2 cold storage model. Cell viability, as expressed by WST-8, in the active moesin binding mutant T558D was significantly greater compared to the inactive moesin mutant T558A (Figure 5A). After 2 and 3 days of cold exposure, there was a 4-5 fold increase in hepatocyte viability with the active T558D mutation, relative to the inactive T567A mutation. Figure 5B shows that hepatocytes expressing the T558A moesin mutation

became significantly more detached after cold exposure compared to the T558D mutants. In fact, the active T558D moesin mutants remained completely attached at all levels of cold ischemic exposure.

Discussion

Cold ischemia time is the strongest risk factor for primary graft non function (PNF) and graft loss in clinical liver transplantation [22]. Solving PNF caused by cold preservation injury is a major step towards promoting the broadest possible use of available livers and for expanding the donor pool. However, since the advent of the University of Wisconsin (UW) organ flush solution in 1987, there has been no further significant advances in solving the problem of hypothermic liver preservation injury. This is mainly because the basic molecular mechanisms of the injury are still poorly understood. Our overall goal is to understand these mechanisms in order to rationally target the injury.

To facilitate the search for molecular mechanisms involved in liver preservation injury, we developed, tested, and validated novel cell based models. Using human hepatocyte and sinusoidal endothelial cells, it was possible to clearly demonstrate a preservation injury phenotype that closely paralleled preservation injury in the whole liver. Markers of viability in the cell models were proportionally reduced as the length of the cold preservation time increased. Similar changes in liver functional outcomes were also seen during progressive exposure to cold ischemia in the whole liver. The cell and liver preservation models were validated since both the models and human livers used for clinical transplantation demonstrate preservation injury proportional to the time of hypothermic exposure. We were also able to demonstrate biochemical changes in moesin function during cold storage in both cell models that paralleled changes seen in the whole cold stored liver model. Thus, the hepatocyte (HepG2) and SEC cell preservation models developed for these studies may be very useful in dissecting out the role of moesin functionality in liver preservation injury since they behave functionally and biochemically similar to whole liver models and because they are amenable to genetic manipulations of moesin.

Our previous work in preservation sensitive renal proximal tubule epithelial cells indicate that the sublamellar cytoskeletal system is involved in preservation injury. Previously it was believed that breakdown of the cell cytoskeletal system during ischemia was a terminal epiphenomenon of ischemic cell death rather than a causal mechanism of its occurrence. Based on our previous work with ezrin in hypothermic preservation injury in renal tubular epithelium [16;24;25], we now believe that failure of the sub-lamellar cytoskeletal system during cold ischemia causally mediates ischemic injury rather than the converse. In the liver, the effect is even more pronounced. It is clear from the data that having more moesin in hepatocytes is better than having less during cold storage injury. The mechanism of how moesin protects cells under these conditions is not fully appreciated yet. Our hypothesis has been that membrane failure commonly seen with cold storage injury in many organs contributes to preservation injury by loss of cell polarity, loss of ultrastructure (microvilli), and loss of cell adhesion. Loss of cell adhesion in the liver by either hepatocyte or SEC populations could also signal loss of survival and apoptotic death because interaction of the matrix components with cell surface molecules produces survival signaling. Therefore, it

was reasonable to suggest that ERM proteins, like moesin in hepatocytes, are protective because of their linker role, which is essential for cell adhesion to the matrix. Specifically, these proteins function by holding the plasma membrane and essential components embedded on the plasma membrane (like adhesion molecules, receptors, and signaling molecules) in place by binding these components to actin microfilaments and stress fibers.

To test that hypothesis further, the linker functionality of these proteins must be shown to be important during preservation injury. Linker functionality is the ability of ERM proteins to assume a linear active configuration so that their N-terminus FERM domains are exposed for membrane binding and their C-terminus ABD domains are exposed for actin binding. For moesin, this functionality is controlled by T558 phosphorylation, which opens and activates the molecule. Dephosphorylation allows the FERM and ABD domains to bind together, which makes them unavailable for active binding to the plasma membrane and actin. Mutations of the moesin molecule at this control point (T558) were used to test the binding configuration hypothesis during preservation injury in hepatocytes. These data clearly support the binding hypothesis since constitutively active moesin mutants (T558D) are multiples of magnitude more protected in our hypothermic preservation models compared to the constitutively inactive moesin mutants (T558A). Furthermore, hepatocytes expressing active moesin do not detach from their substrates during prolonged periods of hypothermic ischemia, compared to the cells expressing the inactive moesin mutant. The correlation between binding and viability in cold stored hepatocytes expressing the active moesin mutant may represent cause and effect since hepatocyte survival is known to be dependent on integrin binding to the matrix. Adhesion to ECM is essential for the growth and survival of hepatocytes. When they detach from the matrix and disengage their integrin adhesion molecules, they undergo dedifferentiation and apoptosis, also known as anoikis [6]. Since T558D moesin affords better binding, it seems reasonable to suggest that this then causes improved cell viability by maintaining binding-induced cell survival signaling, possibly via a β_1 integrin pathway [6;19].

The major questions still remaining are what other cell types are involved and how do they interact in the whole liver? These questions will be answered by using both knockout and knock-in moesin mutant mice. Another question is what signaling mechanisms control moesin binding functionality during hypothermia and ischemia? Although this is unresolved, it may depend highly on the Lysophosphatidic acid (LPA), RhoA (RhoA), and Rho Kinase (ROK) signaling circuits [18;21;27] but non-Rho pathways (protein kinase-C and -A) have also been described in ERM activation [27]. Pharmacological pressure applied to those circuits (LPA, RhoA, ROK, or PKC) may be salutary in liver preservation injury in the clinic by activating moesin or radixin phosphorylation pathways. Such potential strategies may be effective when used as donor pretreatments where they are given hours before liver retrieval.

Moesin is differentially expressed in actively hibernating ground squirrel intestinal brush border membranes, relative to summer active ground squirrels [8]. It seems reasonable to suggest that moesin expression is an adaptation to hypothermia, which is consistent with our observations of the moesin defect in cold stored livers from non-hibernating mammals. In fact, livers retrieved from actively hibernating ground squirrels were determined to be

resistant to cold storage injury, relative to cold stored livers retrieved from either non-hibernating summer active ground squirrels or from other non-hibernating rodent species like rats [14]. It is not known from these studies whether hibernating ground squirrels also have higher expression levels of moesin in the liver, like they do in their intestine, or if they express a more heavily phosphorylated moesin protein in the liver that would provide for more structural functionality. The previous data in the hibernator, which are by definition resistant to hypothermia, also serve to support our moesin hypothesis.

Protective responses to cold storage were observed with the analogous protein ezrin in renal tubules exposed to cold ischemia [24;25]. This suggests that ERM proteins may be redundant in membrane protection from hypothermia with regional differences due to tissue expression preferences of individual ERM proteins (ezrin in kidney and moesin in liver). A role for radixin in hypothermic preservation in liver was not determined in this study. However, radixin is expressed in hepatocytes and radixin knock-out mice show defects in bile secretion [10], which may indicate that they are also sensitive to preservation injury. Studies using cells and livers from both moesin and radixin knockout mice are needed to clarify their roles in hypothermic preservation injury in the liver.

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Reference List

1. Adam R, Bismuth H, Diamond T, Ducot B, Morino M, Astarcioglu I, Johann M, Azoulay D, Chiche L, Bao YM. Effect of extended cold ischaemia with UW solution on graft function after liver transplantation. *Lancet*. 1992; 340:1373–1376. [PubMed: 1360089]
2. Clavien PA. Sinusoidal endothelial cell injury during hepatic preservation and reperfusion. *Hepatology*. 1998; 28:281–285. [PubMed: 9695988]
3. Compagnon P, Lindell S, Ametani MS, Gilligan B, Wang HB, D'Alessandro AM, Southard JH, Mangino MJ. Ischemic preconditioning and liver tolerance to warm or cold ischemia: experimental studies in large animals. *Transplantation*. 2005; 79:1393–1400. [PubMed: 15912109]
4. Fehon RG, McClatchey AI, Bretscher A. Organizing the cell cortex: the role of ERM proteins. *Nat Rev Mol Cell Biol*. 2010; 11:276–287. [PubMed: 20308985]
5. Fouassier L, Duan CY, Feranchak AP, Yun CH, Sutherland E, Simon F, Fitz JG, Doctor RB. Ezrin-radixin-moesin-binding phosphoprotein 50 is expressed at the apical membrane of rat liver epithelia. *Hepatology*. 2001; 33:166–176. [PubMed: 11124833]
6. Frisch SM, Screaton RA. Anoikis mechanisms. *Curr Opin Cell Biol*. 2001; 13:555–562. [PubMed: 11544023]
7. Furukawa H, Todo S, Inventarza O, Casavilla A, Wu YM, Scotti-Foglieni C, Broznick B, Bryant J, Day R, Starzl TE. Effect of cold ischemia time on the early outcome of human hepatic allografts preserved with UW solution. *Transplantation*. 1991; 51:1000–1004. [PubMed: 2031256]
8. Gorham DA, Bretscher A, Carey HV. Hibernation induces expression of moesin in intestinal epithelial cells. *Cryobiology*. 1998; 37:146–154. [PubMed: 9769165]
9. HRSA OPTN. Report: Current U S Waiting List. 3-23-0013 Ref Type: Online Source.
10. Kikuchi S, Hata M, Fukumoto K, Yamane Y, Matsui T, Tamura A, Yonemura S, Yamagishi H, Keppler D, Tsukita S, Tsukita S. Radixin deficiency causes conjugated hyperbilirubinemia with loss of Mrp2 from bile canalicular membranes. *Nat Genet*. 2002; 31:320–325. [PubMed: 12068294]
11. Lee CY, Mangino MJ. Preservation methods for kidney and liver. *Organogenesis*. 2009; 5:105–112. [PubMed: 20046672]

12. Li S, Stratta RJ, Langnas AN, Wood RP, Marujo W, Shaw BW Jr. Diffuse biliary tract injury after orthotopic liver transplantation. *Am J Surg.* 1992; 164:536–540. [PubMed: 1443384]
13. Lindell S, Williams N, Brusilovsky I, Mangino MJ. Mouse IPK: A Powerful Tool to Partially Characterize Renal Reperfusion and Preservation Injury. *Transplantation Journal.* 2011; 5:15–22.
14. Lindell SL, Klahn SL, Piazza TM, Mangino MJ, Torrealba JR, Southard JH, Carey HV. Natural resistance to liver cold ischemia-reperfusion injury associated with the hibernation phenotype. *Am J Physiol Gastrointest Liver Physiol.* 2005; 288:G473–G480. [PubMed: 15701622]
15. Louvet-Vallee S. ERM proteins: from cellular architecture to cell signaling. *Biol Cell.* 2000; 92:305–316. [PubMed: 11071040]
16. Mangino MJ, Tian T, Ametani M, Lindell S, Southard JH. Cytoskeletal involvement in hypothermic renal preservation injury. *Transplantation.* 2008; 85:427–436. [PubMed: 18301334]
17. McKeown CM, Edwards V, Phillips MJ, Harvey PR, Petrunka CN, Strasberg SM. Sinusoidal lining cell damage: the critical injury in cold preservation of liver allografts in the rat. *Transplantation.* 1988; 46:178–191. [PubMed: 3043774]
18. Neisch AL, Speck O, Stronach B, Fehon RG. Rho1 regulates apoptosis via activation of the JNK signaling pathway at the plasma membrane. *J Cell Biol.* 2010; 189:311–323. [PubMed: 20404112]
19. Newsome PN, Tsiaoussis J, Masson S, Buttery R, Livingston C, Ansell I, Ross JA, Sethi T, Hayes PC, Plevris JN. Serum from patients with fulminant hepatic failure causes hepatocyte detachment and apoptosis by a beta(1)-integrin pathway. *Hepatology.* 2004; 40:636–645. [PubMed: 15349902]
20. Nowak G, Ungerstedt J, Wernerson A, Ungerstedt U, Ericzon BG. Hepatic cell membrane damage during cold preservation sensitizes liver grafts to rewarming injury. *J Hepatobiliary Pancreat Surg.* 2003; 10:200–205. [PubMed: 14605976]
21. Speck O, Hughes SC, Noren NK, Kulikauskas RM, Fehon RG. Moesin functions antagonistically to the Rho pathway to maintain epithelial integrity. *Nature.* 2003; 421:83–87. [PubMed: 12511959]
22. Stahl JE, Kreke JE, Malek FA, Schaefer AJ, Vacanti J. Consequences of cold-ischemia time on primary nonfunction and patient and graft survival in liver transplantation: a meta-analysis. *PLoS One.* 2008; 3:e2468. [PubMed: 18575623]
23. Tector AJ, Mangus RS, Chestovich P, Vianna R, Fridell JA, Milgrom ML, Sanders C, Kwo PY. Use of extended criteria livers decreases wait time for liver transplantation without adversely impacting posttransplant survival. *Ann Surg.* 2006; 244:439–450. [PubMed: 16926570]
24. Tian T, Lindell SL, Henderson SC, Mangino MJ. Protective effects of ezrin on cold storage preservation injury in the pig kidney proximal tubular epithelial cell line (LLC-PK1). *Transplantation.* 2009; 87:1488–1496. [PubMed: 19461485]
25. Tian T, Lindell SL, Lam M, Mangino MJ. Ezrin functionality and hypothermic preservation injury in LLC-PK1 cells. *Cryobiology.* 2012; 65:60–67. [PubMed: 22554620]
26. Vreugdenhil PK, Rankin MA, Southard JH. Cold storage sensitizes hepatocytes to oxidative stress injury. *Transpl Int.* 1997; 10:379–385. [PubMed: 9287404]
27. Yonemura S, Matsui T, Tsukita S, Tsukita S. Rho-dependent and -independent activation mechanisms of ezrin/radixin/moesin proteins: an essential role for polyphosphoinositides in vivo. *J Cell Sci.* 2002; 115:2569–2580. [PubMed: 12045227]

Abbreviations

ERM	Ezrin Radixin Moesin
IPL	Isolated Perfused Liver
UW	University of Wisconsin solution
SEC	Sinusoidal endothelial cell
LDH	Lactate dehydrogenase

FERM	protein 4.1, ezrin, radixin, moesin
LPA	Lysophosphatidic acid
PKC	Protein kinase C
ROK	Rho Kinase

Figure 1.

The isolated perfused liver (IPL) was used to assess mouse liver functional outcomes after progressive cold storage periods, which was used to index the extent of preservation injury. This provided a reference point to assess moesin function and expression from mouse livers that were cold stored under identical conditions. Mouse livers flushed with cold UW solution were stored at 2 degrees C for 0, 24 or 48 hours and then reperused for 1 hour on the IPL with warm oxygenated Krebs buffer. Liver bile production (A), LDH release (B), vascular resistance (C), and sinusoidal endothelial cell death (D, Trypan blue dye exclusion) are shown. The data represent the mean and SD of 6 experiments. * $P < 0.05$ relative to the time zero control values.

Figure 2.

Moesin disassociation from the actin cytoskeleton was measured by a differential tissue extraction and western blot technique in liver biopsies obtained after progressive cold storage times (A) and after IPL reperfusion (B). The free moesin is shown in the figures (A and B) and signals from a western blot example is shown in panel C. Data represent the mean densitometry values of the free moesin signal $\pm$ SD, n=6. * P < 0.05, relative to the value at 0 hrs of cold storage.

Figure 3.

A hypothermic preservation injury model was developed using murine (mSEC) and human sinusoidal endothelial cells (hSEC). Cells were cold stored by placing the plated cells in a nitrogen purged box on ice in a refrigerator followed by reperfusion for an hour in the CO₂ incubator. Cell viability after reperfusion was assessed to index preservation injury by measuring the % LDH release (A) and WST-8 dye conversion (B). Free moesin was also measured over time in both cell models (C). Values are mean $\pm$ SD for 4 independent experiments performed in quadruplicate wells per condition. * $P < 0.05$, relative to all other time points for both cell types.

Figure 4.

Hypothermic preservation injury in a human hepatocyte (HepG2) cell model was developed using WST-8 dye conversion as the endpoint (A). Three populations of HepG2 cells were used in this study, which included cells expressing the wild type moesin (Control), HepG2 cells transfected with a moesin expressing plasmid (Moesin transfected), and cells transfected with a moesin specific siRNA knock-down duplex (Moesin siRNA). Values are mean $\pm$ SD for 4 independent experiments conducted in quadruplicate wells, ** $P < 0.05$, relative to all of the groups in the corresponding storage time. * $P < 0.05$, between the Moesin Transfection group and both the Control and Moesin siRNA groups. A sample of total cell moesin expression by western blot is shown in (B) to verify moesin over-expression (Moesin plasmid) and moesin knock-down (Moesin siRNA), relative to the control cells.

Figure 5.

Results showing hypothermic preservation injury in a human hepatocyte (HepG2) cell model using WST-8 dye conversion as the endpoint (A). Two populations of HepG2 cells were used, which included cells expressing the inactive moesin mutant (Moesin T558A) and those expressing the active moesin mutant (Moesin T558D). Values are mean $\pm$ SD for 4 independent experiments conducted in quadruplicate wells, * $P < 0.05$ relative to the corresponding values for the T558A group. Hepatocyte adherence is shown in panel B. HepG2 cells expressing the active (T558D) or inactive (T558A) moesin mutants were grown adherent to polystyrene culture plates and then cold stored for 1-3 days, stained with DAPI, and imaged with a fluorescence microscope to visualize the effects of hypothermic ischemia on substrate adhesion. The effects of moesin functionality on cell adhesion in the cold can be seen clearly.