

THE DEVELOPMENT OF AN IN VITRO MODEL TO EXAMINE AND MODULATE  
HEPATIC ISCHEMIA AND REPERFUSION RESPONSES

by

Kimberley Savage

Submitted in partial fulfilment of the requirements  
for the degree of Master of Science

at

Dalhousie University  
Halifax, Nova Scotia  
July 2011

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DALHOUSIE UNIVERSITY  
DEPARTMENT OF PATHOLOGY

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Dated: July 5, 2011

Supervisor: \_\_\_\_\_

Readers: \_\_\_\_\_

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DALHOUSIE UNIVERSITY

DATE: July 5, 2011

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TITLE: THE DEVELOPMENT OF AN IN VITRO MODEL TO EXAMINE AND  
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RESPONSES

DEPARTMENT OR SCHOOL: Department of Pathology

DEGREE: M.Sc. CONVOCATION: October YEAR: 2011

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## ABSTRACT

Transplantation is the optimal form of therapy for patients with end-stage liver disease; however, the use of organs with hepatic steatosis is often associated with increased risks for poor function and graft loss. In addition, ischemia reperfusion (IR) injury leads to cellular damage that can culminate in functional impairment and loss of graft. Furthermore, IR injury is aggravated by pre-existing steatosis and may involve additional mechanisms and mediators of cellular damage. Current models to study IR *in vitro* are not well defined and may overlook periods of injury that are involved in transplantation. In this thesis, I present an *in vitro* model for IR injury that includes multiple phases of injury and leads to the upregulation of heme oxygenase-1 (HO-1), and possibly enhances the expression of matrix metalloproteinase-9 (MMP-9). As graft HO-1 expression correlates positively with reduced injury, but MMP-9 expression is associated with increased injury, I therefore examined the utility of *in vitro* gene therapies to affect the expression of these proteins. We conclude that the *in vitro* model of ischemia and reperfusion is a promising tool to study the cellular response to IR and may provide a platform for the development of future therapies which could have clinical applications.

## LIST OF ABBREVIATIONS USED

Ab	Antibody
ACO	Acyl-coenzyme A oxidase
ADAM	A disintegrin and metalloproteinase
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
BMI	Body mass index
BSA	Bovine serum albumin
cDMEM	Complete Dulbecco's Modified Eagle's Medium
CI	Cold ischemia
CIP	Calf intestinal phosphatase
CO	Carbon monoxide
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethylsulfoxide
EBM	Endothelial Basal Medium
ECD	Extended criteria donor
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EGM	Endothelial Growth Medium
FA	Fatty acid
FAS	Fatty acid synthase
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase

GFP	Green fluorescent protein
H & E	Haematoxylin and eosin
HO	Heme oxygenase
HRP	Horseradish peroxidase
Hsp	Heat shock protein
HTK	Histidine-Tryptophan-Ketoglutarate
HUVEC	Human umbilical vein endothelial cells
IL	Interleukin
IR	Ischemia reperfusion
KC	Kupffer cells
mAb	Monoclonal antibody
miRNA	MicroRNA
MMP	Matrix metalloproteinase
MOI	Multiplicity of infection
MTT	3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide
NAFLD	Nonalcoholic fatty liver disease
NASH	Nonalcoholic steatohepatitis
NH	Normal human
NP-40	Nonidet P-40
NR	Normal rat
OLT	Orthotopic liver transplantation
ORO	Oil red O
PAS	Periodic Acid-Schiff

PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PPAR	Peroxisome proliferator-activated receptor
PVDF	Polyvinylidene fluoride
RISC	RNA-induced silencing complex
ROS	Reactive oxygen species
RT	Room temperature
RT-PCR	Reverse transcription PCR
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
SEC	Sinusoidal endothelial cell
SEM	Standard error of the mean
SH	Steatotic human
shRNA	Short hairpin RNA
siRNA	Small interfering RNA
SR	Steatotic rat
SREBP	Sterol regulatory element binding protein
TBS	Tris-buffered saline
TBST	TBS containing Tween-20
TEMED	Tetramethylethylenediamine
TIMP	Tissue inhibitors of metalloproteinase
TNF	Tumour necrosis factor
U	Units

V	Volts
WI	Warm ischemia
WST-1	4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate

## ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr. Ian Alwayn for giving me the opportunity to work and learn in such a positive environment. Your experience and perspective were important to my understanding of the relevance of this work. I would also like to thank my co-supervisor Dr. Tim Lee who was always willing to share his time and experience when I was in need of extra direction. Next, I would like to thank Jennie Parker for starting this journey together as the first members of a brand new lab, for being there to work things through with, and for her wonderful technical assistance. I would also like to thank Dr. David Woodhall, who has brought new perspectives and experience to our lab, in addition to expert technical assistance and constant support. To the rest of the members of the lab, past and present, you have been so many things: supportive, entertaining, thought-provoking and opinion-altering: Nicole, Mryanda, Alison, Mike, Scott, Alec, Chelsey, Amr, Jill, Melvin, Laura, Tanya, Julie, Jen, Ann Marie, Latitia, and Brenda. It was a pleasure working with every single one of you. Lastly, I would like to thank Dave and all the other members of my family, here and away, who have supported me unconditionally through this experience.

# CHAPTER 1: INTRODUCTION

## 1.0 Overview

There are over 100 known forms of liver disease, and while there have been major advances in the treatment of these diseases, there are currently no cures [1]. However, since the first orthotopic liver transplantation (OLT) was performed by Dr. Thomas Starzl in 1963 [2], it has become the principal treatment for end-stage liver disease, with over 400 of these operations performed every year in Canada [3]. Unfortunately, the demand for organs far outweighs the supply, therefore resulting in a significant number of deaths of patients on the waiting list each year [3]. In recent years, one of the strategies to expand the donor pool has been to use organs from extended criteria donors (ECD), which includes those organs having prolonged ischemic times and extensive fatty liver disease [4]. The use of such organs is often minimized, as they are generally expected to pose a greater risk of post-transplant complications [5]. In addition, although fatty liver disease is one of most preventable forms of liver disease, it is increasing in prevalence due to a worldwide rise in obesity. This has a dual impact on the donor pool: progression of fatty liver disease will likely necessitate organ transplantation for treatment, and a greater proportion of available donor organs will show significant fatty infiltration. Therefore, the effective expansion of the donor pool must examine methods to counteract the detrimental effects associated with the transplantation of fatty livers, or define ways to reduce the burden of disease within donor livers.



## 1.1 Hepatic Steatosis

The World Health Organization reports that worldwide obesity has more than doubled since 1980 and that being overweight and being obese are leading risks for global deaths [6]. Classification of an individual as obese or overweight is based on the body mass index (BMI) which is calculated by dividing weight in kilograms by height in metres squared [6, 7]. In 2010, 17.5 % of Canadians were considered obese (BMI of 30.00 or higher) and an additional 31 % were classified as overweight (BMI of 25.00 - 29.99) [8]. A major consequence of obesity is the deposition of triglycerides in the liver, or hepatic steatosis [9].

Hepatic steatosis is a benign condition and is one of a spectrum of conditions described by the term nonalcoholic fatty liver disease (NAFLD). When the steatosis is associated with cellular injury and inflammation, it is termed nonalcoholic steatohepatitis (NASH), and this may progress to fibrosis, cirrhosis and end stage liver disease requiring transplantation [10]. The increasing proportion of the population that is overweight or obese means that a greater number of potential organ donors will have significant hepatic steatosis. Steatosis is classified as being either macrovesicular (a common finding involving one or very few large fatty droplets within the hepatocyte cytoplasm that displace the nucleus to the edge of the cell) or microvesicular (a rare finding involving multiple smaller fat vacuoles within the cytoplasm that surround a centrally located nucleus) [11]. Prior to transplantation, the amount of (macrovesicular) hepatic steatosis is generally assessed visually and by palpation of the organ [12]. Those livers having a severe amount of steatosis (> 60 %) are normally considered unsuitable for

transplantation, while organs having mild amounts of steatosis (0 - 30 %) are generally acceptable for use [13]. There is currently no consensus, however, regarding the use of livers with moderate amounts of fatty infiltrate (30 - 60 %), as these moderately steatotic livers are frequently associated with poor initial function following transplant [4, 5, 12-16], and retrospective studies have correlated the degree of hepatic steatosis with an increase in rates of graft loss [12, 13, 17]. Therefore, the disparity between organ supply and demand is expected to continue to grow, as livers with fatty infiltration are often discarded [14, 18].

## **1.2 Mechanisms and Mediators of Hepatic Steatosis**

In mammals, the liver is the principal organ responsible for the conversion of excess dietary carbohydrates into triglycerides [19]. Fatty acid (FA) metabolism is a strictly controlled process that involves regulation at several levels: uptake, oxidation, *de novo* synthesis and export to the circulation. Biosynthesis of FAs is executed by a set of lipogenic genes which includes fatty acid synthase (FAS), a multifunctional enzyme that participates in the catabolism of carbohydrates. The overall result of this biochemical process is the production of fatty acids which are essential biological components and important stores of energy. Degradation of FAs to provide metabolic fuel is accomplished by a set of lipolytic genes which includes acyl-coenzyme A (CoA) oxidase (ACO), an enzyme involved in peroxisomal  $\beta$ -oxidation of fatty acids [20]. Hepatic steatosis results from an imbalance in these processes which are normally tightly regulated by a number of transcription factors and their target genes.

Fatty acid synthesis in the liver occurs when blood glucose is elevated, for instance after feeding, in order to generate energy stores. When insulin is released in response to a meal, it stimulates the breakdown of carbohydrates, which provides acetyl-CoA, the substrate for FA synthesis. Specifically, insulin leads to increased levels of sterol regulatory element binding protein-1c (SREBP-1c) mRNA [21], a transcription factor that binds to genes involved in FA metabolism, including FAS. Support for this is provided by Shimomura *et al.* who showed that insulin treatment reversed a near-total reduction in SREBP-1c mRNA observed in a rat model of chemically-induced diabetes [21]. In addition, Bennett *et al.* showed that SREBP-1c binds to the promoter region of FAS and regulates its expression [22]. Moreover, transgenic mice expressing high levels of SREBP-1c exhibited triglyceride-enriched livers when compared to wild-type [23]. Furthermore, Yahagi *et al.* demonstrated that disruption of SREBP-1 gene expression in genetically obese (*ob/ob*) mice led to a reduced hepatic triglyceride burden [19]. Fatty acid synthesis in response to carbohydrate ingestion is therefore largely attributable to the actions of SREBP-1c.

Hepatic FA catabolism occurs in order to provide a source of energy during periods of fasting. Free FAs that are released into the bloodstream from adipose tissue are taken up by the liver and this signals their own degradation. Specifically, these free FAs activate the transcription factor peroxisome proliferator-activated receptor alpha (PPAR $\alpha$ ) [24-26], which binds to and activates genes involved in a variety of pathways, including fatty acid catabolism. Evidence to support this is provided by several studies showing that FAs act as ligands for PPAR $\alpha$  [24-26]. In addition, Tugwood *et al.*

determined that PPAR binds to the promoter region of the ACO gene and upregulates its expression [20]. Moreover, PPAR $\alpha$ -deficient mice exhibited increased hepatic FA content compared to that of wild-type [27]. Furthermore, chemical activation of PPAR $\alpha$  has been shown to both prevent the development of, and ameliorate previously established diet-induced steatohepatitis [28, 29]. Fatty acid breakdown in response to free FAs is therefore mainly mediated by the actions of PPAR $\alpha$ .

In addition to affecting the progression of hepatic steatosis, FA accumulation has a negative impact on the hepatocellular condition. The end product of hepatic FA metabolism is predominantly palmitic acid, a saturated FA; however oleic acid, a monounsaturated FA, and stearate, a polyunsaturated FA, are also produced. When cultured hepatocyte cell lines are exposed to both palmitic and oleic acids, there is an accumulation of cytoplasmic lipids and an increase in triglyceride concentration; however there is also an increase in the rate of apoptosis as compared to that seen in control cultures [30]. In addition, rat hepatocyte cell cultures exposed to palmitate or stearate showed increased markers of endoplasmic reticulum stress and decreased viability [31], and exposure to palmitate has been shown to induce DNA fragmentation and cell death [32]. Steatosis of the liver also affects the expression of the proinflammatory cytokine tumour necrosis factor alpha (TNF- $\alpha$ ); gene expression is higher in cells isolated from steatotic livers than in those isolated from healthy controls, and serum TNF- $\alpha$  is elevated in patients with steatosis when compared to matched controls [33, 34]. Alternatively, exogenous TNF- $\alpha$  induces steatosis of the mouse liver by enhancing the expression of SREBP-1c and FAS [35]. Taken together these observations provide evidence that an

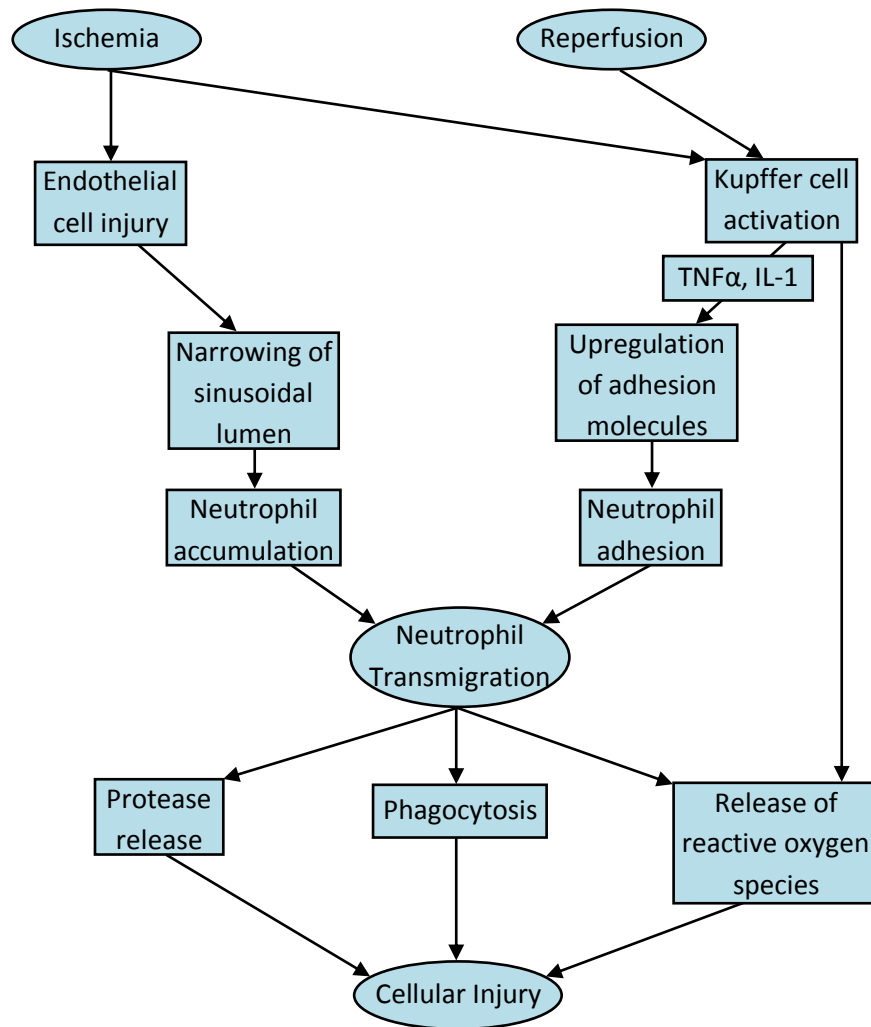
overabundance of hepatic fat is detrimental and cytotoxic. Furthermore, organs containing a higher percentage of fatty infiltrate are more severely injured by the process of transplantation, as measured by assessments of liver function in recipients [15, 16].

### **1.3 Ischemia Reperfusion Injury**

Ischemia reperfusion (IR) injury is an unavoidable consequence of the organ transplantation process [36], and is a chief contributing factor in early graft dysfunction [37], causing up to 10 % of early organ failure [38]. Ischemia is the reduction of blood flow to a tissue or organ, while reperfusion is the restoration of blood flow to that organ or tissue. Injury from IR is mediated by several processes that lead to cellular damage (Figure 1) [39-41] and consists of four phases of injury to the organ: pre-preservation, cold preservation, rewarming and reperfusion injuries [42]. Pre-preservation injury results from either pre-existing disease or from factors associated with death of the donor. Both cause injury to the organ prior to harvest, and can significantly influence the damage from subsequent ischemic injury [43]. Hepatic steatosis is an example of pre-existing liver disease because, as mentioned, it is damaging to the donor organ.

Cold preservation injury occurs as a result of the combination of ischemia and hypothermia of the tissue. Hypothermia is applied in conjunction with ischemia in order to reduce metabolic activity and energy demand on the tissue during storage [43].

Ischemia is initiated when the donor organ is excised and the supply of oxygen and nutrients is inhibited. The organ is then flushed with a pre-cooled preservation solution and stored on ice in order to maintain a temperature of between 1 and 4°C [44]. Cellular



**Figure 1. Mechanisms Involved in Ischemia Reperfusion Injury.** Ischemia leads to sinusoidal endothelial cell (SEC) injury, compression of the sinusoidal space and leads to the accumulation of neutrophils. In addition, ischemia activates the Kupffer cells (KC) to influence damage during reperfusion. Reperfusion also causes injury to the SEC, and activates the KC to release tumour necrosis factor alpha (TNF- $\alpha$ ) and interleukin-1 (IL-1) which leads to SEC expression of adhesion molecules and promotes neutrophil adhesion. Activated KC also release reactive oxygen species (ROS) in response to reperfusion contributing to cell injury and death. Following neutrophil accumulation and adherence to SEC, they transmigrate into the parenchyma causing cellular injury through the release of ROS and proteolytic enzymes. Adapted from Banga *et al.*, 2005 [41]

damage occurs as a result of oxygen deprivation and energy depletion which can both cause parenchymal cell (hepatocyte) death [36]. Ischemia and hypothermia cause cell swelling, the production of precursors of reactive oxygen species (ROS) and protease activation [43, 45], all of which are damaging to the organ. In addition, endothelial cells upregulate the surface expression of adhesion molecules during cold ischemia [36]. These biochemical changes caused by ischemia predispose the tissue to further damage once the blood supply is restored [37]. While the parenchymal cells of the liver are highly resistant to even extreme periods (days) of cold ischemia, the nonparenchymal cells are particularly susceptible to this injury [46]. This is supported by the detection of sinusoidal endothelial cells (SEC) which are viable, but exhibit rounding and partial detachment from the hepatocyte plate following cold ischemia [42, 47-49]. Furthermore, cold ischemia activates the resident macrophages of the liver (Kupffer cells; KC) [49, 50], priming these phagocytes to influence subsequent damage upon reoxygenation [51].

During implantation and the restoration of vascular supply by the surgeon, rewarming injury occurs during ischemia that is no longer hypothermic. This period of rewarming is accompanied by substantial metabolic activity [52], however the tissue is still deprived of oxygen and therefore unable to replenish energy stores diminished during cold ischemia [42, 43]. Additionally, significant hepatocyte vacuolization has been observed in livers after warm ischemia following cold preservation [48]. Every effort is therefore made to minimize these periods of ischemia in order to lessen their damaging downstream effects.

Reperfusion injury occurs with the reintroduction of oxygenated blood to the transplanted organ. While this is necessary for survival of the organ, the restoration of blood flow initiates a series of events that cause cellular damage [43]. Immediately following the initiation of reperfusion, graft endothelial and parenchymal cells produce proinflammatory cytokines including TNF- $\alpha$  and interleukin-1 (IL-1) [50, 53, 54]. These cytokines stimulate the vascular endothelium to produce chemokines that will attract leukocytes, predominantly neutrophils, into the post-ischemic tissue [54]. In addition, the vascular endothelium upregulates expression of adhesion molecules and this will allow neutrophils to adhere during reperfusion [36, 42]. These neutrophils can cause tissue damage by producing ROS, including the superoxide radical, hydroxyl, and hydrogen peroxide [38], and by secreting proteolytic enzymes; both products are capable of causing endothelial cell death either alone, or in combination [55]. In the liver, while neutrophils are activated and observed to accumulate in post-ischemic tissues, these early events do not correlate with impaired graft function post-transplant [56]. Neutrophils are however, important mediators of later hepatocyte injury and death, contributing to the overall impact of ischemia and reperfusion in the liver [57, 58]. The Kupffer cells are considered the chief effectors of injury in the period immediately following reperfusion; during that time they undergo degranulation and release hydrolytic enzymes that contribute to cell injury and death [59]. In addition, KC are the main producers of ROS in early reperfusion [60], which creates an environment of oxidative stress that exacerbates the sublethal SEC injury established during ischemia [42, 47-49], triggering apoptosis [47].



Studies of IR injury *in vitro* have used a variety of methods to approximate the loss of an oxygenated blood supply and its subsequent restoration [61-66]. Lee *et al.* designed a parallel-plate flow chamber to provide a continuous flow of culture medium over human microvascular endothelial cells; ischemia is simulated by the cessation of flow for 0.5 h or 1 h and reperfusion is initiated with flow restoration [61]. This model of IR resulted in increased levels of chemokine and adhesion molecule mRNA, indicating endothelial cell activation in response to blood flow alteration [61]. Emadali *et al.* assessed the functional consequences of changes to bile canaliculi integrity in hepatocyte cell lines subjected to *in vitro* IR [62]. Cultures are incubated for 1 h in prechilled University of Wisconsin preservation solution supplemented with antimycin A to chemically deplete cellular adenosine triphosphate (ATP) and induce ischemia; the exchange of this solution with prewarmed culture medium approximates reperfusion [62]. This model combines chemical anoxia with thermal shock and results in a loss of hepatocyte viability following treatment [62]. Using an *in vivo* model of renal IR injury, Baldwin *et al.* observed increased mRNA expression of the ephrin receptor, EphA2 and subsequently detected upregulation of this receptor in renal tubular epithelial cell lines following two different models of *in vitro* IR [63]. In one model, cells are incubated for 1 to 4 h in a glucose-free buffer supplemented with both antimycin A and 2-deoxyglucose to deplete cellular energy stores and mimic ischemia; reperfusion is imitated when fresh complete medium is provided to the cells [63]. Otherwise, renal cells in culture medium are incubated for 2 h at 37°C in a modular incubator chamber flushed with nitrogen to simulate ischemia; the removal of the cells from the hypoxic environment initiates reoxygenation and models reperfusion [63].

Inauen *et al.* studied IR injury of isolated coronary microvasculature endothelial cells by exposing cultures to an anoxic environment for 0.5 to 4 h at 37°C to produce ischemia; reperfusion was represented by reoxygenation of the cells and exchange of the culture medium with buffered saline [64]. This treatment of oxygen deprivation and reintroduction results in cellular lysis and detachment, indicating injury. [64]. Umansky *et al.* assessed the ability of two compounds to inhibit apoptosis of primary rat neonatal cardiomyocytes induced by IR [65]. In this model, cells in serum- and glucose-free medium were incubated in an airtight chamber containing oxygen-free gas for 8 h at 37°C to mimic ischemia and reperfusion was initiated when cultures were provided complete medium and returned to a normoxic environment [65]. Using hepatocytes isolated from transgenic mice, Suzuki *et al.* examined the effect of free radical scavenger activity on peroxide product production, a marker of cell injury, following *in vitro* IR [66]. Ischemia is simulated when cells in serum-free medium are placed in an environment which is subsequently rendered anaerobic for 1.5 h and reperfusion is approximated by the reoxygenation of these cultures [66].

These models of *in vitro* IR do not incorporate the various phases of injury which occur in the clinical situation of organ transplantation and include a significant period of cold ischemia followed by a shorter period of warm ischemia and subsequent reperfusion. The model of flow cessation and restoration consists of a brief period of media deprivation in a normoxic, 37°C environment and does not examine the cellular response to changes in oxygen concentration or temperature [61]. Two different models supplemented glucose-free solutions with inhibitors of cellular respiration to produce a

brief period of ischemia followed by the reintroduction of complete culture medium [62, 63]. Chemical depletion of ATP mimics the reduction in cellular energy stores which occurs during preservation, however, ischemia is brief in both of these models (not longer than 4 h) [62, 63]. When this ischemia is combined with hypothermia, there is not also a period of warm ischemia prior to the initiation of reperfusion [62]. Four models simulate ischemia by placing cultured cells in an enclosed environment which is depleted of oxygen and kept at 37°C [63-66]; two of these models also incorporate nutrient deprivation during this period [65, 66]. Reperfusion is initiated by the return of the cultures to a normoxic environment, accompanied by the provision of fresh medium or saline in some instances. As the oxygen deprivation is not accompanied by hypothermia, these models may not provide sufficient information about the injury caused during cold preservation of organs. There is currently no model that incorporates cold preservation, warm ischemia and reperfusion; multiple periods of injury that occur during the process of organ transplantation.

There are factors, in addition to those already discussed, that affect IR injury in livers with substantial steatosis as compared to non-steatotic grafts. Fatty infiltration of parenchymal cells causes their enlargement, which compresses the area within the sinusoidal space [67, 68]. This has the twofold effect of decreased blood flow to the tissue prior to organ removal, contributing to pre-preservation injury, as well as impaired flow during reperfusion, prolonging tissue ischemia for some areas [67-69]. Additionally, FA accumulation can impair cellular functions, as evidenced by reduced oxidative phosphorylation activity of mitochondria isolated from steatotic rat livers when compared

to those isolated from non-steatotic grafts after cold preservation and reperfusion [67]. Furthermore, while Kupffer cell phagocytic activity was increased in both normal and steatotic grafts following cold preservation and reperfusion, it is enhanced in those livers having significant fatty infiltration [68]. In addition, while hepatocytes in normal livers are resistant to injury from cold preservation, significant hepatocyte death is observed in fatty livers subjected to the same length of cold preservation [69]. This evidence supports the observation that steatotic grafts are more severely affected by the process of IR, and therefore at greater risk of functional impairment or loss of graft following OLT [4, 5, 12, 15, 16, 70]. Moreover, when steatosis is alleviated or prevented by administration of pharmacologic strategies, genetic manipulation of animals or by the administration of peptide therapy techniques, studies report a reduction in the injury caused by ischemia and reperfusion [71-73].

#### **1.4 Heme Oxygenase-1**

The production of ROS is a cytotoxic condition that stimulates cellular defense mechanisms against oxidative damage. One such response to oxidative stress is the induction of heme oxygenase-1 (HO-1), also known as heat shock protein 32 (Hsp32), which Keyse and Tyrrell reported as the predominant stress protein induced in response to exogenous hydrogen peroxide treatment [74]. Additionally, HO-1 induction by the toxic trace metal cadmium is preceded by an initial oxidative stress in rat livers [75]. Furthermore, HO-1 expression was induced in hepatocytes when associated with disease conditions such as fatty degeneration or liver malignancies [76]. In addition, following ischemia and reperfusion, HO-1 gene expression, protein expression and enzymatic

activity were significantly increased when compared to controls [77-79]. HO-1 is an inducible, rate-limiting enzyme involved in the catabolism of the heme portion of the hemoglobin molecule [40, 80], consequently producing the bile pigment bilirubin as a by-product [80], which is transported in the blood bound to plasma albumin [81]. This albumin-bound bilirubin is a free radical scavenger, and is therefore protective against cellular damage from ROS due to its antioxidant activity [79, 82]. An additional by-product of heme catabolism is carbon monoxide (CO), which is also known to be protective following hypothermic ischemia [83]. HO-1 expression has therefore been associated with IR in liver transplants, which causes oxidative stress [84]. Specifically, the degree of HO-1 upregulation in human livers following OLT is important in conferring hepatoprotection during transplantation. Evidence for this was presented by Geuken *et al.* who showed that an increase in HO-1 expression from pre-transplant to post-reperfusion resulted in fewer indicators of hepatic injury than an initially high level of HO-1, which decreased slightly but remained high post-transplant (no induction of HO-1 expression during the process of OLT) [84].

Further evidence exists to support a beneficial role for HO-1 in transplantation. For example, when HO-1 is upregulated as a consequence of pharmacological or chemical therapies, significant reduction in post-IR necrotic areas and sinusoidal congestion, as well as attenuation of hepatic microcirculation disturbances have resulted [85, 86]. Additionally, upregulation of HO-1, due to manipulation of an upstream gene product, was associated with reduced hepatic IR injury and improved overall survival [87]. Gene transfer of HO-1 directly into donor grafts also increases HO-1 protein

expression and enzymatic activity, and results in a reduced number of apoptotic cells, decreased expression of adhesion molecules, inhibition of chronic rejection, and prolonged post-transplant survival [88-90]. Researchers have also demonstrated that exogenous HO-1 can be protective in a model of cardiac IR injury [91], and that gene transfer of HO-1 into donor rats with hepatic steatosis prior to OLT improves recipient survival rates and markers of liver functions [92]. Moreover, the administration of methylene chloride, which increases endogenous production of CO, also resulted in protection from IR injury [88, 90], while inhibition of HO-1 results in a loss of hepatoprotection from IR injury [87, 89].

### **1.5 Matrix Metalloproteinases**

In addition to HO-1 playing a pivotal role in injury associated with organ transplantation, the matrix metalloproteinases (MMPs) have also been shown to be released and activated during ischemia and reperfusion [45, 93] and are therefore an important factor in mediating IR injury. The MMPs are members of the metzincins superfamily of zinc-based proteases, which also includes a disintegrin and metalloproteinases (ADAMs) and ADAM with thrombospondin motif (ADAMTS) [94]. The MMPs are a diverse family of proteases which degrade all components of the extracellular matrix (ECM), including collagen, fibronectin, gelatin, and elastin [94, 95]; they are, therefore, important regulators of physiological processes such as embryonic development, wound healing and angiogenesis, as well as cell migration, invasion, proliferation and apoptosis [94, 96]. Various factors have been shown to influence their transcription, including hormones, growth factors, oncogenes and cytokines [95]. All

MMPs share structural homology including an N-terminal propeptide domain, a catalytic domain, and most have a C-terminal hemopexin/vitronectin domain, while the membrane-type MMPs also have a transmembrane domain (Figure 2) [95, 96]. The MMPs are synthesized and secreted as proenzymes (zymogens), becoming activated after cleavage of the propeptide sequence [94]. The interaction of a cysteine residue within the propeptide and the zinc portion of the catalytic site is thought to maintain the enzyme in an inactive form [96]. Activation of the enzyme occurs when disruption of this interaction triggers the cysteine switch mechanism [94, 96], typically by extracellular protease activity [95], although ROS are able to activate several MMPs *in vitro* [97]. Additional regulation of MMPs is accomplished by the tissue inhibitors of metalloproteinases (TIMPs), which can bind to both the latent and active forms, thereby inhibiting MMP activation, or enzymatic activity [95]. In the liver, MMP expression is detectable in all cell populations, including the chief producer of ECM, the stellate cells (SC), the SEC, the KC, and the parenchymal cells (the hepatocytes) [98].

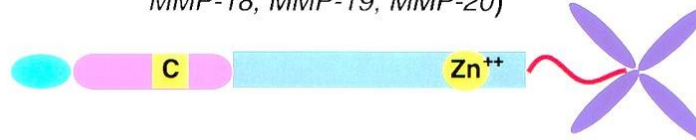
Matrix metalloproteinase activity also plays a fundamental role in various pathological conditions such as tumour invasion and metastasis and hepatic fibrosis [94, 95, 98]. While most MMP-knockout animals develop normally, when challenged with injury or other stimuli, their inflammatory response is greatly altered [99]. In addition to the degradation of the ECM, MMPs can have an effect on the biological functions of chemokines and cytokines, including TNF- $\alpha$  [94, 99]. Biologically active TNF- $\alpha$  is released following proteolytic cleavage of the membrane-anchored TNF- $\alpha$  precursor pro-TNF- $\alpha$ ; this is achieved predominantly by ADAM-17, which is also known as TNF- $\alpha$

A MMPs with minimal domain (*MMP-7*)

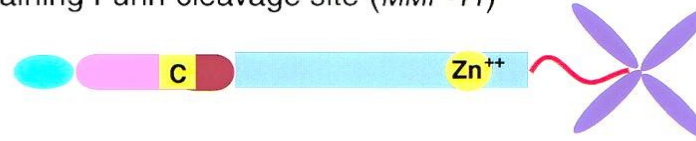


B MMPs with Hemopexin/Vitronectin domain

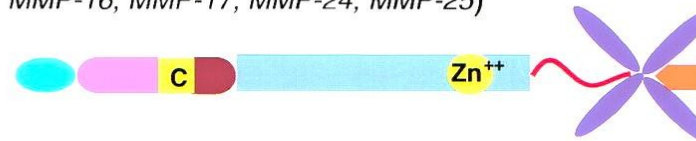
Simple (*MMP-1, MMP-3, MMP-8, MMP-10, MMP-12, MMP-13, MMP-18, MMP-19, MMP-20*)



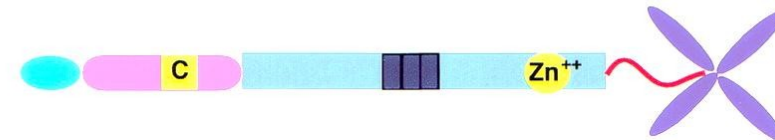
Containing Furin-cleavage site (*MMP-11*)



Transmembrane with Furin-cleavage site (*MMP-14, MMP-15, MMP-16, MMP-17, MMP-24, MMP-25*)



Containing Fibronectin type II repeat (*MMP-2, MMP-9*)



**Figure 2. The Structural Domains of the Matrix Metalloproteinases.** The MMPs share structural homology including a propeptide domain (pro-domain) that contains a conserved sequence with a cysteine residue which interacts with the catalytic domain maintaining the enzyme in latent form (cysteine switch). The fibronectin type II repeats in the catalytic domain of the gelatinases (*MMP-2* and *MMP-9*) bind gelatin. Except for *MMP-7*, the MMPs also have a C-terminal hemopexin/vitronectin domain, while some also have a transmembrane domain. Adapted from Vu and Werb, 2000 [96]



converting enzyme (TACE) [94, 99], but can also be performed by additional MMPs including MMP-1, MMP-7 and MMP-9 [100]. Furthermore, MMP expression is induced by TNF- $\alpha$  during injury [98], indicating their central role in the progression of inflammatory conditions [99]. Evidence to support this is provided by Wielockx *et al.* who demonstrated that inhibition of MMPs prevented death of animals that had been given a lethal dose of TNF- $\alpha$  [101]. Additionally, MMPs have been implicated in the development of hepatic steatosis, as inhibition of MMPs resulted in increased expression and activation of PPAR $\alpha$ , unaltered levels of SREBP-1c, improved hepatic clearance of fat as compared to steatotic livers and decreased levels of liver injury markers [102].

MMPs have also been implicated in IR injury [45, 47, 93, 103]; protease activity is detectable in effluents from cold preserved livers and increases with increasing time of preservation [45], and MMP activity is increased in the serum of animals following warm hepatic IR [93]. Moreover, MMP expression is likely mediated by oxidative stress during IR, as evidenced by a reduction in serum MMP activity when the oxygen free radical scavenger N-acetylcysteine was administered prior to warm hepatic IR [93]. The injury to liver SEC caused during ischemia and hypothermia is attributed to the activity of proteases [47], as isolated SEC secrete MMPs when cultured in cold preservation solution [45]. Furthermore, when MMP activity is inhibited during hepatic IR, the indicators of organ damage and cell death are reduced [104]. Broad inhibition of MMPs is also hepatoprotective during periods of prolonged cold ischemia followed by warm reperfusion [105]. In 2002, Cursio *et al.* examined the expression profile of MMPs following reperfusion in a rat model of liver IR and observed that select MMPs

(including MMP-9) had increased expression immediately after IR, while a different set of proteases (including MMP-2) showed a delayed increase in expression [104]. They therefore proposed that MMPs which were upregulated immediately following IR were likely to be influencing detrimental processes such as inflammation, and consequently cell death, while those enzymes which were induced much later following IR, were more likely to be affecting beneficial processes like liver repair or regeneration [104].

### **1.6 Matrix Metalloproteinase-9**

The expression profile of MMPs in response to hepatic IR [104] implicated matrix metalloproteinase-9 (MMP-9) in the progression of post-reperfusion inflammation and resultant cell death. MMP-9 expression and activity, in particular, was upregulated in models of hepatic and myocardial IR injury [45, 93, 106-110] and is also associated with metastatic tumours [111]. Furthermore, Wielockx *et al.* observed that a genetic deficiency in several MMPs, including MMP-9, prevented death of animals that had been given a lethal dose of TNF- $\alpha$  [101]. Matrix metalloproteinase-9 is synthesized as a 92 kDa inactive proenzyme which requires cleavage of the propeptide sequence for activation [112]. The resultant 82 kDa gelatinase is specific for substrates such as gelatin types I and V, and collagen types IV and V [95], and physiologically, MMP-9 has a significant role in cell migration during development [96]. Elevated levels of MMP-9 expression have also been found in connection with liver regeneration following hepatectomy [113], and hepatocyte proliferation *in vitro* [114].

## 1.7 The Role of Matrix Metalloproteinase-9 in Ischemia Reperfusion Injury

Although MMP-9 expression is associated with beneficial processes including liver regeneration, it has also frequently been implicated in the injury caused by IR [45, 93, 106-109]. The catalytic domain structure of pro-MMP-9 is highly conserved with that of pro-MMP-2, the other gelatinase; therefore, inhibition of MMP-9 enzymatic activity would likely also affect MMP-2 activity [112]. Several studies have, therefore, assessed gelatinase activity following hepatic IR injury in order to determine their individual roles. In 2006, Khandoga *et al.* noted enhanced MMP-9 activity following IR, while MMP-2 activity was undetectable [106], and in 2010, Ma *et al.* observed increased expression and activity of MMP-9 after OLT using small-for-size grafts in rats, while MMP-2 levels were unaffected by this type of IR injury [108]. Both groups also noted that inhibition of gelatinase activity during IR reduces hepatocellular necrosis and markers of liver injury and improves post-IR survival, concluding that the protective effects were due specifically to decreased MMP-9 activity [106, 108]. In support of these conclusions, murine investigations with animals that were deficient for MMP-9 had previously been shown to provide myocardial protection from IR injury [109]. Furthermore, Hamada *et al.* assessed the level of protection against injury in a murine model of hepatic IR using three different methods of MMP-9 inhibition and noted that genetic deficiency of MMP-9 or treatment with a neutralizing antibody against MMP-9 conferred superior protection against injury than did mixed inhibition of both MMP-2 and MMP-9 [107]. Specific inhibition of MMP-9 is therefore a worthy goal for therapies designed to reduce injury caused by ischemia and reperfusion.

## 1.8 Research Rationale

The accumulation of fat in hepatocytes is a pathological condition that can cause a downstream inflammatory response. Additionally, organ transplantation causes injury from the processes of ischemia and reperfusion. A standardized model for studying *in vitro* IR is not currently in use; therefore the first aim of this research is to design a model to study the effect of preservation or ischemia followed by reperfusion on cellular mRNA and protein profiles. Furthermore, the model is designed to incorporate the use of hepatocyte cell lines which have pre-existing steatosis in order to determine specific injury that could be attributed to the accumulation of fat prior to IR.

The method of transplantation, specifically the *ex vivo* storage of the organ, provides a unique opportunity for the application of therapies aimed at reducing the disease burden or IR stress to these organs. This research aims to produce gene therapy options designed to increase hepatoprotection or decrease liver injury following IR. Initially, lentivirus vectors were designed and constructed to overexpress HO-1 sequences, then additional vectors that contained short hairpin RNA (shRNA) sequences and small interfering RNA (siRNA) were purchased to knockdown MMP-9 gene expression. These gene therapy techniques were assessed *in vitro* for their ability to affect gene and protein expression, as an indication of their potential to be applied during organ transplantation.

## **CHAPTER 2: MATERIALS AND METHODS**

### **2.1 Cell Lines**

HepG2 human hepatocellular carcinoma cells and McA-RH7777 rat hepatoma cells were purchased from American Type Culture Collection (ATCC; Manassas, VA). Human umbilical vein endothelial cells (HUVEC) were provided by Dr. J-F. L egar e (Dalhousie University, NS), J774 mouse macrophage cells were provided by Dr. Tim Lee (Dalhousie University, NS) and 293T human kidney fibroblast cells were provided by Dr. Craig McCormick (Dalhousie University, NS).

### **2.2 Culture Medium**

The HepG2s, McA-RH7777s, J774s and 293Ts were cultured in Dulbecco's Modified Eagle's Medium (DMEM; ATCC, Manassas, VA and Gibco, Grand Island, NY) supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS; HyClone, Logan, UT), 100 U/mL of penicillin (Gibco) and 100 µg/mL of streptomycin (Gibco) (complete DMEM, cDMEM). The HUVECs were cultured in Endothelial Basal Medium (EBM™) containing supplements provided by the supplier (Lonza Inc., Walkersville, MD) that included hydrocortisone, hEGF, FBS, VEGF, hFGF-B, R3-IGF-1, ascorbic acid, heparin and gentamicin/amphotericin-B (Endothelial Growth Medium, EGM™).

### **2.3 Cell Culture**

All cell cultures were grown in 75cm<sup>2</sup> tissue culture flasks (Corning Incorporated, Corning, NY) and were maintained at 37°C in a 5% CO<sub>2</sub> humidified atmosphere. The

HepG2 cells were passaged at 70-90% confluency using 0.05 % trypsin with EDTA•4Na (trypsin; Gibco) for 7 min at 37°C, and the McA-RH7777 cells were passaged before 70% confluency using trypsin for 2 min at 37°C. The HUVECs and 293Ts were passaged at 70-90% confluency using trypsin for 2 min at 37°C. The J774s were passaged at 70-90% confluency using trypsin for 2 min at room temperature (RT), followed by gentle scraping of the flask surface with a cell scraper.

## **2.4 Reagents**

$\beta$ -Mercaptoethanol, Tris Base, and Tetramethylethylenediamine (TEMED) were purchased from Bio-Rad Laboratories Ltd. (Mississauga, ON). Ethylenediaminetetraacetic acid (EDTA), sodium fluoride (NaF), bovine serum albumin (BSA), dimethylsulfoxide (DMSO) and isopropanol were purchased from Sigma-Aldrich Canada (Oakville, ON). Phosphate buffered saline (PBS) was purchased from Gibco, sodium chloride (NaCl) was purchased from EMD Biosciences (San Diego, CA), sodium dodecyl sulphate (SDS) was purchased from Boehringer Mannheim, (Germany) and SOC Outgrowth Medium was purchased from New England Biolabs, Ltd. (Pickering, ON). Sodium deoxycholate (Sigma-Aldrich) and Nonidet P-40 (NP-40; Fluka Biochemika, Buchs, Switzerland) were generously provided by Dr. Craig McCormick.

## **2.5 Antibodies**

Mouse anti- $\beta$ -actin monoclonal antibody (mAb) and mouse anti- $\beta$ -tubulin mAb were purchased from Sigma-Aldrich. Rabbit anti-GAPDH polyclonal antibody (Ab) was

purchased from Open Biosystems (Huntsville, AL). Rabbit anti-HO-1 Ab was purchased from Stressgen Biotechnologies Corporation (Victoria, BC). Rabbit anti-MMP-9 Ab was purchased from Millipore Corporation (Temecula, CA). Rabbit anti-PPAR $\alpha$  Ab was purchased from Abcam Inc. (Cambridge, MA). Rabbit anti-SREBP-1 Ab was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG and HRP-conjugated horse anti-mouse IgG were purchased from Vector Laboratories (Burlingame, CA).

## **2.6 Trypan Blue Exclusion Assay for Cell Viability**

The viability of HepG2 and McA-RH7777 cells was assessed using the Trypan Blue dye exclusion test. In this test, a cell suspension is mixed with Trypan Blue dye and examined; those cells with no dye uptake must possess an intact cell membrane and are counted as viable. Culture medium was discarded from cells that had been plated in a 6-well tissue culture dish (BD Falcon, Franklin Lakes, NJ), monolayers were washed with 1X PBS and treated with 0.5ml trypsin at 37°C for 2-3 min. Trypsin was neutralized with 0.5ml cDMEM and the cells were collected by centrifugation at 1000  $\times$  g for 5 min. The cell pellet was resuspended in 1ml cDMEM; 20 $\mu$ l of the cell suspension was diluted 1:5 in Trypan Blue Stain (Sigma-Aldrich), and cell counts were performed using a Haemocytometer. Live cells were counted and percent viability was calculated as  $(\text{number cells treatment}) / (\text{number cells control}) \times 100$ .

## 2.7 MTT Assay for Cell Viability

The colorimetric 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay was used to determine cell viability. This system measures the reduction of the tetrazolium component (MTT) into an insoluble, coloured formazan product by the mitochondria of viable cells. Following incubation with the MTT reagent, cells are lysed and the formazan is solubilized using a detergent solution. The absorbance is measured spectrophotometrically and the amount of colour produced is directly proportional to the number of viable cells. Supernatants were removed from cells that had been plated in a 96-well flat bottom tissue culture dish (Corning Incorporated), and 20  $\mu$ l of MTT solution (Sigma-Aldrich; 5 mg/ml in PBS) was added to each well, as well as in triplicate empty wells for control. Plates were then covered with foil and incubated for 2.5 h at 37°C in a 5% CO<sub>2</sub> humidified atmosphere. The MTT solution was exchanged for 100  $\mu$ l of DMSO (Sigma-Aldrich), the plate was re-wrapped in foil and shaken for 5 min at RT and the absorbance was measured at a wavelength of 490 nm using an ELx800 Microplate Reader (Bio-Tek Instruments, Inc., Winooski, VT) and associated Gen5 software. Percent viability was calculated as (mean absorbance treatment) / (mean absorbance control)  $\times$  100.

## 2.8 WST-1 Assay for Cell Viability

The colorimetric 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (WST-1) assay (Roche Applied Science, Mannheim, Germany) was used to determine cell viability. This assay measures the production of a soluble formazan product following the reduction of a tetrazolium salt (WST-1) occurring



primarily at the cell surface of viable cells. Following incubation with the WST-1 reagent, the absorbance is measured directly and the amount of colour produced is directly proportional to the number of viable cells. Ten microlitres of WST-1 reagent was added to each well of a 96-well flat bottom tissue culture dish that contained cells, as well as to 3 wells containing only culture medium. Cultures were then incubated for 3.5 h at 37°C, shaken for 1 min, and the absorbance was measured at a wavelength of 420 - 480 nm using a Bio-Tek microplate reader and a reference wavelength of > 600 nm. Percent viability was calculated as (mean absorbance treatment) / (mean absorbance control) × 100.

## **2.9 Preparation of Total Cellular Lysates**

Total cellular protein content of HepG2, McA-RH7777, HUVEC, J774 and 293T cells was collected from whole cell lysates. Cellular supernatants were removed, monolayers were washed once with 4°C 1X PBS, treated with a volume of 4°C RIPA lysis buffer (50 mM Tris (pH 7.4), 1 % NP-40, 0.25 % sodium deoxycholate, 150 mM NaCl, 1 mM EDTA (pH 8.0), 0.1 % SDS and 50 mM NaF) appropriate to the size of dish being used, and rocked on ice for 15 min. Lysates were pulled through a 21-gauge needle 2 - 3 times, incubated on ice for 30 min and subjected to centrifugation at 4°C at 10,000 × g for 10 min. The protein-containing supernatants were collected and stored at -20°C.

## **2.10 Western Blot Analysis**

The Western blotting method was used to compare the amount of a specific

protein produced by control or treated cells. Cellular lysates are subjected to gel electrophoresis under denaturing, reducing conditions to separate proteins by size. Separated proteins are then transferred onto a membrane and antibodies are used to indirectly detect a target protein. This technique can provide information about the relative amount of a protein of interest when the intensity of a detected protein is compared to the intensity of a structural protein. The concentration of protein was measured for each cellular lysate collected, using a Bradford protein assay (Bio-Rad Laboratories Ltd.). An equal amount of protein (10 - 25  $\mu$ g) was resolved on a 10 or 12 % SDS-polyacrylamide gel, depending on the size of the protein being detected. The gel was subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) for 1 - 2 h at 115 V for 10 % gels or 150 V for 12 % gels. The proteins were then transferred onto a polyvinylidene fluoride (PVDF; Millipore Corporation) membrane using an electrophoretic tank transfer system (Bio-Rad Laboratories Ltd.), at 115 V for 75 min with gentle stirring. Membranes were then blocked for 30 min at RT in Tris-buffered saline (TBS; 10 mM Tris (pH 8.0), 150 mM NaCl) containing 0.05 % Tween-20 (TBST) and 5 % (w/v) skim milk powder (Carnation). The membranes were washed once with TBST for 5 min at RT and incubated in the appropriate primary Ab overnight at 4°C, or for 3 h at RT. Primary antibodies were prepared as follows, using primary antibody dilution buffer (5 % BSA in TBS, containing 0.05 % sodium azide): anti-GAPDH Ab as a 1:2000 dilution; anti-HO-1 Ab as a 1:1000 dilution; anti-MMP-9 Ab as a 1:2000 dilution; anti-PPAR $\alpha$  Ab as a 1:100 dilution; and anti-SREBP-1 Ab as a 1:200 dilution. Membranes were then washed 3 times for 5 min each with TBST and probed with the HRP-conjugated goat anti-rabbit IgG Ab, diluted 1:10,000 in TBST containing 5 % (w/v)

skim milk powder, for 1 h at RT. The membranes were again washed 3 times with TBST for 5 min, followed by a single 5 min wash using TBS. Protein bands were visualized using an enhanced chemiluminescence (ECL) Western blotting detection reagent (ECL Plus; GE Healthcare, Baie d'Urfe, QC) and a Kodak Image Station 4000mm Pro (Carestream Molecular Imaging, Woodbridge, CT) provided by Dr. Craig McCormick. As a control for equal protein loading, blots were re-probed with either anti- $\beta$ -actin mAb (1:10,000) or anti- $\beta$ -tubulin mAb (1:5000) for 3 h at RT, followed by washing, probing with the HRP-conjugated horse anti-mouse IgG Ab (1: 20,000), washing and visualization as described above. Expression of protein of interest was determined relative to the expression of either  $\beta$ -actin or  $\beta$ -tubulin by densitometry assessment of images having no band saturation using AlphaEase<sup>®</sup> FC software (Alpha Innotech Corporation, now Cell Biosciences, Inc., Santa Clara, CA). Percent change in protein expression due to treatment was calculated as  $(\text{mean treatment} / \text{mean control}) \times 100$ .

### **2.11 Zymogram Gel Analysis**

Substrate zymography was used to assess the gelatinolytic activity of proteins produced by control or treated cells. Cellular lysates or supernatants are subjected to gel electrophoresis under denaturing, nonreducing conditions to separate proteins by size within a gelatin-containing polyacrylamide gel. The separated proteins are able to renature and recover their activity during a wash step while the development period allows enzymatic degradation of the substrate to occur. Staining of the gel reveals clear bands in areas of gelatin digestion against a background of darkly stained undegraded substrate. Equal amounts of protein (15 - 25  $\mu\text{g}$ ) were resolved on a 10 % SDS-

polyacrylamide gel containing gelatin (Bio-Rad Laboratories Ltd.), using SDS-PAGE for 2 - 3 h at 120 V. Gels were then washed twice with rinse buffer (2.5% Triton X-100) for 15 min at RT, 3 times (briefly) with distilled water, and incubated in a 1:10 dilution of 10X Zymogram Development Buffer (50 mM Tris-HCl (pH 7.5), 200 mM NaCl, 5 mM CaCl<sub>2</sub>, 0.02% Brij-35; Bio-Rad Laboratories Ltd.) at 37°C for 16 or 40 h. Gels were then stained using Coomassie staining solution composed of 0.5 % Coomassie Brilliant Blue G-250 stain (Bio-Rad Laboratories Ltd.), 40 % methanol (Fisher Scientific Company, Ottawa, ON) and 10 % acetic acid (BDH Inc., Toronto, ON) for at least 1 h at RT. Gels were then destained in a solution of 40 % methanol and 10 % acetic acid, for 30 - 60 min at RT. Gels were visualized using an AlphaImager<sup>®</sup> (Alpha Innotech Corporation) according to manufacturer's recommendations.

## **2.12 Steatosis Treatment of Liver Cell Lines**

HepG2 and McA-RH7777 cells were incubated with a steatosis-inducing medium in order to allow accumulation of lipids in the cytoplasm. Steatosis medium was prepared using DMEM, and contained 100 U/mL of penicillin, 100 µg/mL of streptomycin, 1 % (w/v) Fatty Acid Free BSA (Sigma-Aldrich), 0.66 mM Oleic acid sodium salt (Sigma-Aldrich) and 0.33 mM Palmitic acid sodium salt (Sigma-Aldrich). Liver cells were either plated into a chamber slide using manufacturer's recommendations, or transferred to a fresh 75cm<sup>2</sup> tissue culture flask, and allowed to adhere for 24 - 30 h. Culture medium was exchanged for either Serum-Free medium (DMEM containing 100 U/mL of penicillin and 100 µg/mL of streptomycin), or Opti-MEM<sup>®</sup> I reduced serum medium (Gibco) and cells were incubated overnight at 37°C in a 5% CO<sub>2</sub> humidified atmosphere. Treatment

cells then received steatosis medium, while control cells received cDMEM, and all cells were returned to the 37°C environment for 24 h. Following the incubation, cells were fixed for staining, plated into chamber slides, plated in tissue culture dishes, or provided fresh cDMEM, and returned to the 37°C, 5% CO<sub>2</sub> humidified atmosphere.

### **2.13 Histological Analysis of Liver Cell Lines**

Liver cells that had been treated with steatosis medium and their respective controls were plated in the wells of a chamber slide and allowed to adhere overnight; otherwise HepG2 and McA-RH7777 cells that had been previously plated in chamber slides received a control or steatosis treatment. For general histology slides were stained with Harris' Haematoxylin and 0.5% Eosin (H & E) using a standard protocol; for glycogen content slides were stained with a Periodic Acid-Schiff (PAS) kit (Sigma-Aldrich) following manufacturer's protocol. For lipid content, slides were stained using Oil Red O (ORO; Sigma-Aldrich) where cells were fixed at RT using 10 % formalin for 5 min, then fresh 10 % formalin for at least 1 h. Slides that were not immediately stained were kept at 4°C in 10 % formalin until use. These slides were rinsed with tap water, washed with a 60 % isopropanol solution, and stained with a freshly prepared 60 % ORO solution for 15 min at RT. Slides were washed with 60 % isopropanol, stained using Mayer's Haematoxylin, rinsed in distilled water and mounted using an aqueous medium. Images were captured using a Zeiss Imager.A1 light microscope and attached Canon PowerShot A640 digital camera.

## **2.14 *In Vitro* Cold Preservation Injury or Ischemia and Reperfusion Assays**

For the cold preservation injury assay, HepG2 and McA-RH7777 cells were plated in a 6-well tissue culture dish (BD Falcon) at a density of  $3 \times 10^5$  cells/well and incubated overnight at 37°C in a 5% CO<sub>2</sub> humidified atmosphere. The cell culture medium was then replaced with either 4°C cDMEM, or 4°C Histidine-Tryptophan-Ketoglutarate (HTK) organ preservation solution (Custodiol, Methapharm Inc., Brantford, ON, provided by Dr. Ian Alwayn) and incubated at 4°C in room air for 1 h. Control plates were given warmed cDMEM and returned to the 37°C, 5% CO<sub>2</sub> humidified atmosphere for 1 h. Pre-warmed cDMEM was then provided to all plates and cells were returned to the 37°C, 5% CO<sub>2</sub> humidified atmosphere for 2 h to recover.

For the cold ischemia and reperfusion assay, HepG2 and McA-RH7777 cells were plated either in triplicate wells of a 96-well flat bottom tissue culture dish (Corning Incorporated) at a density of  $5 \times 10^4$  cells/well or in a 6-well tissue culture dish at a density of  $3 \times 10^5$  cells/well and incubated for 16 - 24 h at 37°C in a 5% CO<sub>2</sub> humidified atmosphere in order to adhere. Cells were then treated with HTK organ preservation solution that had been preconditioned by incubation at 4°C in an anoxic gas mixture (4.98 % CO<sub>2</sub>, 5.22 % H<sub>2</sub> and 89.8 % N<sub>2</sub>; Praxair Inc., Danbury, CT), placed in a modular incubator chamber (Billups-Rothenberg, Del Mar, CA) which was then flushed with the anoxic gas mixture (20 L/min) for 4 min, and incubated at 4°C for 2 - 72 h. Control plates were given warmed cDMEM and returned to the 37°C, 5% CO<sub>2</sub> humidified atmosphere for 2 - 72 h. All plates were then provided with cDMEM and returned to the 37°C environment for 2 - 24 h of reperfusion.

For the cold ischemia plus warm ischemia and reperfusion assay, HepG2s, McA-RH7777s, or liver cells having received a steatosis treatment and their respective controls were plated at a density of  $3 \times 10^5$  cells/well in a 6-well tissue culture dish and allowed to adhere for 16 - 24 h. Cells were then either provided with anoxic, 4°C HTK and incubated in an anoxic gas mixture (cold ischemia), or provided with normoxic, 4°C HTK and incubated in air (cold preservation). Both treatments were maintained at 4°C for either 6 or 9 h before being transferred to 37°C for either 30 min or 1 h of warm ischemia (or warm preservation). HTK solution was exchanged for either warmed cDMEM, or warmed Opti-MEM<sup>®</sup> I reduced serum medium, and plates were returned to the 37°C, 5% CO<sub>2</sub> humidified atmosphere for 3 h of reperfusion. Control cells were provided with warmed cDMEM and incubated for either 9.5 or 13 h at 37°C in a 5% CO<sub>2</sub> humidified atmosphere. Alternatively, control cells were incubated in warmed cDMEM for the initial 6.5 h, provided with warmed Opti-MEM<sup>®</sup> I reduced serum medium, and returned to the 37°C, 5% CO<sub>2</sub> humidified atmosphere for the final 3 h.

## **2.15 Generation of Lentiviral Plasmids for HO-1 Expression**

Plasmid pLEX-MCS (Open Biosystems) was used to create lentiviral plasmids which would express either human HO-1 (pLEX-H-HO-1), or rat HO-1 (pLEX-R-HO-1) in mammalian cells. Lentiviral plasmids are delivered to target cells and the sequence of interest irreversibly integrates into the genome of the host cell, providing long-term expression of the coded material. These plasmids were generated by cloning the entire HO-1 DNA coding sequence from an HO-1 expression vector into the pLEX-MCS vector by way of an intermediary TOPO TA cloning step. The expression vectors used were

human HO-1 expression vector (pOTB7-HO-1; IMAGE ID 3504480; ATCC) and rat HO-1 expression vector (pExpress-1-HO-1; IMAGE ID 7384448; ATCC). Primers used to direct amplification of H-HO-1 from the pOTB7 vector using polymerase chain reaction (PCR) were Forward Primer: 5' CCT CGA GCC CGC TCA TTA GGC 3' and Reverse Primer: 5' CCT CGA GCC GCG TTC TAA CGT CG 3'. Primers used to PCR amplify R-HO-1 from the pExpress-1 vector were Forward Primer: 5' CAG CGA GTG GAG CCT GCC 3' and Reverse Primer: 5' GCT CGA GTT CAC AGC CTC TGG G 3'. Amplification was done using a *Taq* polymerase chain reaction (PCR) kit from New England Biolabs with the protocol: 4 min at 95°C, 30 cycles of (30 s at 95°C, 45 s at 60°C and 1.5 min at 72°C), followed by 10 min at 72°C. PCR product was then purified using QIAquick PCR purification kit (Qiagen, Mississauga, ON) before insertion into pCR<sup>®</sup>4-TOPO<sup>®</sup> intermediary cloning vector using a TOPO TA Cloning<sup>®</sup> Kit from Invitrogen (Burlington, ON). TOPO-H-HO-1 was then digested with *SpeI* (New England Biolabs) and *NotI* (New England Biolabs) restriction endonucleases and TOPO-R-HO-1 was digested with *XhoI* (New England Biolabs) restriction endonuclease following the manufacturer's instructions. Plasmid pLEX-MCS was also cut with *SpeI* and *NotI* together, or *XhoI* alone, and treated with an alkaline phosphatase, (calf intestinal; CIP; New England Biolabs) as per manufacturer's instructions. Digestion products were separated using migration through a 1 % agarose (Invitrogen) gel. Gel extraction of H-HO-1, R-HO-1 and linearized pLEX-MCS was performed using either QIAquick or QIAEX II gel extraction kits (Qiagen) as required for DNA fragment size. Extracted H-HO-1 insert was ligated into double-cut pLEX-MCS, while extracted R-HO-1 insert was ligated into CIP-treated, gel extracted pLEX-MCS in a 7:1 or 5:1 ratio, using T4 DNA



ligase (New England Biolabs). Plasmids were amplified by transformation of either Prime Plus™ (Dr. Craig McCormick) or One Shot® TOP10 (Invitrogen) chemically competent *E. coli* using recommended transformation procedure, and DNA was recovered using a PureLink Quick Plasmid Miniprep Kit (Invitrogen).

## **2.16 Production and Use of Lentiviral Particles**

The Trans-Lentiviral™ GIPZ Packaging System (Open Biosystems) was used to deliver shRNA targeted to MMP-9 into HepG2, McA-RH7777 and HUVEC cells. Lentiviral particles were produced in 293T cells using the Trans-Lentiviral™ GIPZ Packaging System and recommended reagents and protocols; particles contained one of 5 pGIPZ plasmids encoding a green fluorescent protein (GFP) sequence, a puromycin resistance gene and a shRNAmir sequence (Open Biosystems) that would integrate into a target cells genome following transduction. The shRNAmir sequence is adapted from the human microRNA (miRNA), miR-30 and is therefore designed to follow the pathway for the generation of naturally occurring miRNA encoded within the genome. The sequence coding for the shRNA is transcribed by RNA polymerase II, resulting in a primary transcript containing a hairpin like stem-loop structure which resembles the long primary precursor of endogenous miRNA. This primary transcript is processed in the nucleus by the dsRNA-specific RNase, Drosha, to form a pre-shRNA molecule; this is similar to the processing of a primary miRNA to generate the pre-miRNA intermediate. The hairpin RNA is exported to the cytoplasm where is it further processed by Dicer, an RNase which removes the loop of the hairpin and produces a double-stranded siRNA duplex. Duplexes interact with components of the RNA-induced silencing complex (RISC) and allow

selective incorporation of the antisense strand; gene silencing using the miRNA pathway occurs at the levels of transcription and translation as well as through degradation of the mRNA transcript. Clone Ids for shRNA against MMP-9 were: Human homology, SH-1 (V3LHS\_303164), SH-2 (V3LHS\_303168), SH-3 (V3LHS\_303167), and rat homology, R-1 (V2LMM\_50831), R-2 (V3LMM\_431868). Harvested supernatants containing the viral particles were stored at -80°C, and were titred according to manufacturer's protocol using the appropriate target cells. Human and rat liver cell lines, as well as HUVEC cells from passages 3 - 7 were plated at a density of  $2.4 - 3.8 \times 10^5$  cells/well in a 6-well tissue culture dish and allowed to adhere for 16 - 24 h. Culture medium was exchanged for viral supernatant and cells were incubated for 6 - 8 h at 37°C in a 5% CO<sub>2</sub> humidified atmosphere. Supernatants were added at a Multiplicity of Infection (MOI) of 1, 5, 10, 15 or 20, where MOI is the number of transducing units of virus per target cell. Following incubation, cells were washed once with complete medium (cDMEM or EGM-2), provided with fresh complete medium and incubated for 48 h. Following incubation, medium was exchanged for selective medium containing puromycin dihydrochloride (Mediatech, Inc., Manassas, VA) at a concentration as determined by a puromycin kill curve (manufacturer's directions): HUVEC cells - 1.5 µg/ml, HepG2 cells - 2.0 µg/ml and McA-RH7777 cells - 12.0 µg/ml. Selective medium was replaced every 2 - 3 days until GFP expression, visualized using a Zeiss Axiovert 200 fluorescence microscope and AxioCam camera (Carl Zeiss, Thornwood, NY), reached 100 % in target cells by visual inspection (2 - 4 days). Total cellular lysates were prepared as described above, and protein expression was analyzed by Western blotting as described above.

### **2.17 Transfection of siRNA**

Stealth Select RNAi™ siRNA duplexes against human MMP-9 were purchased from Invitrogen, and included Clone Ids: H-1 (HSS106625), H-2 (HSS106626) and H-3 (HSS181135). Stealth RNAi™ siRNA negative control Hi GC duplex was also purchased from Invitrogen. Synthetic siRNA enters the cytoplasm and requires no processing before interacting with the RISC; the two strands of the duplex are separated, the antisense strand is retained and RNA interference occurs solely through mRNA cleavage and degradation. HepG2 cells were seeded at a density of  $3 - 6 \times 10^4$  cells/well in a 24-well tissue culture dish (Corning Incorporated) and allowed to adhere for 16 - 24 h. siRNA was complexed in combination or alone, at varying concentrations, to Oligofectamine™ transfection reagent (Invitrogen) using Opti-MEM® I reduced serum medium and manufacturer's recommendations. Cell monolayers were washed once with Opti-MEM® I medium, provided fresh Opti-MEM® I medium; siRNA:Oligofectamine complexes were added and cells were incubated at 37°C in a 5% CO<sub>2</sub> humidified atmosphere for 4 - 6 h. Following incubation, transfection medium was exchanged for cDMEM, and cells were incubated for 24 - 72 h in a 37°C, 5% CO<sub>2</sub> humidified atmosphere. Alternatively, siRNA:Oligofectamine complexes were not removed, and cells were incubated for 24 - 72 h in a 37°C, 5% CO<sub>2</sub> humidified atmosphere. Total cellular lysates were prepared and protein expression was analyzed by Western blotting, as described above. Alternatively, RNA was extracted and gene expression was analyzed by RT-PCR, as described below.

### **2.18 Isolation of Total Cellular RNA**

Total cellular RNA was isolated from HepG2, McA-RH7777, and 293T cells

using Invitrogen's TRIzol<sup>®</sup> reagent or Qiagen's RNeasy Plus Mini Kit. Cells were plated at an unspecified density in a 6-well tissue culture dish, allowed to adhere for 16 - 24 h and used if monolayer had reached 70 % confluence. For Invitrogen's TRIzol<sup>®</sup> extraction, cellular supernatants were removed and 1 ml of TRIzol<sup>®</sup> was added, cells were incubated at RT for 5 min, mixed well to ensure total lysis, and snap frozen in liquid nitrogen. Samples were stored at -80°C before RNA was precipitated following manufacturer's directions. For Qiagen's RNeasy Plus Mini Kit, total cellular RNA was isolated from HepG2, McA-RH7777, and 293T cells following manufacturer's instructions. RNA quality was examined using a 1 % agarose gel, while purity was assessed using a NanoDrop 2000 Micro-Volume UV-Vis Spectrophotometer (Thermo Scientific, Waltham, MA) and NanoDrop 2000 software.

## 2.19 RT-PCR Analysis

Reverse transcription PCR analysis was performed by synthesizing cDNA from extracted RNA using a Superscript III First Strand Synthesis SuperMix kit (Invitrogen) and the manufacturer's instructions. PCR was performed on 25 ng of cDNA template using *Taq* DNA polymerase (Fermentas Canada Inc., Burlington, ON) and recommended reagents (1X *Taq* buffer with (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 1.5 mM MgCl<sub>2</sub>, 0.4 mM dNTP mix, 0.26 mM each forward and reverse primers, and 1.25 U *Taq* DNA polymerase). Several primer sets were used to amplify either the human or rat MMP-9 gene, or the human tubulin gene, as a control. Primer sets for each gene were designed to span or flank introns to avoid co-amplification of genomic DNA. Set H-MMP-9-1 included Forward: 5' TCT TCC CTG GAG ACC TGA GA 3' and Reverse: 5' TCA AAG GTT TGG AAT CTG CC 3' and was

expected to amplify a 244 bp sequence of the human MMP-9 gene. Set H-MMP-9-2 included Forward: 5' GAG GTT CGA CGT GAA GGC GCA GAT G 3' and Reverse: 5' CAT AGG TCA CGT AGC CCA CTT GGT C 3' and was expected to amplify a 200 bp sequence of the human MMP-9 gene. Set R-MMP-9-1 included Forward: 5' ATA TGG TTT CTG CCC CAG TG 3' and Reverse: 5' AGT TGC CCC CAG TTA CAG TG 3' and was expected to amplify a 224 bp sequence of the rat MMP-9 gene. Set H-Tubulin included Forward: 5' ATC AAG ACC AAG CGT ACC ATC C 3' and Reverse: 5' CCA ACG TAC CAG TGA ACA AAG G 3' and was expected to amplify a 227 bp sequence of the human tubulin gene. Amplification was performed using the general protocol: 2 min at 94°C, 30 - 40 cycles of (30 s at 94°C, 45 s at annealing temperature and 45 s at 72°C), followed by 10 min at 72°C. The annealing temperature was determined for each set of primers and was based on the melting temperatures provided by the manufacturer. PCR products were examined on a 1.5 % or 2 % agarose gel and visualized with an AlphaImager<sup>®</sup> HP imaging system according to manufacturer's recommendations.

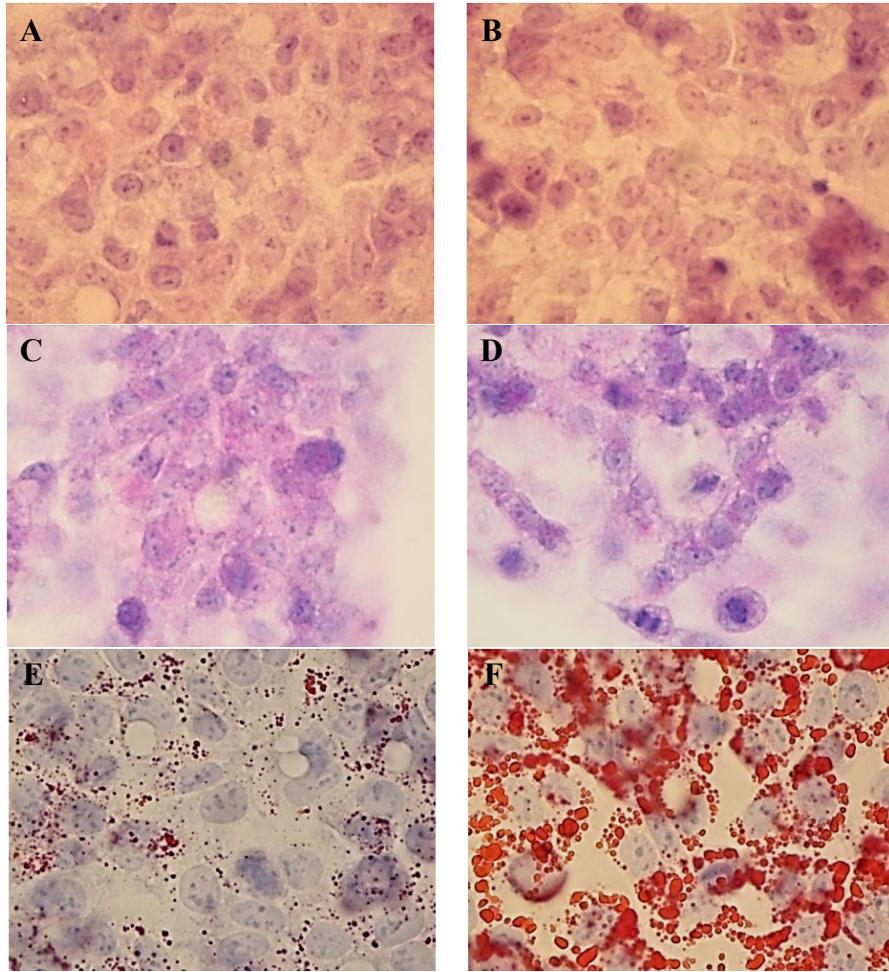
## **2.20 Statistical Analysis**

Data were analyzed using GraphPad Prism statistics program, version 5.00 for Windows (GraphPad Software Inc., San Diego, CA). Statistical comparisons were performed by the unpaired t-test, one-way analysis of variance (ANOVA) with Dunnett's post-test, or two-way ANOVA with the Bonferroni post-test;  $p < 0.05$  was considered statistically significant.

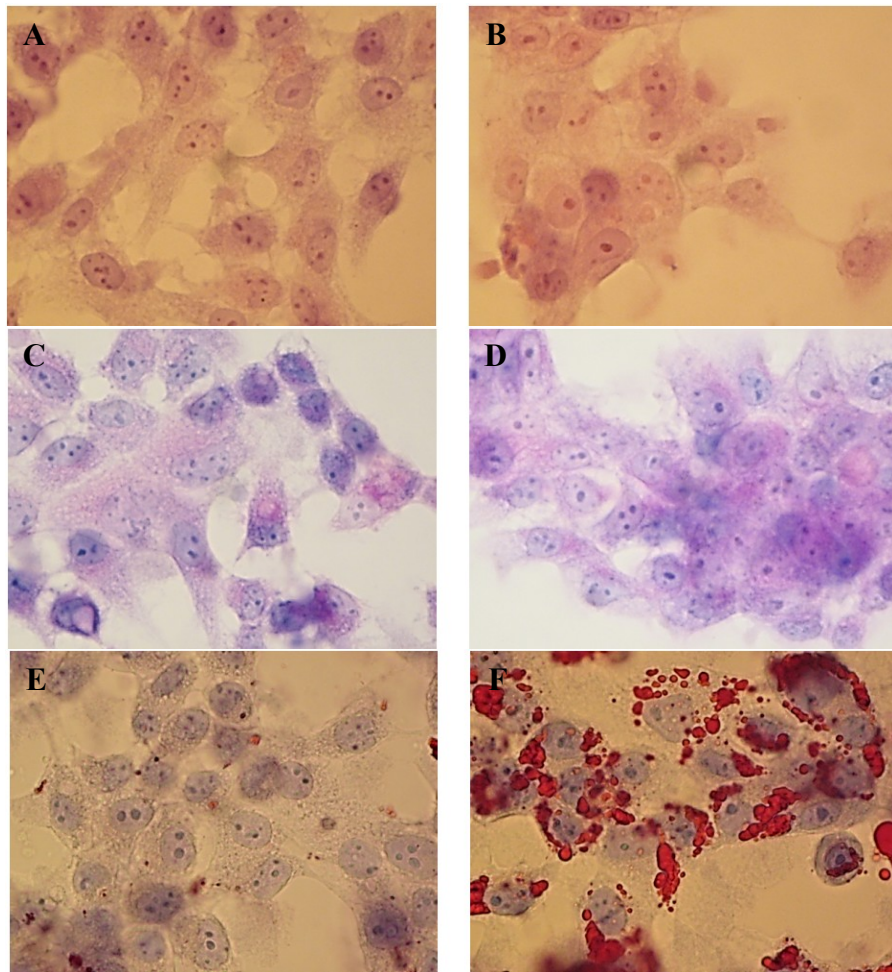
## CHAPTER 3: RESULTS

### 3.1 Generation of Steatotic Liver Cell Lines

The purpose of this research was to develop an *in vitro* model to study the effects of combining pre-existing hepatic steatosis and ischemia reperfusion (IR) injury. To model this, human and rat liver cell lines were incubated in steatosis medium prior to being subjected to the cold preservation injury assay, cold ischemia and reperfusion assay, or cold ischemia plus warm ischemia and reperfusion assay. H & E staining for general histology of both human (Figure 3A, B) and rat (Figure 4A, B) cell lines revealed centrally located, light blue nuclei with prominent nucleoli and a pink cytoplasm containing numerous small vacuoles in both control treated (Figures 3A and 4A) and steatosis treated (Figures 3B and 4B) cells. There were no large, prominent vacuoles or displaced nuclei evident in cells from either treatment group. PAS staining for glycogen content of human (Figure 3C, D) and rat (Figure 4C, D) liver cells also revealed blue centrally located nuclei, prominent nucleoli and diffuse light purple glycogen within the cytoplasm of both control treated (Figures 3C and 4C) and steatosis treated (Figures 3D and 4D) cells. Glycogen content appeared similar in cells from both treatment groups. The ORO staining for lipid content of human (Figure 3E, F) and rat (Figure 4E, F) cells revealed centrally located, blue nuclei with prominent nucleoli and red lipids stained throughout the cytoplasm in both the control and steatosis treated cells. Cells incubated in steatosis medium, however, (Figures 3F and 4F) showed significant intracellular lipid accumulation when compared, visually, to control treated (Figures 3E and 4E) cells.



**Figure 3. Generation of Normal and Steatotic Human Liver Cells.** Analysis of HepG2 human hepatocellular carcinoma cells following (A, C, E) control treatment or (B, D, F) steatosis treatment using, respectively, H & E staining, PAS stain and ORO stain. Images taken at a magnification of 630X.



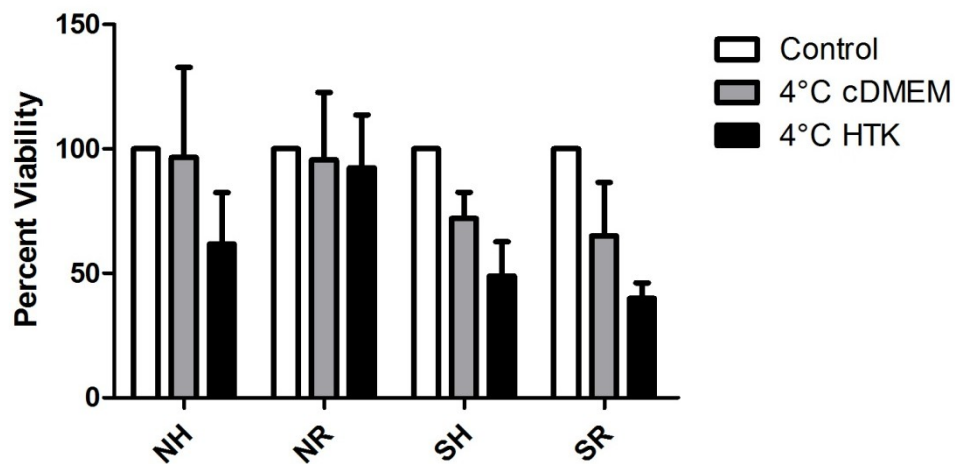
**Figure 4. Generation of Normal and Steatotic Rat Liver Cells.** Analysis of McA-RH7777 rat hepatoma cells following (A, C, E) control treatment or (B, D, F) steatosis treatment using, respectively, H & E staining, PAS stain and ORO stain. Images taken at a magnification of 630X.



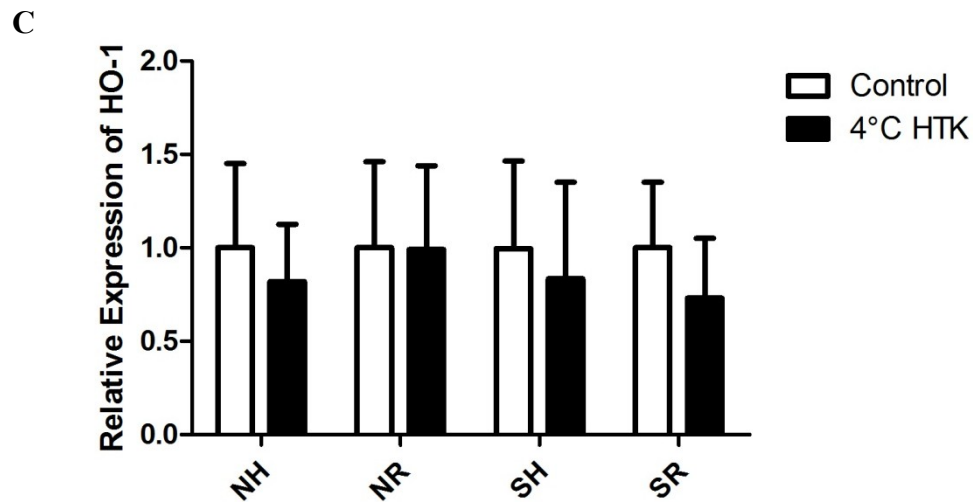
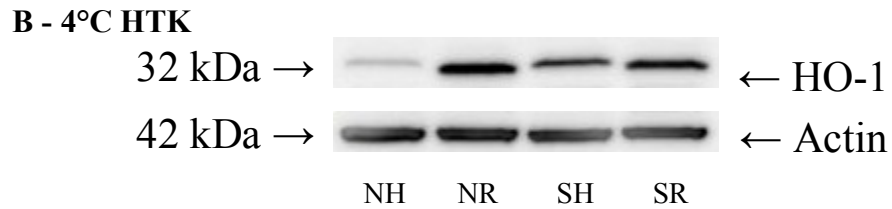
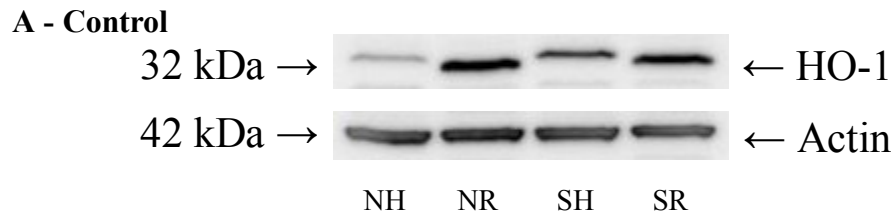
### **3.2 Cold Preservation Does Not Affect Cell Viability or Expression of HO-1 in Liver Cell Lines**

To study the effect of cold preservation on HepG2 and McA-RH7777 viability, cells were treated with control medium [normal human (NH) and normal rat (NR)] or steatotic medium [steatotic human (SH) and steatotic rat (SR)], subjected to the cold preservation injury assay and allowed to recover at 37°C in cDMEM for 2 h. Cell number was quantified using the Trypan Blue exclusion assay and percent viability was determined relative to the control treatment (Figure 5). The viability of normal human cells was 97 % (SEM 36 %) following incubation in chilled medium (4°C cDMEM) and 62 % (SEM 21 %) following incubation in chilled preservation solution (4°C HTK). The viability of normal rat cells incubated in 4°C cDMEM was 96 % (SEM 27 %) and was 92 % (SEM 21 %) after incubation in 4°C HTK. The viability of steatotic human cells that were incubated in 4°C cDMEM was 72 % (SEM 11 %) and was 49 % (SEM 14 %) following incubation in 4°C HTK. The viability of steatotic rat cells following incubation in 4°C cDMEM was 65 % (SEM 22 %) and was 40 % (SEM 6 %) after incubation in 4°C HTK. Viability of both cold incubated (4°C cDMEM) and cold preserved (4°C HTK) cells was not significantly different from that of control treated cells (Figure 5).

To observe the effect of cold preservation on protein expression, cells were subjected to the cold preservation injury assay and total cell lysates were analyzed using Western blotting. As HO-1 is expected to increase significantly in response to cellular stress [74-79], its expression was measured following cold preservation (Figure 6B) and control (Figure 6A) treatments. Relative expression of HO-1 (Figure 6C) was not



**Figure 5. Cold Preservation Does Not Affect Viability of Normal and Steatotic Human and Rat Liver Cells.** Human HepG2 and rat McA-RH7777 cells treated with control medium [normal human (NH) and normal rat (NR)] or steatotic medium [steatotic human (SH) and steatotic rat (SR)] were incubated for 1 h in chilled complete medium (4°C cDMEM), chilled preservation solution (4°C HTK) or 37°C complete medium (control) for the cold preservation injury assay. Cell viability was quantified after 2 h recovery in 37°C cDMEM using the Trypan Blue exclusion assay. Data are an average of 3 experiments, shown as percent mean viable cells + SEM expressed relative to control treatment.



**Figure 6. Cold Preservation Does Not Affect HO-1 Protein Expression in Normal and Steatotic Human and Rat Liver Cell Lines.** Human HepG2 and rat McA-RH7777 cells treated with control medium [normal human (NH) and normal rat (NR)] or steatotic medium [steatotic human (SH) and steatotic rat (SR)] were incubated for 1 h in chilled preservation solution (4°C HTK) or 37°C complete medium (control) for the cold preservation injury assay. Total cell lysates were analyzed after 2 h recovery in 37°C cDMEM using Western blotting to detect HO-1 expression in (A) control treated or (B) cold preserved cells. Equal loading was verified through analysis of actin expression. Data from a representative experiment of 3 are shown along with the corresponding densitometric analysis (C) shown as mean values + SEM (n=3).

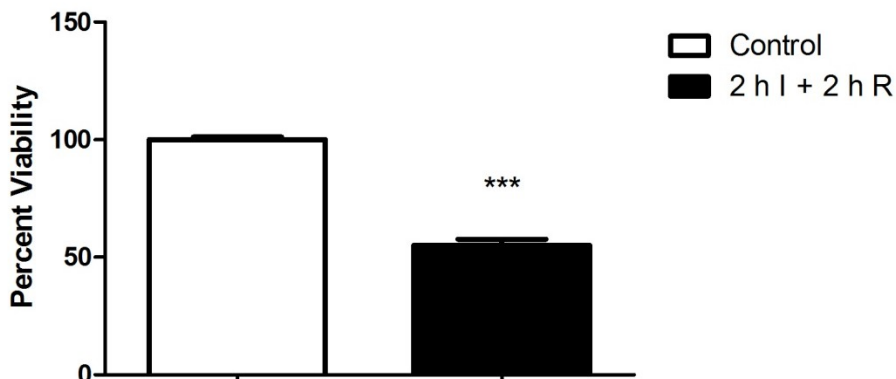
significantly changed in cells incubated in chilled preservation solution (4°C HTK) when compared to cells incubated in 37°C cDMEM (control).

### **3.3 Development of the Cold Ischemia and Reperfusion Assay**

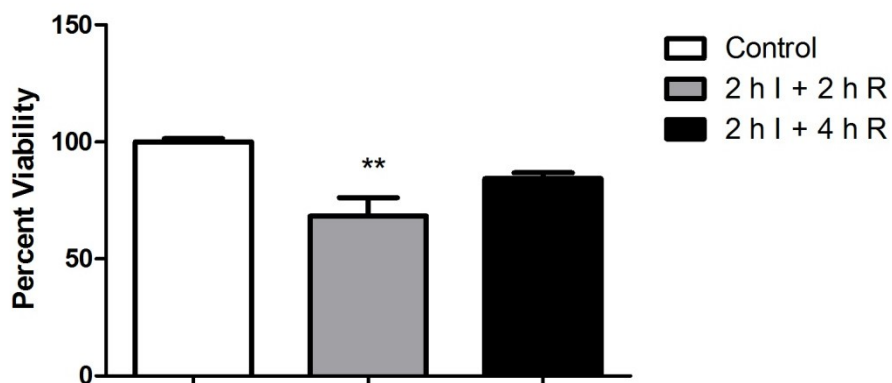
To study the effect of cold ischemia on cell viability, HepG2 cells were subjected to the cold ischemia and reperfusion assay for varying lengths of time; the resulting effects on cell number were quantified using MTT or WST-1 assays and percent viability was determined relative to the control treatment (Figure 7). The viability of HepG2 cells following an incubation in 4°C anoxic HTK for 2 h and reperfusion with 37°C cDMEM for 2 h was 55 % using the indirect MTT assay (Figure 7A). Using the direct WST-1 assay, the viability of HepG2 cells following 2 h of ischemia was 68 % when assessed after 2 h of reperfusion, while cell viability was not significantly reduced compared to control cells when assessed 4 h after the initiation of reperfusion (Figure 7B).

To study the effect of cold ischemia on HepG2 and McA-RH7777 cells that had received a steatosis treatment, normal and steatotic human and rat cells were subjected to varying lengths of cold ischemia and reperfusion in order to determine an IR combination that affected the greatest change in viability. Ischemia ranged from 2 - 72 h and was combined with 0 - 24 h of reperfusion; the resulting effects on cell number were quantified using the one-step, direct WST-1 assay and are expressed as percent viability of the control treated cells (Table 1 and Figure 8). As there was no appreciable pattern, the intermediate time of 48 h cold ischemia and 4 h reperfusion affected the greatest variety of cell types in a consistent manner, and was therefore used for subsequent assessments of *in vitro* IR.

### A - MTT



### B - WST-1

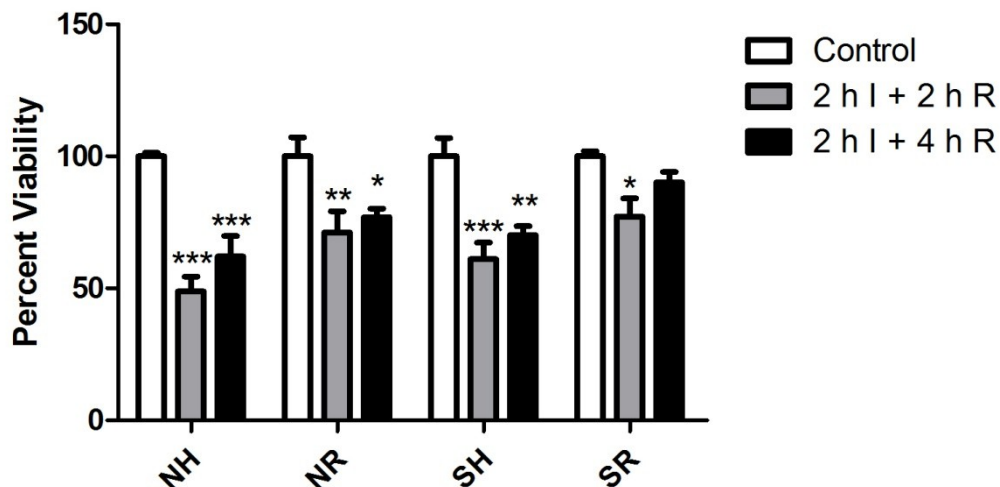


**Figure 7. Cold Ischemia and Reperfusion Reduces Viability of HepG2 Cells.** HepG2 cells were subjected to 2 h of ischemia followed by either 2 h or 4 h of reperfusion and changes in cell viability were quantified by (A) MTT assay or (B) WST-1 assay. Data are shown as mean viability + SEM (n=3). Statistical significance in comparison to the control treatment was determined by (A) the unpaired t-test for a single treatment group or by (B) one-way ANOVA with Dunnett's post-test when there were multiple treatment groups; \*\* denotes  $p < 0.01$ , \*\*\* denotes  $p < 0.001$ .

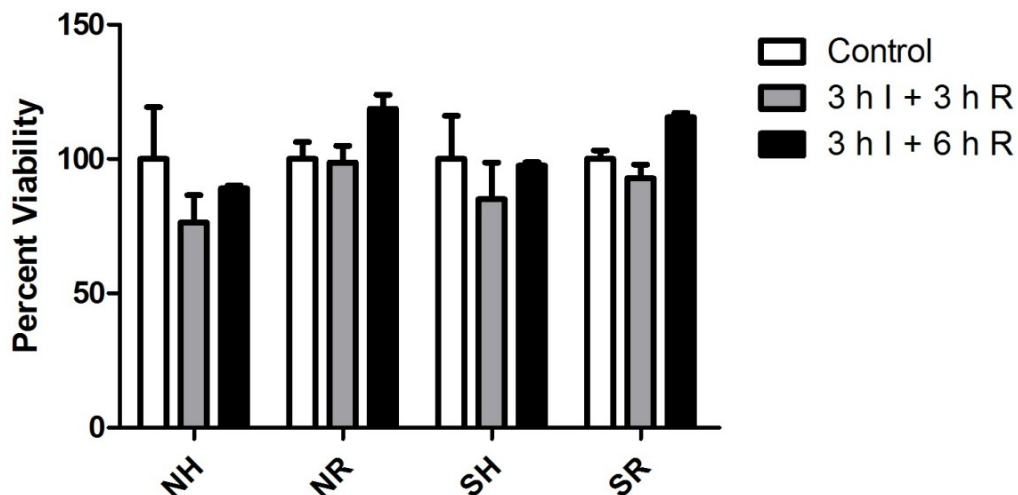
**Table 1. Normal and Steatotic Human and Rat Liver Cell Viability Following Cold Ischemia and Reperfusion**

	Percent Viability of Control Treatment Mean $\pm$ SEM (n=3); * denotes significance			
	Normal Human	Normal Rat	Steatotic Human	Steatotic Rat
<b>2 h ischemia (Figure 8A)</b>				
2 h reperfusion	49 $\pm$ 6 *	71 $\pm$ 8 *	61 $\pm$ 6 *	77 $\pm$ 7 *
4 h reperfusion	62 $\pm$ 8 *	77 $\pm$ 3 *	70 $\pm$ 3 *	90 $\pm$ 4
<b>3 h ischemia (Figure 8B)</b>				
3 h reperfusion	76 $\pm$ 10	99 $\pm$ 6	85 $\pm$ 14	93 $\pm$ 5
6 h reperfusion	89 $\pm$ 1	119 $\pm$ 5	98 $\pm$ 1	116 $\pm$ 2
<b>5 h ischemia (Figure 8C)</b>				
3 h reperfusion	82 $\pm$ 1 *	93 $\pm$ 3 *	93 $\pm$ 1 *	54 $\pm$ 2 *
24 h reperfusion	92 $\pm$ 2 *	97 $\pm$ 1	101 $\pm$ 2	59 $\pm$ 1 *
<b>24 h ischemia (Figure 8D)</b>				
0 h reperfusion	88 $\pm$ 1	96 $\pm$ 4	59 $\pm$ 4 *	97 $\pm$ 3
2 h reperfusion	97 $\pm$ 0	98 $\pm$ 6	74 $\pm$ 3 *	105 $\pm$ 2
<b>48 h ischemia (Figure 8E)</b>				
0 h reperfusion	32 $\pm$ 2 *	81 $\pm$ 3 *	40 $\pm$ 1 *	96 $\pm$ 3
4 h reperfusion	33 $\pm$ 0 *	82 $\pm$ 1 *	45 $\pm$ 2 *	106 $\pm$ 2
7 h reperfusion	37 $\pm$ 2 *	63 $\pm$ 2 *	50 $\pm$ 2 *	100 $\pm$ 5
<b>72 h ischemia (Figure 8F)</b>				
0 h reperfusion	85 $\pm$ 6	71 $\pm$ 6 *	54 $\pm$ 5 *	93 $\pm$ 3
2 h reperfusion	104 $\pm$ 8	89 $\pm$ 4	57 $\pm$ 7 *	108 $\pm$ 5

**A - 2 h Ischemia**

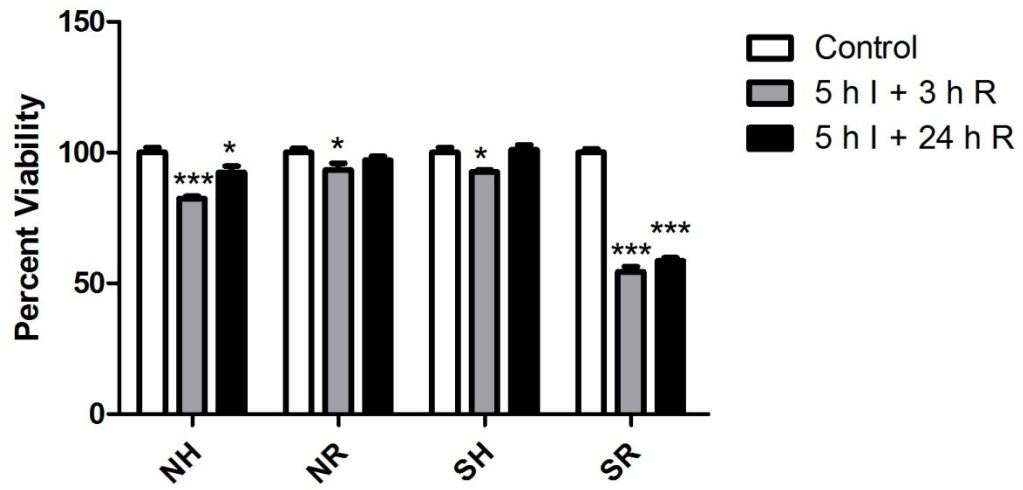


**B - 3 h Ischemia**

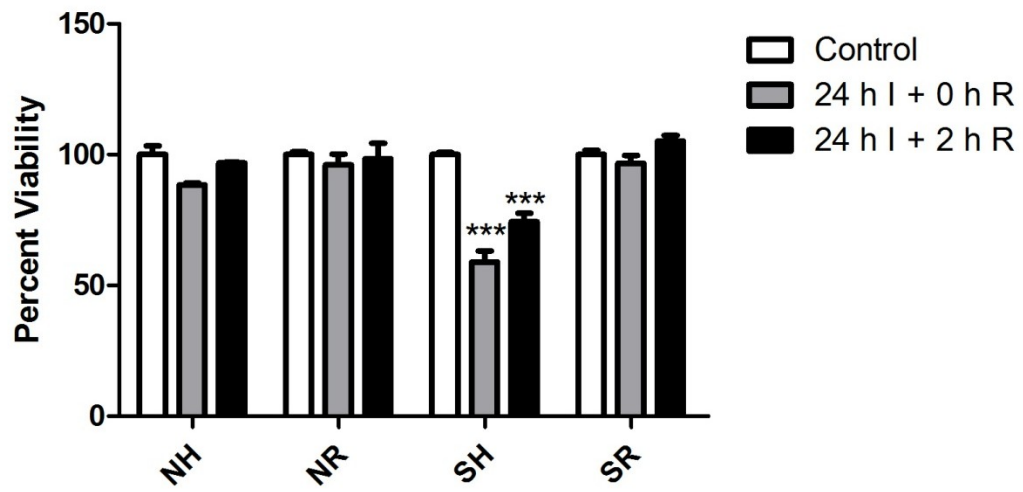


**Figure 8. Cold Ischemia and Reperfusion Have Variable Effects on the Viability of Normal and Steatotic Human and Rat Liver Cell Lines.** HepG2 and McA-RH7777 cells treated with control medium [normal human (NH) and normal rat (NR)] or steatotic medium [steatotic human (SH) and steatotic rat (SR)] were subjected to varying lengths of ischemia (I) and reperfusion (R) or for matched lengths of time in complete medium (control) and changes in cell viability were quantified by WST-1 assay. (A) 2 h I followed by 2 h or 4 h R (B) 3 h I followed by 3 h or 6 h R (C) 5 h I followed by 3 h or 24 h R (D) 24 h I followed by 0 h or 2 h R (E) 48 h I followed by 0 h, 4 h or 7 h R (F) 72 h I followed by 0 h or 2 h R. Data are shown as mean viability + SEM (n=3). Statistical significance in comparison to the control treatment was determined by two-way ANOVA with Bonferroni post-test; \* denotes  $p < 0.05$ , \*\* denotes  $p < 0.01$ , \*\*\* denotes  $p < 0.001$ .

C - 5 h Ischemia

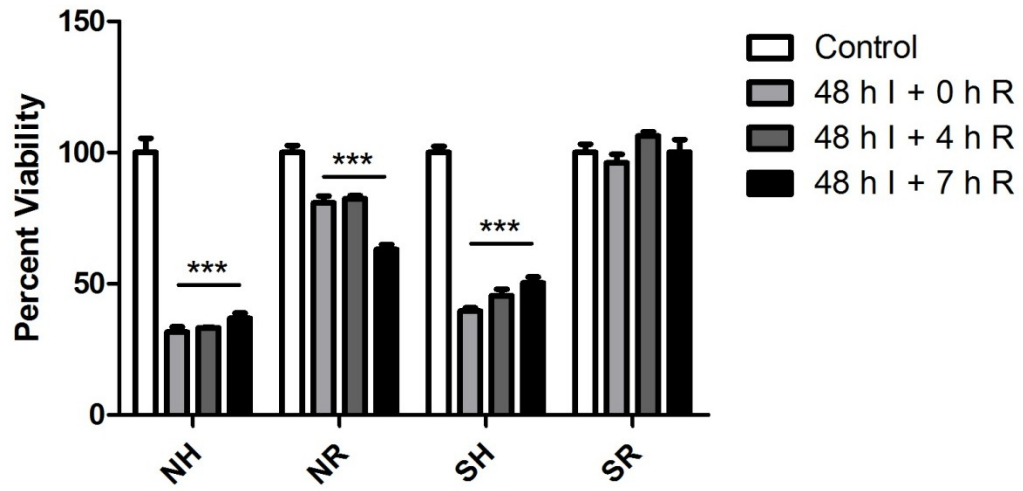


D - 24 h Ischemia

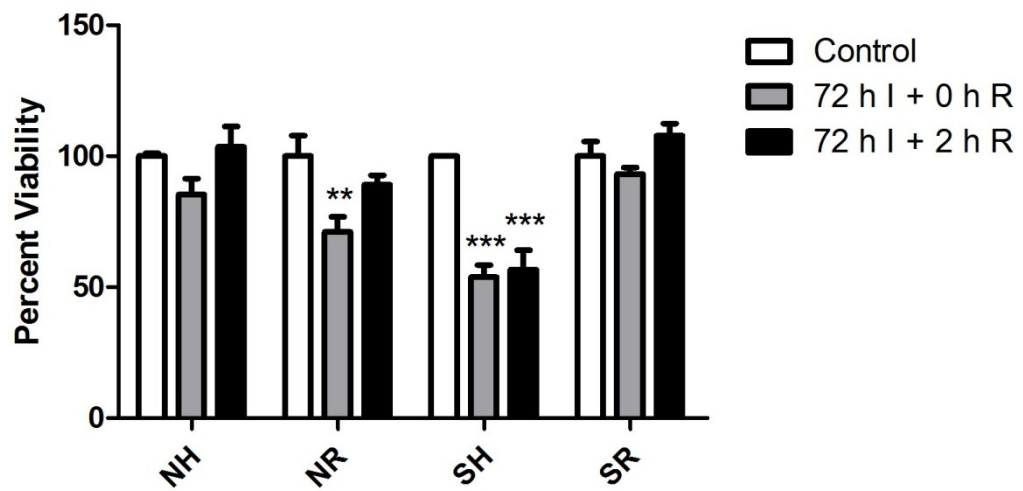




E - 48 h Ischemia



F - 72 h Ischemia

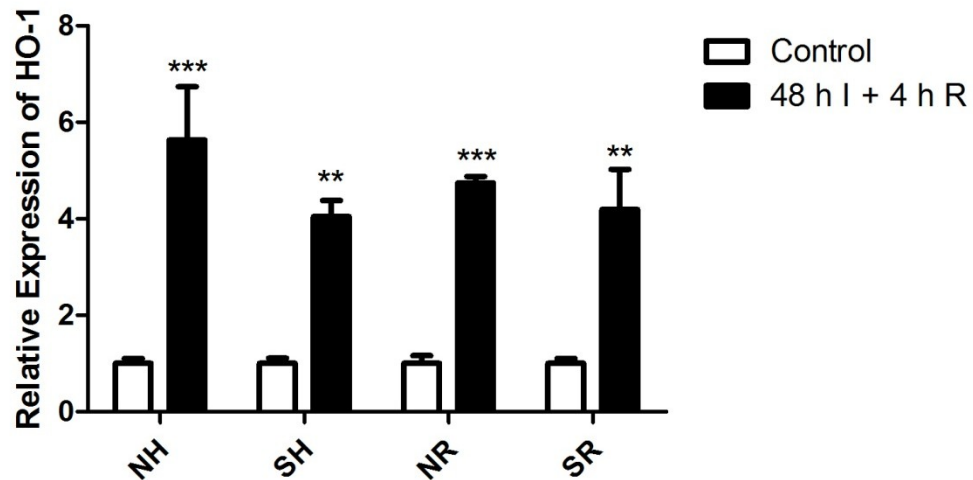
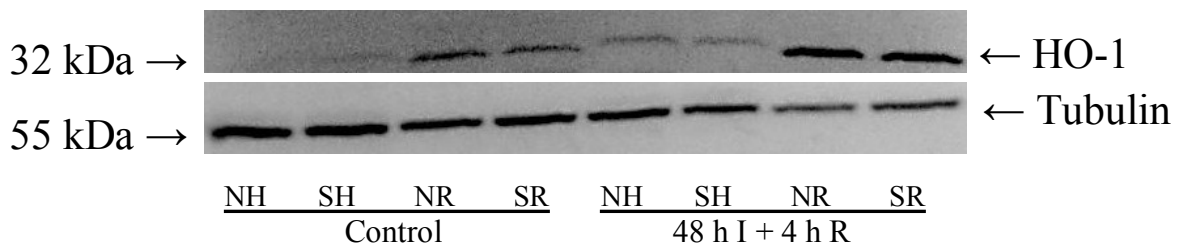


### **3.4 Cold Ischemia Induces HO-1 Expression in Normal and Steatotic Liver Cell Lines, but Does Not Affect the Expression of MMP-9**

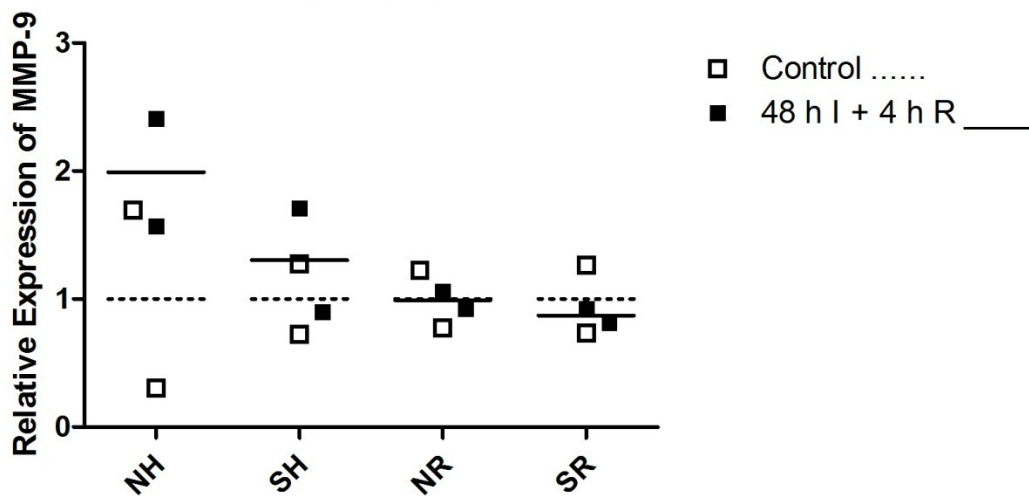
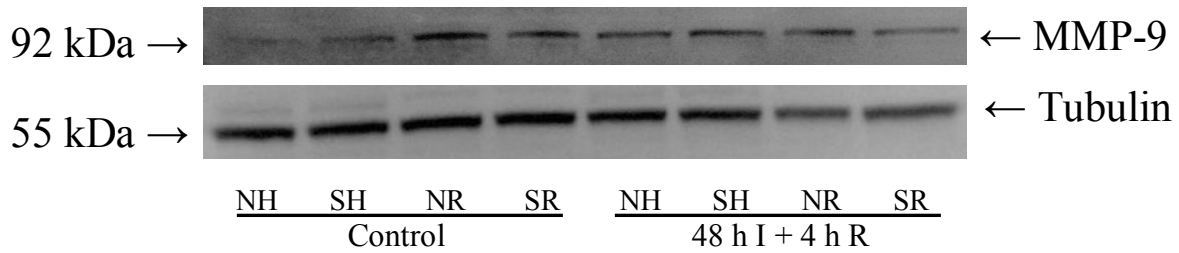
To observe any effect that cold ischemia had on protein expression, HepG2 and McA-RH7777 cells that had received a steatosis treatment were subjected to the cold ischemia and reperfusion assay. Western blotting analysis of total cell lysates revealed that HO-1 expression increased significantly in all cell populations in response to 48 h ischemia and 4 h reperfusion (Figure 9). As MMP-9 is increased following hepatic injury [45, 93, 106-109], its expression was also measured following *in vitro* IR, however there was no appreciable change in treated populations when compared to controls (Figure 10).

### **3.5 Cold Ischemia plus Warm Ischemia Does Not Significantly Affect Protein or RNA Expression of HO-1 and MMP-9 in HepG2 Cells**

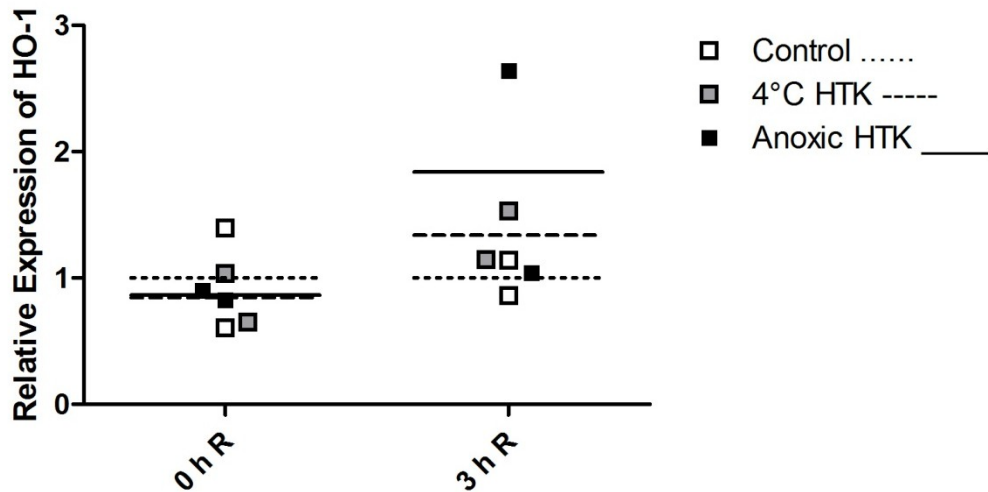
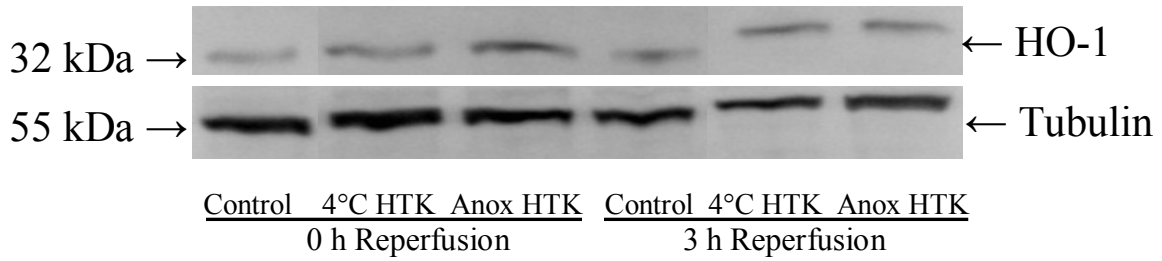
Transplanted livers are usually preserved for a maximum of approximately 10 h, followed by a rewarming period of 30 - 60 min before being reperfused. To mimic this treatment, HepG2 cells were subjected to the cold ischemia (CI) with warm ischemia (WI) and reperfusion assay and assessed after 9 h of CI, 1 h of WI and 3 h of reperfusion. To ascertain the effect of the additional period of warm ischemia on protein expression, total cell lysates were collected following *in vitro* IR and analyzed using the Western blot technique. The expression of HO-1 (Figure 11) was not significantly changed in response to incubation in chilled preservation solution (4°C HTK) or chilled preservation solution in an anoxic chamber (anoxic HTK) when compared to expression levels in control cells. The expression of MMP-9 (Figure 12) was also not significantly different in cells incubated in chilled preservation solution (4°C HTK) or chilled preservation solution in an anoxic chamber (anoxic HTK) when compared to expression levels in control cells.



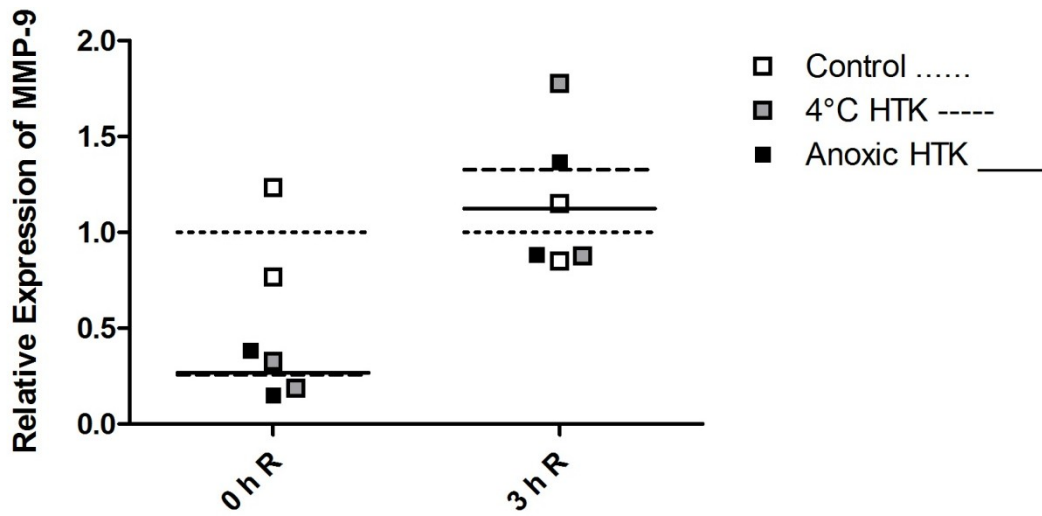
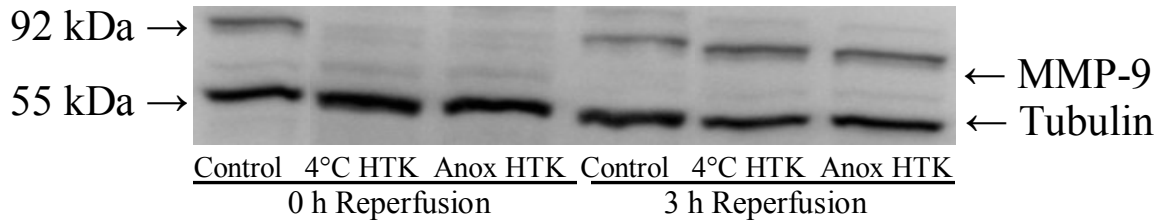
**Figure 9. Cold Ischemia and Reperfusion Induces HO-1 Protein Expression in Normal and Steatotic Human and Rat Liver Cell Lines.** HepG2 and McA-RH7777 cells treated with control medium [normal human (NH) and normal rat (NR)] or steatotic medium [steatotic human (SH) and steatotic rat (SR)] were subjected to 48 h of ischemia (I) or incubation in complete medium (control) followed by 4 h of reperfusion (R) and total cell lysates were analyzed using Western blotting to detect HO-1 expression. Equal loading was verified through analysis of tubulin expression. Data from a representative experiment of 3 are shown along with the corresponding densitometric analysis shown as mean values + SEM (n=3). Statistical significance in comparison to the control treatment was determined by two-way ANOVA with Bonferroni post-test; \*\* denotes  $p < 0.01$ , \*\*\* denotes  $p < 0.001$ .



**Figure 10. Cold Ischemia and Reperfusion Does Not Affect MMP-9 Protein Expression in Normal and Steatotic Human and Rat Liver Cell Lines.** HepG2 and McA-RH7777 cells treated with control medium [normal human (NH) and normal rat (NR)] or steatotic medium [steatotic human (SH) and steatotic rat (SR)] were subjected to 48 h of ischemia (I) or incubation in complete medium (control) followed by 4 h of reperfusion (R) and total cell lysates were analyzed using Western blotting to detect MMP-9 expression. Equal loading was verified through analysis of tubulin expression. Data from a representative experiment of 2 are shown along with a scatter plot of mean values from the corresponding densitometric analysis.



**Figure 11. Cold Ischemia Plus Warm Ischemia and Reperfusion Does Not Affect HO-1 Protein Expression in HepG2 Cells.** HepG2 cells were incubated for 9 h in chilled preservation solution (4°C HTK) or chilled preservation solution in an anoxic chamber (anoxic HTK) and transferred to 37°C for 1 h followed by either 0 h or 3 h reperfusion (R). Additional cells were incubated for matched lengths of time in 37°C complete medium (control) and total cell lysates were analyzed using Western blotting to detect HO-1 expression. Equal loading was verified through analysis of tubulin expression. Data from a representative experiment of 2 are shown along with a scatter plot of mean values from the corresponding densitometric analysis.



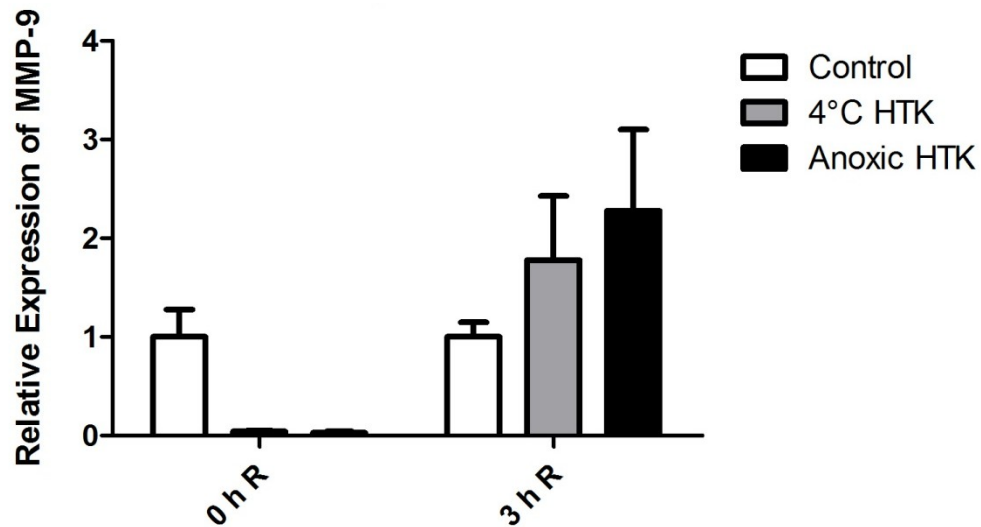
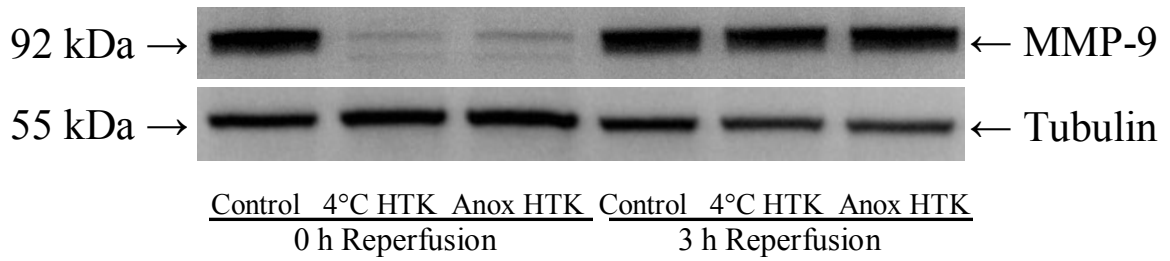
**Figure 12. Cold Ischemia Plus Warm Ischemia and Reperfusion Does Not Affect MMP-9 Protein Expression in HepG2 Cells.** HepG2 cells were incubated for 9 h in chilled preservation solution (4°C HTK) or chilled preservation solution in an anoxic chamber (anoxic HTK) and transferred to 37°C for 1 h followed by either 0 h or 3 h reperfusion (R). Additional cells were incubated for matched lengths of time in 37°C complete medium (control) and total cell lysates were analyzed using Western blotting to detect MMP-9 expression. Equal loading was verified through analysis of tubulin expression. Data from a representative experiment of 2 are shown along with a scatter plot of mean values from the corresponding densitometric analysis.

MMP-9 expression was very low in cells incubated in HTK when assessed after 0 h of reperfusion (Figure 12). Ischemic and preservation times were then reduced to 6 h of cold, followed by 30 min of warm and 3 h of reperfusion to further assess MMP-9 expression in response to *in vitro* IR (Figure 13). MMP-9 expression was again very low when assessed after 0 h reperfusion (Figure 13) and was also not significantly increased in response to incubation in chilled preservation solution (4°C HTK) or chilled preservation solution in an anoxic chamber (anoxic HTK) when compared to expression levels in control cells.

To assess the effect of cold plus warm ischemia on gene expression, HepG2 cells were subjected to 6 h of CI, 30 min of WI and 3 h of reperfusion, total RNA was isolated, cDNA was reverse transcribed, and MMP-9 was detected using PCR. While an initial assessment indicated that MMP-9 gene expression was increased following the anoxic HTK treatment and reperfusion (Figure 14), further RT-PCR was unable to validate this observation. HepG2 cells treated with control medium (NH) and with steatosis medium (SH) were also subjected to 6 h of CI, 30 min of WI and 3 h of reperfusion. When compared to expression levels in control cells, a preliminary analysis of mRNA levels (Figure 15) indicated MMP-9 gene expression is increased in response to incubation in chilled preservation solution in an anoxic chamber (anoxic HTK) but not in response to incubation in chilled preservation solution in room air (4°C HTK).

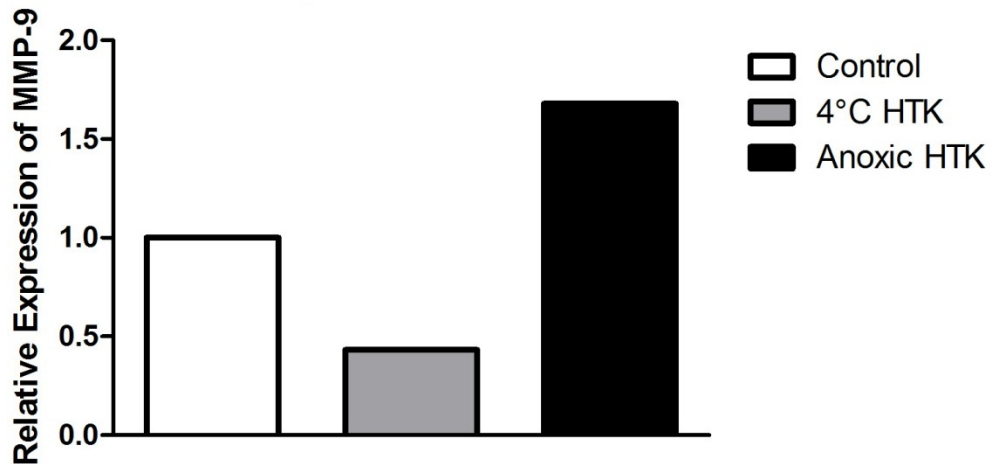
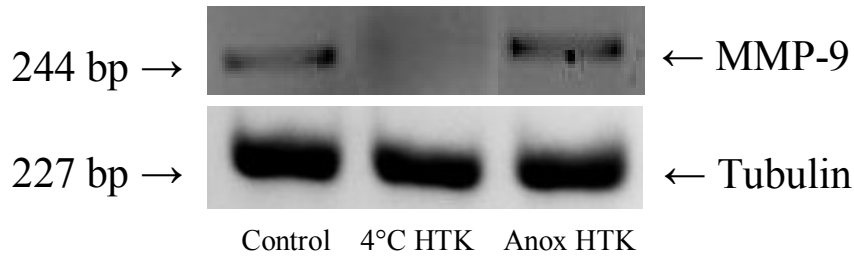
### **3.6 Generation of Lentiviral Plasmids for HO-1 Expression**

There is evidence that supplying exogenous HO-1 to donor grafts can result in



**Figure 13. Cold Ischemia Plus Warm Ischemia and Reperfusion Does Not Affect MMP-9 Protein Expression in HepG2 Cells.** HepG2 cells were incubated for 6 h in chilled preservation solution (4°C HTK) or chilled preservation solution in an anoxic chamber (anoxic HTK) and transferred to 37°C for 30 min followed by either 0 h or 3 h reperfusion (R). Additional cells were incubated for matched lengths of time in 37°C complete medium (control) and total cell lysates were analyzed using Western blotting to detect MMP-9 expression. Equal loading was verified through analysis of tubulin expression. Data from a representative experiment of 3 are shown along with the corresponding densitometric analysis shown as mean values + SEM (n=3).



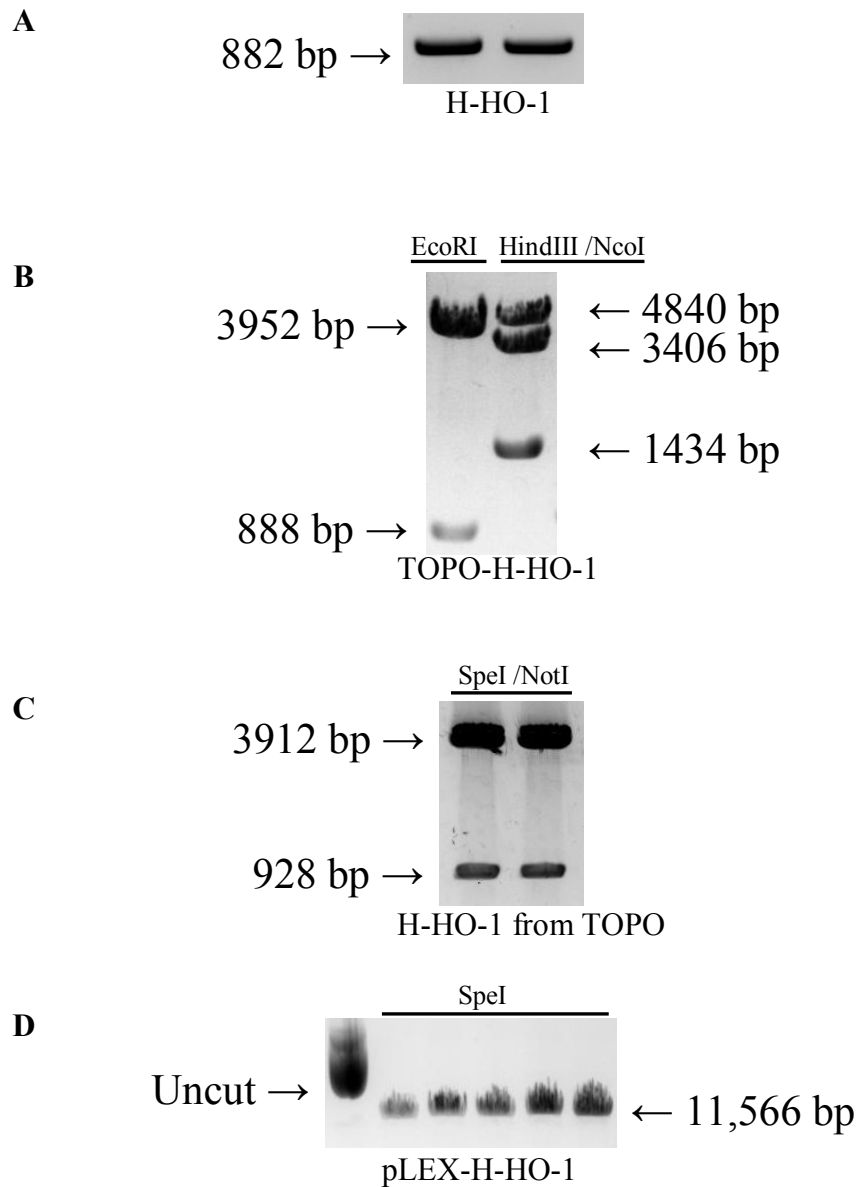


**Figure 14. Cold Ischemia Plus Warm Ischemia and Reperfusion May Affect MMP-9 Gene Expression in HepG2 Cells.** HepG2 cells were incubated for 6 h in chilled preservation solution (4°C HTK) or chilled preservation solution in an anoxic chamber (anoxic HTK) and transferred to 37°C for 30 min followed by 3 h reperfusion. Additional cells were incubated for an equal time in 37°C complete medium (control). Total mRNA was extracted and analyzed using RT-PCR to detect MMP-9 expression. Relative gene expression was assessed compared to tubulin expression. Data are from a single experiment, shown along with the corresponding densitometric analysis.

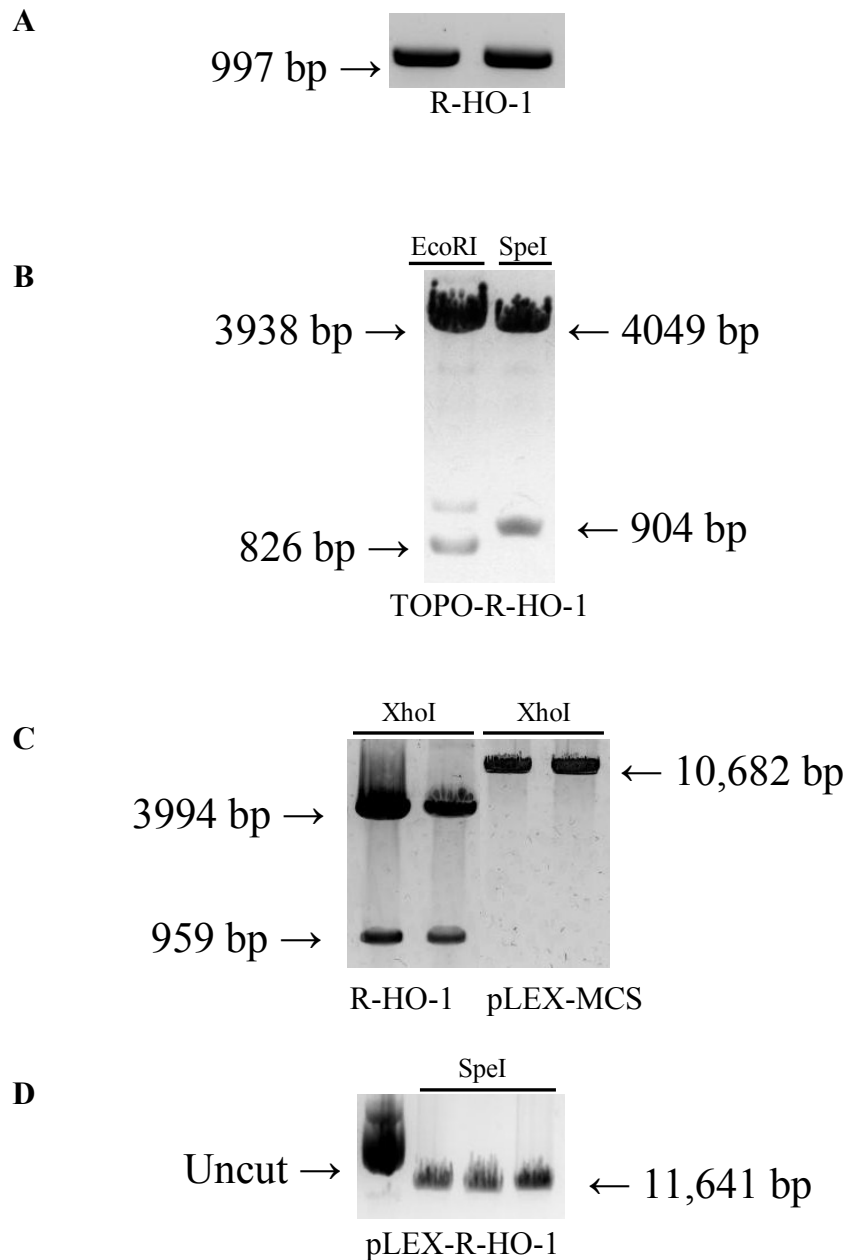


beneficial effects following transplantation [88-92]. To test whether supplying a source of HO-1 protein to cells provides protection against *in vitro* IR injury, HO-1 gene sequences were cloned into a lentiviral expression vector that would be able to be delivered to cells in culture. The human HO-1 (H-HO-1) sequence was PCR-amplified from pOTB7-HO-1 expression vector (Figure 16A). Purified PCR product was then inserted into a pCR<sup>®</sup>4-TOPO<sup>®</sup> cloning vector; endonuclease digestion with EcoRI yielded 3952 bp and 888 bp fragments while cuts with HindIII and NcoI restriction endonucleases resulted in undigested 4840 bp plasmid plus 3406 bp and 1434 bp fragments revealing properly oriented TOPO-H-HO-1 clones (Figure 16B). The H-HO-1 sequence was then excised from the TOPO vector using SpeI and NotI endonucleases, separated on an agarose gel, and correctly sized bands of 928 bp were extracted (Figure 16C). The vector pLEX-MCS was also cut using SpeI and NotI endonucleases to allow the 928 bp H-HO-1 insert to be ligated (data not shown). The resultant 11,566 bp pLEX-H-HO-1 clones showed linearization when digested with SpeI (Figure 16D).

Rat HO-1 (R-HO-1) sequence was PCR-amplified from pExpress-1-HO-1 expression vector (Figure 17A). Purified PCR product was then inserted into a pCR<sup>®</sup>4-TOPO<sup>®</sup> cloning vector; endonuclease digestion with EcoRI resulted in 3938 bp and 826 bp fragments with an additional very faint 189 bp fragment (not shown), while digestion with SpeI restriction endonuclease yielded 4049 bp and 904 bp fragments revealing properly oriented TOPO-R-HO-1 clones (Figure 17B). The R-HO-1 sequence was then excised from the TOPO vector using XhoI endonuclease; pLEX-MCS was also cut using XhoI, these digestion products were separated on an agarose gel, and correctly sized



**Figure 16. Generation of Lentiviral Plasmid for Human HO-1 Expression.** A) Human HO-1 (H-HO-1) cDNA was PCR amplified from pOTB7-HO-1 vector and cloned into (B) pCR4-TOPO and diagnostic cuts were made on resultant TOPO-H-HO-1 clones with restriction endonucleases EcoRI, HindIII and NcoI. (C) H-HO-1 was excised from pCR4-TOPO vector using SpeI and NotI endonucleases and the 928 bp fragment was cloned into pLEX-MCS that had been digested with SpeI and NotI (not shown) and (D) diagnostic cuts were done on the resultant pLEX-H-HO-1 clones using SpeI.



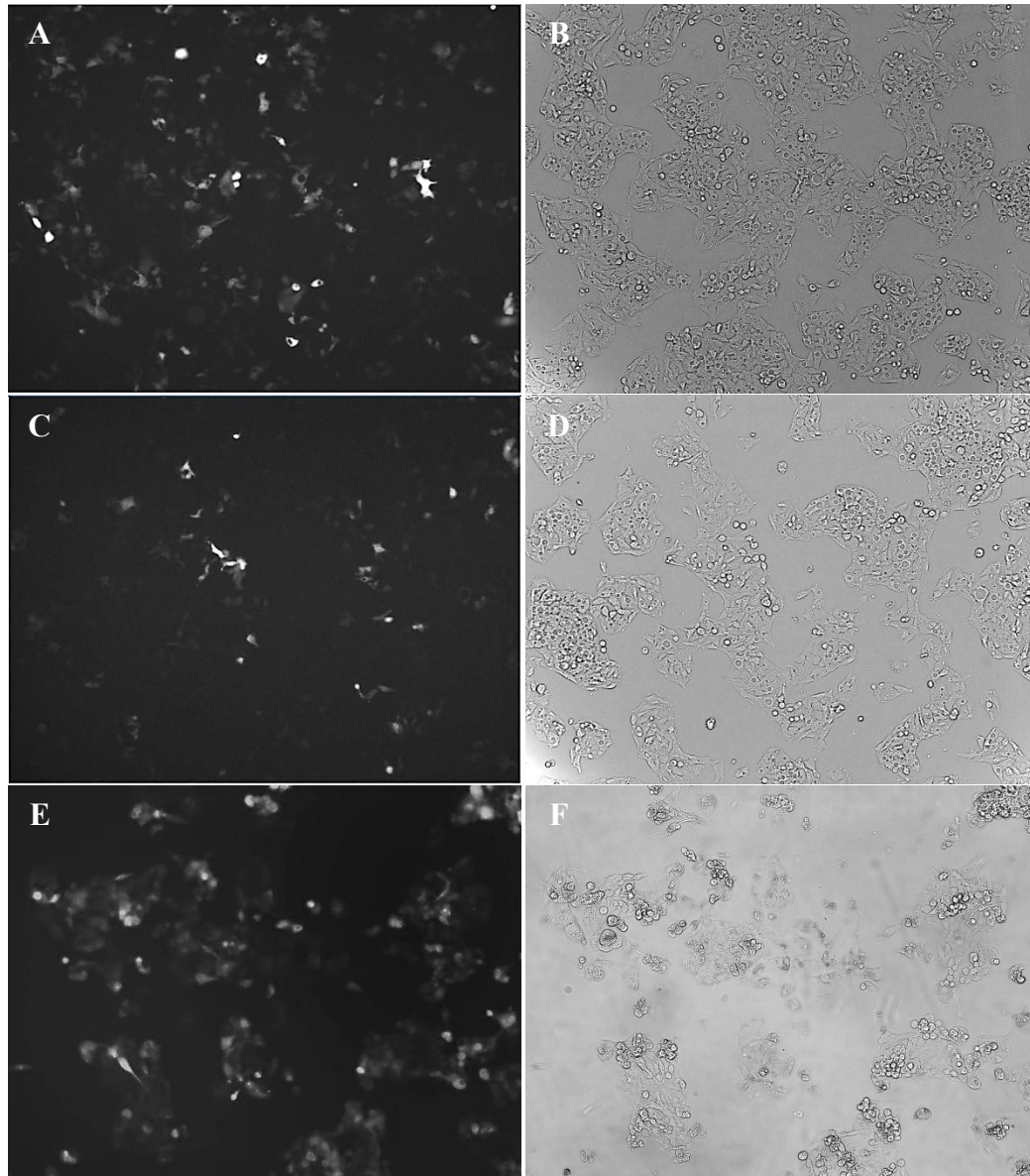
**Figure 17. Generation of Lentiviral Plasmid for Rat HO-1 Expression.** A) Rat HO-1 (R-HO-1) cDNA was PCR amplified from pExpress-1-HO-1 vector and cloned into (B) pCR4-TOPO and diagnostic cuts were made on resultant TOPO-R-HO-1 clones with restriction endonucleases EcoRI and SpeI. (C) R-HO-1 was excised from pCR4-TOPO vector using XhoI endonuclease and the 959 bp fragment was cloned into pLEX-MCS digested with XhoI and (D) diagnostic cuts were done on the resultant pLEX-R-HO-1 clones using SpeI.

bands of 959 bp (R-HO-1) and 10,682 bp (pLEX-MCS) were extracted (Figure 17C). The R-HO-1 insert was then ligated into pLEX-MCS and the resultant 11,641 bp pLEX-R-HO-1 clones showed linearization when digested with SpeI (Figure 17D).

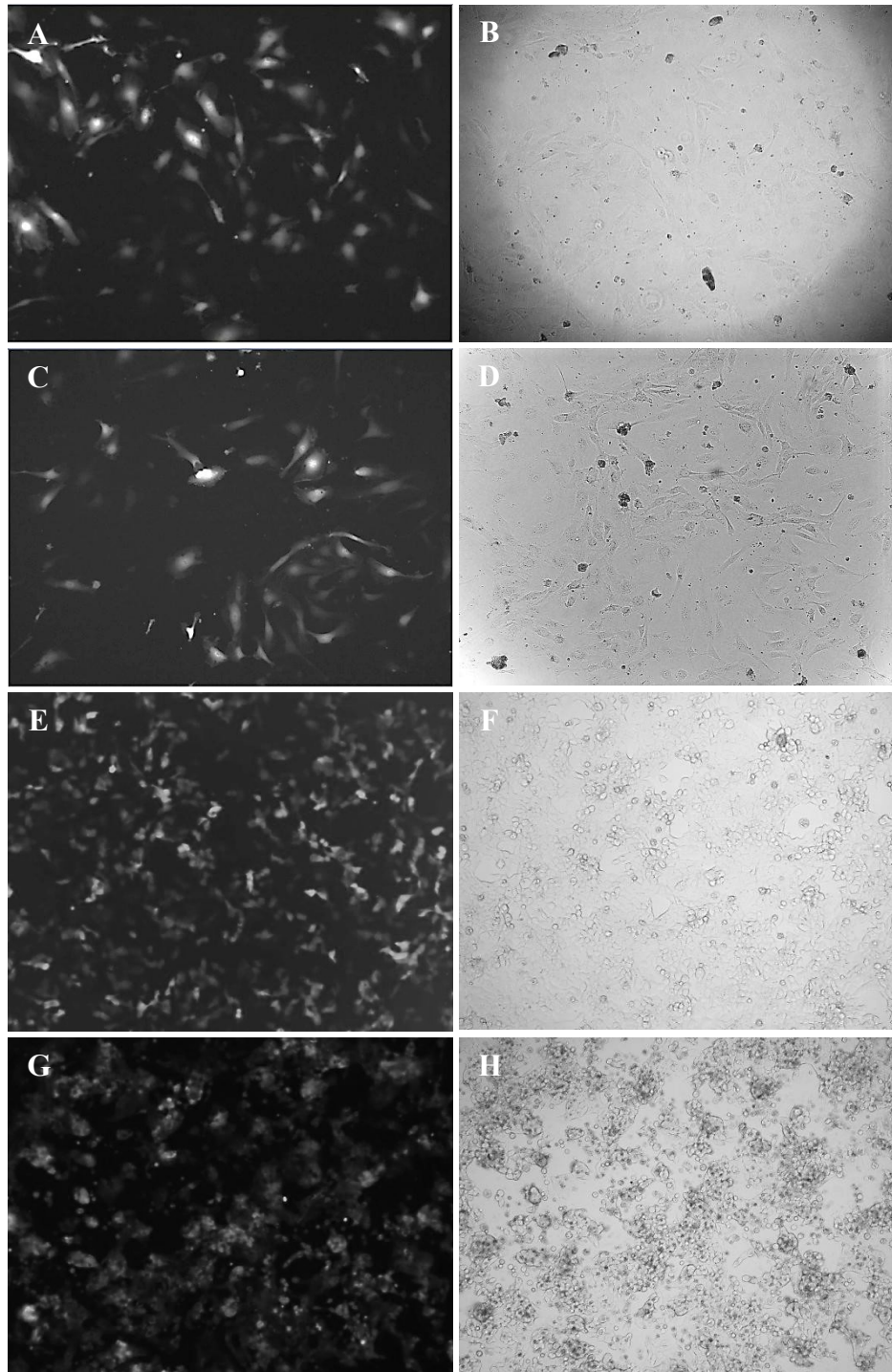
### **3.7 Transduction of Lentivirus Encoding shRNA Against MMP-9 and GAPDH Did Not Affect Target Protein Expression**

The specific inhibition of MMP-9 gene or protein expression is reported to reduce injury following an ischemic insult in an *in vivo* setting [107, 109]. Several lentiviral plasmids were therefore purchased which encoded shRNA against human and rat MMP-9 gene sequences. A lentiviral plasmid encoding shRNA against the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was also purchased for control transduction purposes. These plasmids were packaged in lentiviral particles, and the particles were used to transduce cultured human and rat cells. Effective transduction of HepG2 (Figure 18), HUVEC (Figure 19A - D), McA-RH7777 (Figure 19E - H) and 293T cells (data not shown) was observed by detection of GFP expression in the target cells. Transduction efficiency varied among cell and virus particle type (Figures 18 and 19), however, cells used for future experiments were maintained in selective medium containing puromycin until all surviving cells expressed GFP.

To gauge the ability of the shRNA delivered by lentivirus to knock down expression of their target genes in hepatocytes, HepG2 cells were transduced with lentiviral particles containing non-silencing, MMP-9-targeted or GAPDH-targeted shRNA sequences and total cell lysates were analyzed using the Western blot technique



**Figure 18. GFP is Expressed in HepG2 Cells Following Transduction with Lentiviral Particles.** A, C, E) Black and white fluorescence microscopy and (B, D, F) differential interference contrast microscopy images of HepG2 cells transduced with lentivirus containing, respectively, non-silencing, MMP-9-targeted and GAPDH-targeted shRNA sequences. Images taken at a magnification of 200X.



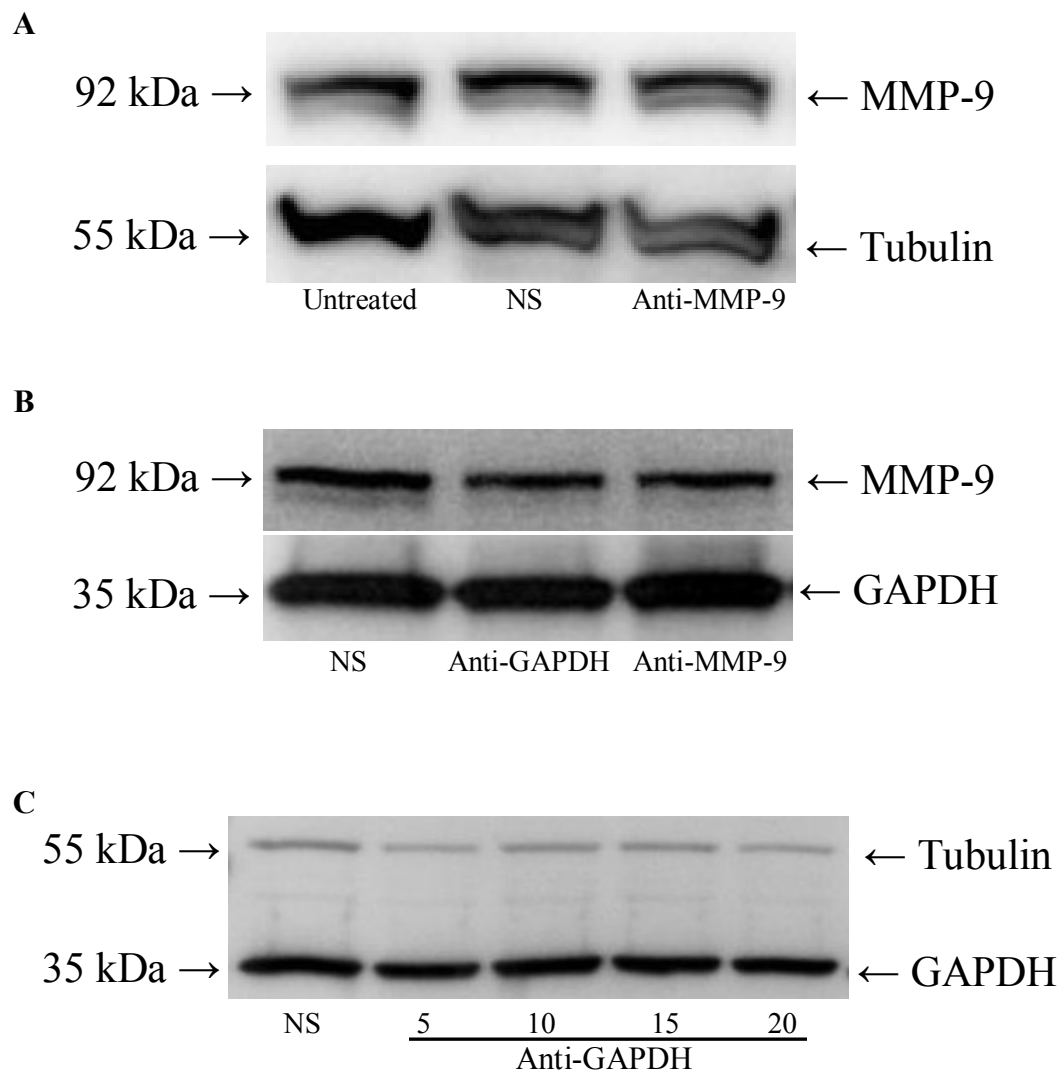
**Figure 19. GFP is Expressed in Human Cell Lines Following Transduction with Lentiviral Particles.** A - D) HUVEC and (E - H) McA-RH7777 cells were imaged using (A, C, E, G) black and white fluorescence microscopy and (B, D, F, H) differential interference contrast microscopy after cells were transduced with lentivirus containing (A, B, E, F) non-silencing and (C, D, G, H) MMP-9-targeted shRNA sequences. Images taken at a magnification of 200X.



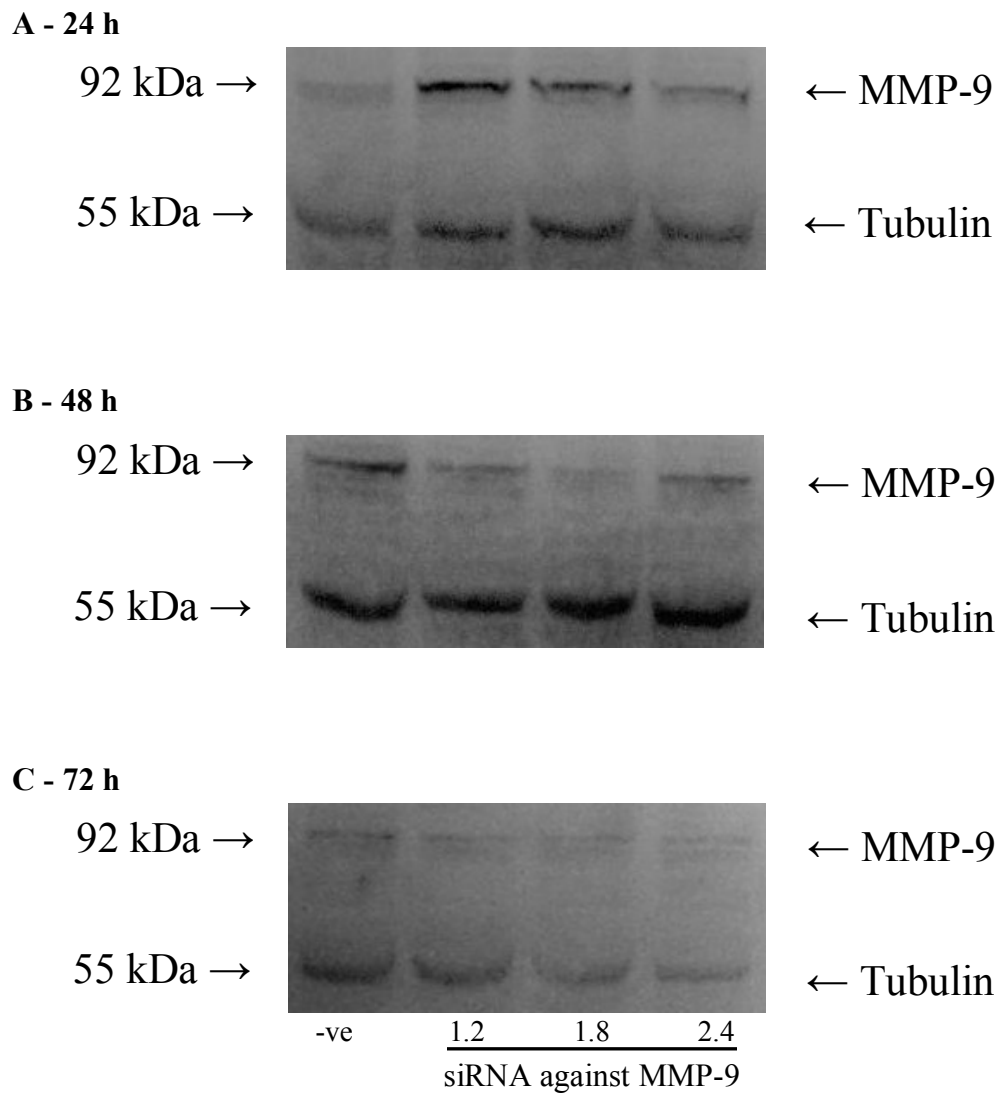
(Figure 20). Levels of MMP-9 protein were similar in lysates from cells that were untreated and from cells that were transduced (MOI of 1) with either non-silencing or MMP-9-targeted shRNA (Figure 20A). Additionally, levels of MMP-9 and GAPDH protein were not visually different in cells transduced with non-silencing shRNA or with shRNA targeted to MMP-9 or GAPDH at an MOI of 1 (Figure 20B). Furthermore, expression of GAPDH was comparable in cells transduced with the non-silencing shRNA sequence and with shRNA targeted to GAPDH, using increasing MOIs (Figure 20C).

### **3.8 Transfection of siRNA Against MMP-9 Did Not Affect Protein or Gene Expression in HepG2 Cells**

MMP-9 knock down through small interfering RNA (siRNA) does not require virions for delivery to the target cell and instead relies on a less invasive, lipid-based reagent for transfection. As an alternative to lentiviral-mediated silencing of MMP-9, siRNA duplexes which targeted the human MMP-9 gene sequence, as well as a negative control siRNA duplex, were purchased. To assess the capability of the oligonucleotide duplexes to knock down expression of their target genes in hepatocytes, HepG2 cells were transfected with siRNA duplexes. Total cell lysates were analyzed using the Western blot technique. Following transfection with a combination of all three siRNA duplexes targeted to MMP-9 (H-1, H-2 and H-3) at concentrations of 1.2, 1.8 and 2.4 pmol/ $\mu$ l siRNA, MMP-9 expression after 24 h appeared greater than that seen in cells transfected with the siRNA negative control duplex at a concentration of 100 nM (Figure 21A). After 48 h (Figure 21B) and 72 h (Figure 21C), MMP-9 expression appeared to decrease in response to transfection with H-1, H-2 and H-3 siRNA when compared to



**Figure 20. Protein Expression in HepG2 Cells is Unchanged Following Transduction of Lentiviral Particles.** A&B) HepG2 cells were either left untreated or transduced at an MOI of 1 with lentivirus containing non-silencing (NS), MMP-9-targeted (Anti-MMP-9) or GAPDH-targeted (Anti-GAPDH) shRNA sequences. (C) HepG2 cells were transduced at an MOI of 1 with lentivirus containing a non-silencing shRNA sequence or at several MOI (5, 10, 15, 20) with lentivirus containing a GAPDH-targeted shRNA sequence. Total cell lysates were analyzed using Western blotting to detect MMP-9, GAPDH or tubulin expression. Data are representative images from 2 experiments.



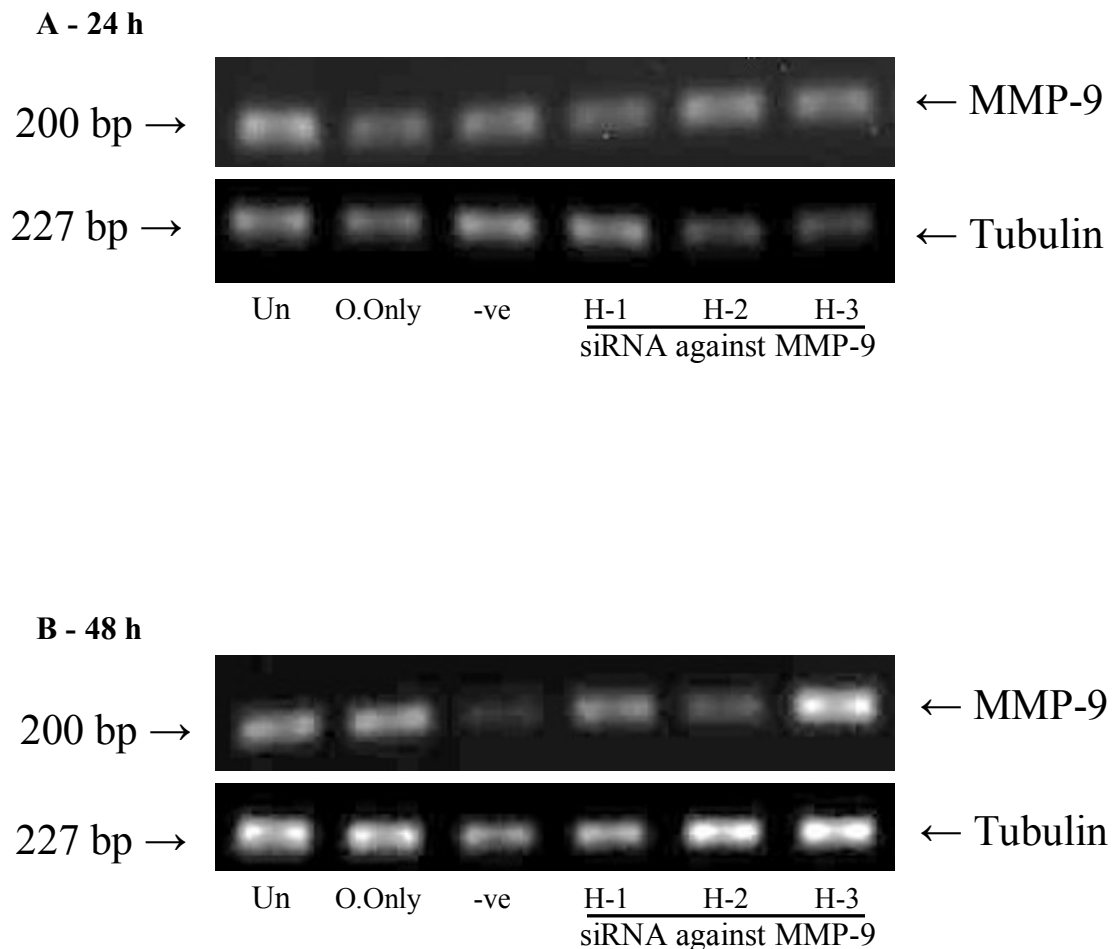
**Figure 21. MMP-9 Protein Expression in HepG2 Cells was Not Affected by Transfection with siRNA Duplexes.** HepG2 cells were transfected with an siRNA negative control duplex (-ve) at a concentration of 100 nM or a combination of three siRNA duplexes targeted to human MMP-9 at concentrations of 1.2, 1.8 and 2.4 pmol/ $\mu$ l siRNA. Total cell lysates were analyzed after (A) 24 h, (B) 48 h or (C) 72 h using Western blotting to detect MMP-9 and tubulin expression. Data are representative images from 2 experiments.

protein levels from cells transfected with the siRNA negative control duplex. Tubulin expression, however, was inconsistent in cell lysates at all time points examined (Figure 21).

To determine the potential of MMP-9-targeted siRNA duplexes to inhibit gene expression, HepG2 cells were transfected with individual duplexes targeted to MMP-9 at a concentration of 1.2 pmol/ $\mu$ l siRNA or with the siRNA negative control duplex (-ve) at a concentration of 100 nM. Total RNA was then isolated, cDNA was reverse transcribed, and MMP-9 was detected using PCR. Levels of MMP-9 gene expression were similar in cells that were untreated, treated with the transfection reagent alone (o.only), transfected with the negative control (-ve) and transfected with siRNA duplexes H-1, H-2 or H-3 when assessed after 24 h (Figure 22A). MMP-9 gene expression did not appear to decrease in response to transfection with siRNA duplexes H-1, H-2 or H-3, when compared to untreated, o.only treated and negative control treated HepG2s 48 h after transfection (Figure 22B).

### **3.9 Cold Preservation and Cold Ischemia Do Not Affect Intracellular MMP-9 Activity in Normal and Steatotic Human and Rat Cells**

An increase in MMP-9 activity is reported following IR injury *in vivo* [45, 93] [106, 108], therefore the enzymatic activity of protein lysates collected from cultured cells was examined. To observe protease activity, total cell lysates from HepG2 and McA-RH7777 cultures were assessed by gelatin zymography following the cold preservation injury assay, or cold ischemia and reperfusion assays. Intracellular MMP-9



**Figure 22. MMP-9 Gene Expression in HepG2 Cells was Not Affected by Transfection with siRNA Duplexes.** HepG2 cells were either untreated (Un), incubated with the transfection reagent only (O.Only), transfected with an siRNA negative control duplex (-ve) or one of three siRNA duplexes targeted to human MMP-9. Total mRNA was analyzed after (A) 24 h or (B) 48 h using RT-PCR to detect MMP-9 and tubulin expression. Data are representative images from 2 experiments.

gelatinase activity was not detected in cells subjected to the cold preservation injury assay (Figure 23B) or in their respective control cultures (Figure 23A) as evidenced by a lack of digested gelatin which would have resulted in a clear band at 92 kDa. In addition, there was no MMP-9 enzymatic activity detectable in liver cell lysates after 48 h of cold ischemia followed by 4 h of reperfusion (Figure 23C), while murine macrophage lysates showed considerable intracellular gelatinase activity.

### **3.10 Cold Ischemia plus Warm Ischemia May Affect Activity of MMP-9 Secreted by HepG2 Cells**

To examine the enzymatic activity of MMP-9 secreted by HepG2 in response to cold plus warm ischemia, cells were subjected to 6 h of CI, 30 min of WI and 0h or 3 h of reperfusion and supernatants were collected and analyzed for gelatinase activity. Secreted MMP-9 activity was detected from control-treated cells both before and after reperfusion (Figure 23D), however supernatants from cells incubated in chilled preservation solution in room air (4°C HTK) and in an anoxic chamber (anoxic HTK) only showed gelatinase activity following 3 h of reperfusion.



## CHAPTER 4: DISCUSSION

### 4.1 Summary

OLT is a life-saving treatment for patients in organ failure, but fatty liver disease plays a substantial role in the aggravation of IR injury caused during transplantation [4, 5, 12-16, 18]. Within the fatty acid synthesis pathway, SREBP-1c and PPAR $\alpha$  have emerged as key regulators of this process [20, 22] and are, therefore, potentially crucial players in the development and resolution of hepatic steatosis [19, 23, 27-29]. The effectors of the injury caused by ischemia and reperfusion include an extensive collection of cell types, chemical signals and proteins. Defence against this injury involves the induction of protective proteins such as HO-1 [74-79], which has been identified as playing a significant role in mediating the response to injury [84-92]. MMP-9 has been implicated as a significant mediator of this injury, given that inhibition of its expression or activity alleviates IR injury [101, 107, 109]. The process of organ transplantation is complex so isolating the role of a single gene product in an individual tissue or organ is difficult. Furthermore, donor organs have varying amounts of pre-existing disease [4, 5], presenting a further challenge for deciphering the role of proteins produced in response to IR. Methods for studying the effects of IR include *in vivo* animal studies and *in vitro* cell culture research; however current *in vitro* models [61-66] do not include the multiple phases of injury that are involved in the clinical setting of transplantation [42]. For that reason, the purpose of this dissertation was to develop an *in vitro* system which reproduces the conditions of pre-existing fatty liver disease, cold ischemia, warm ischemia and reperfusion and leads to a pattern of protein expression which is similar to the observed hepatic response to IR.



Injury from ischemia and reperfusion is inevitable, however, minimizing ischemic times can reduce the chances of affecting the organ outcome. Clinical practice requires the administration of drugs to prevent rejection, however there are no therapies aimed at reducing or eliminating the detrimental injury sustained during the transplantation process. Current research into pharmacological or gene therapies to minimize IR injury often require their systemic application to the donor, the recipient, or both [72, 85, 86, 89, 92]. In the clinical situation, donors are not often treatable with therapies, as they are frequently identified immediately prior to organ recovery. Additionally, the systemic application of virally delivered gene therapies is not without potential side effects to the recipient [115]. For that reason, therapies designed for application during the period of cold storage would, likely, decrease the risks of any detrimental off-target effects to the recipient and could prove advantageous during the process of transplantation. The *ex vivo* treatment of organs has been employed for several different kinds of therapy [71, 88, 90, 91, 116] and is thus a promising method for gene therapy in the liver. Investigations into the functions of PPAR $\alpha$  and SREBP-1c have provided evidence that alterations to these gene products could affect hepatic steatosis [19, 28, 29], and there is ample research to suggest that modulations to HO-1 and MMP-9 should affect the extent of IR injury [84-92, 106-109]. The alleviation of either condition would benefit transplant recipients. Here, gene therapy avenues were examined for their potential to affect hepatocytes in culture, as in indication if they could be applied to livers awaiting transplantation.

## 4.2 Generation of an *In Vitro* Model of IR Incorporating Normal and Steatotic Hepatic Cells

Existing models for *in vitro* ischemia and reperfusion vary in their methods used, however, no single system examines cold preservation combined with rewarming and followed by reperfusion. These multiple periods of injury occur during the process of transplantation and may also be aggravated by pre-existing disease within a donor organ. Specifically, the accumulation of intracellular lipids can have an effect on hepatic protein and gene expression, in addition to causing cell injury and death [30-32]. To examine differences in the cellular responses of steatotic and non-steatotic hepatocytes to an *in vitro* model of IR, liver cell lines were incubated in steatosis medium prior to being subjected to cold preservation, cold ischemia with reperfusion or cold plus warm ischemia with reperfusion. Histological assessment of these steatotic cells using H & E staining revealed similar general histology to cells incubated in control medium, including centrally located nuclei and numerous small vacuoles distributed throughout the cytoplasm (Figures 3 and 4; A - B). Cells treated with steatosis medium (Figures 3B and 4B), when examined with this stain, did not show evidence of macrovesicular steatosis which would have presented as large cytoplasmic vacuoles that displaced the nucleus to the edge of the cell [11]. Upon assessment of intracellular glycogen content using PAS staining (Figures 3 and 4; C - D), levels appeared similar in both control treated (Figures 3C and 4C) and steatosis treated (Figures 3D and 4D) cells. Serum starvation during the preparation of these cells, therefore, appears to have resulted in a similar effect on the glycogen content of both treatment groups. Lipid accumulation as detected by ORO staining (Figures 3 and 4; E - F), was greatly increased in the cells that were incubated in steatosis medium (Figures 3F and 4F) than in those that were given

control medium (Figures 3E and 4E) as evidenced by the abundant red staining visible within the cytoplasm of steatotic cells. The presence of many smaller fat droplets surrounding a centrally located nucleus indicates that our steatosis treatment of hepatocytes *in vitro* resulted in microvesicular steatosis [11]. Upon histological analysis with these stains, the protocol for the generation of steatotic hepatocytes resulted in intracellular lipid accumulation as expected, but did not significantly affect general morphology or glycogen content by visual comparison.

Initially, to examine the molecular effects of a period of cold preservation and recovery, we incubated cultured human and rat liver cells in 4°C, nutrient-poor preservation solution (HTK), followed by an exchange for 37°C complete culture medium (cDMEM). Based on the model employed by Emadali *et al.*, [62] the cold preservation solution was left on for 1 h, followed by a recovery period of 2 h. In order to assess the effect of hypothermia alone during storage, cells were also incubated in 4°C cDMEM for the length of preservation. Viability, as assessed by Trypan Blue stain, was not significantly affected by either hypothermia or cold preservation in any of the cell populations used (Figure 5), however, greater numbers could have led to significance. There was no significant effect on cell viability attributable to the accumulation of fat in either HepG2 or McA-RH7777 cells (Figure 5) which was unexpected as the accumulation of intracellular lipids can result in hepatocyte injury and death [30-32]. As HO-1 is upregulated following injury [74-79, 84-92], its expression was assessed in cells subjected to the cold preservation model. Levels of HO-1 protein did not change significantly in response to hypothermia alone or hypothermic preservation (Figure 6).

HO-1 expression was also not significantly changed as a result of steatosis treatment, even though intracellular fat can have an effect on hepatic protein and gene expression. Therefore, this model of cold preservation produced insufficient injury to the cells, failed to induce expression of the stress response protein, HO-1, and was consequently determined to be an inadequate model of IR.

To further develop the model for IR and more closely approximate the loss of blood supply to a tissue, oxygen deprivation was introduced during the time of preservation. Additionally, the period of preservation was increased, as the cold storage of an organ during transplantation is typically much longer than 1 h. Finally, cell viability was assessed at various times following the exchange of the cold storage solution, HTK, for complete culture medium. To deprive the cells of oxygen, we used a modular incubator chamber filled with an anoxic gas mixture, held at 4°C for culture incubations and preconditioned the HTK solution before use. As cultured cells do not receive a blood supply, this anoxia and deprivation of nutrients is only an approximation of ischemia; however for the purposes of this dissertation, this incubation period is referred to as cold ischemia. Similarly, the introduction of oxygenated, nutrient-rich cDMEM is referred to as reperfusion, as it approximates clinical reperfusion where blood flow is restored to newly transplanted organs.

The viability of HepG2 human liver cells decreased when subjected to 2 h of cold ischemia and 2 h of reperfusion, as assessed by both the MTT (Figure 7A) and WST-1 assays (Figure 7B). Cell viability was also decreased in normal human (NH), normal rat

(NR), steatotic human (SH) and steatotic rat (SR) cells following 2 h of ischemia and 2 h of reperfusion (Table 1 and Figure 8A). Cell viability assessed after 4 h of reperfusion indicated a decrease for all populations except SR cells (Table 1 and Figure 8A). However, 3 h of ischemia did not affect the viability of any population when assessed after 3 h or 6 h of reperfusion (Table 1 and Figure 8B). Cold ischemia of 5 h did decrease viability in all populations when assessed following 3 h of reperfusion, but only affected NH and SR cells when assessed after 24 h of reperfusion (Table 1 and Figure 8C). In addition, fatty build-up appeared to affect the viability of rat cells following 5 h of ischemia (SR compared to NR), but not human cells (Table 1 and Figure 8C), however, lipid accumulation did not appear detrimental to cell viability at shorter ischemic times (Table 1 and Figure 8A, B). These brief ischemic times did not correlate well with decreases in viability of liver cell populations and it was decided that ischemia of cultured cells would be extended.

As significant death of HepG2 cells has been noted after 24 h, 48 h and 72 h of hypoxia by other investigators [117, 118], ischemia was increased to include these times and viability was also assessed immediately following ischemia (0 h reperfusion). After 24 h of ischemia, with or without 2 h of reperfusion, SH cells had decreased viability, while all other populations showed no change compared to the control treatment (Table 1 and Figure 8D). This indicates a possible effect on viability due to increased cytoplasmic fat (SH compared to NH). After 48 h of ischemia and 0 h, 4 h, or 7 h of reperfusion, all populations except SR cells showed a significant decrease in cell viability (Table 1 and Figure 8E), with NH and SH cells being affected approximately equally. The 72 h

ischemic treatment, with or without 2 h of reperfusion, decreased the viability of SH cells, but reduced the viability of NR cells only when assessed immediately following ischemia (Table 1 and Figure 8F). The 24 h and 48 h data are somewhat in agreement with assessments of HepG2 cells subjected to glucose deprivation and hypoxia [117], where viability was greatly reduced after 48 h but less severely affected after 24 h. However, the 72 h data are not consistent with the observation that hypoxia significantly reduces viability by the third day [118]. The viability of SH cells was reduced, while the viability of SR cells was unaffected at ischemic times of 24 h and longer, making of the role of fat accumulation in hepatocytes difficult to determine.

As 48 h of ischemia led to a significant decrease in viability of most populations, and 4 h of reperfusion was adequate to detect this effect, these were determined to be the best representation of hepatic IR, and this model was used to investigate the cellular response to injury. As HO-1 is upregulated following injury [74-79, 84-92], and MMP-9 expression and activity are increased in response to injury [45, 93, 104, 106-110], the expression of both proteins was assessed in normal and steatotic human and rat liver cells following 48 h of ischemia and 4 h of reperfusion. The IR significantly increased expression of HO-1 in all cell populations (Figure 9) indicating 48 h of ischemia was a sufficiently stressful condition to increase levels of this heat shock protein. Combined with the observation that viability decreased in both human populations following this treatment (Table 1 and Figure 8E), this indicates that prolonged (48 h) ischemia results in cellular injury. This injury was also evident in NR cells, but not in SR cells suggesting that fatty build-up may confer protection against cell death during long periods of

ischemia (24 h to 72 h). Further studies using cultured rat cells may provide insight into this phenomenon. This model of IR injury did not result in increased MMP-9 expression (Figure 10) in any of the cell populations suggesting it may not play a significant role in mediating hepatocyte death resulting from this prolonged *in vitro* IR.

Cold ischemia of 48 h is quite a bit longer than the time an organ is preserved in the clinical setting, so we reduced the incubation time to fewer than 10 h. In addition, we included an approximation of the brief rewarming period that occurs during organ implantation, by transferring the cold plates of cells to the 37°C, 5% CO<sub>2</sub> environment for a short amount of time, which is referred to as a period of warm ischemia. We decided to examine HO-1 and MMP-9 protein expression in human HepG2 cells following two different combinations of cold ischemia (CI) plus warm ischemia (WI) and reperfusion (R). Additionally, an intermediate level of injury was expected from cold plus warm preservation without concurrent anoxia. Neither HO-1 (Figure 11) nor MMP-9 (Figure 12) expression was increased following the combination of 9 h CI + 1 h WI + 3 h R, so both ischemic times were reduced for further evaluation. The trend observed in MMP-9 expression following 6 h of cold plus 30 min of warm preservation, or 6 h CI + 30 min WI + 3 h R (Figure 13) indicated that increasing stress may affect its expression. In support of this observation, preliminary assessments of mRNA levels indicate increased MMP-9 expression in HepG2 cells (Figure 14), as well as NH and SH cells (Figure 15) subjected to anoxic HTK incubations used to approximate cold ischemia in this *in vitro* model of IR.

### 4.3 Gene Therapy *In Vitro*

The upregulation of HO-1 following 48 h of cold ischemia and 4 h of reperfusion (Figure 9) was not associated with cellular protection in our model, as evidenced by the decreased viability (Table 1 and Figure 8E), although an increase in HO-1 expression is often associated with reduced injury from IR [84-92]. Additionally, although increases in MMP-9 protein levels did not reach significance, the preliminary data indicate that MMP-9 gene expression is upregulated in response to 6 h CI + 30 min WI + 3 h R (Figures 14 and 15); increased MMP-9 expression is implicated in the progression of IR injury [45, 93, 104, 106-110]. Therefore, therapies designed to provide additional HO-1, or to reduce the expression of MMP-9, should reduce the severity of injury from that seen in our *in vitro* model of IR and provide support for their eventual use in organ transplantation, a process which presents an opportunity for the direct and restricted application of these therapies. However, they would need to be administrable during hypothermic storage, and be able to provide continued benefits after being flushed from the organ.

Gene therapies are an attractive option for this application, as researchers have demonstrated successful expression of HO-1 delivered to liver and aortic tissue by adenoviral vector during cold storage [88, 90]. Henry *et al.* have designed lentiviral vectors capable of hepatocellular integration when applied during hypothermic preservation *in vitro* [119]. Moreover, Henry *et al.* have also demonstrated long-term (> 2 weeks) expression of lentiviral shRNA constructs in transduced cells *in vitro* [120]. For this reason, cloning techniques were used here to produce a lentiviral vector that would



result in overexpression of human (Figure 16) or rat (Figure 17) HO-1 in mammalian cells and lentiviral vectors containing shRNA sequences targeted to the human MMP-9 gene were purchased. When shRNA sequences are transcribed, they are processed using the miRNA pathway, involving the nuclear RNase Drosha and the cytoplasmic RNase Dicer before they interact with the RISC complex [121]; this results in suppressed expression of the gene they target at the levels of transcription and translation as well as through degradation of the mRNA transcript. It was therefore expected that lentiviral transduction of human and rat cultured cells would lead to suppression of MMP-9 expression as assessed by Western blotting. The reporter gene coding for green fluorescent protein (GFP) was detected in cells transduced with MMP-9-targeted and GAPDH-targeted lentivirus (Figures 18 and 19), however, protein expression of MMP-9 and GAPDH did not appear to be affected (Figure 20). Lysates from several experiments were collected once visual inspection of transduced cultures indicated GFP expression in all cells, so integration is likely to have occurred; it is possible therefore, that the miRNA processing pathway in HepG2 cells is ineffective for sequences coded by the plasmid pGIPZ. Further research using alternate plasmids having a lentiviral backbone may provide insight into this occurrence.

As an alternative to the long-term suppression of target genes by lentiviral-delivered shRNA, siRNA duplexes can provide short-term suppression without requiring DNA integration which can generate undesired genetic manipulations [115]. Target cells are transfected with siRNA duplexes using a lipid-based reagent and allow for direct interaction with the cytoplasmic RISC complex, leading to RNA interference through

mRNA cleavage and degradation. Three different siRNA duplexes targeted to human MMP-9 were purchased for use in the human hepatocyte cell line; following transfection of HepG2 cells with these duplexes, either in combination or individually, protein expression was assessed by Western blotting and gene expression was assessed by RT-PCR. Protein levels of MMP-9 assessed in cells 24 h after transfection with a combination of the three duplexes appeared higher than in cells transfected with the negative control duplex (Figure 21A). Tubulin expression in transfected cells, however, also appeared greater when compared to the negative control sample (Figure 21A) indicating that MMP-9 expression was likely unaffected by siRNA transfection when assessed after 24 h. MMP-9 expression assessed 48 h after transfection with the combined siRNA duplexes indicated possible knockdown when examined visually, however this result was not consistent between both experiments performed (Figure 21B and data not shown). Protein levels of MMP-9 72 h after transfection also appeared to decrease in response to treatment with the targeted siRNA duplexes (Figure 21C), however, tubulin levels in these cells were also reduced when compared to the negative control treatment. It is possible, therefore, that the method we employed for the transfection of siRNA does not result in adequate RNA interference and decreased levels of MMP-9 protein in HepG2 cells. The level of MMP-9 mRNA did not decrease in the presence of individual siRNA duplexes, relative to the levels detected in the untreated, transfection reagent only treated and negative control treated cultures, when assessed 24 h (Figure 22A) or 48 h (Figure 22B) after transfection. MMP-9 gene expression might also be unaffected by our method of transfecting targeted siRNA into HepG2 cells.

#### 4.4 Strengths and Weaknesses of This Study

In order to study gene therapies for their potential to ameliorate injury caused by hepatic steatosis, ischemia and reperfusion, or both of these combined, we attempted to design and employ an *in vitro* model of IR using hepatocellular carcinoma cell lines. The final model included cold, anoxic preservation (CI) with a period of rewarming (WI), followed by an exchange for nutrient-rich, 37°C culture medium (R). Hepatocyte cell lines were cultured to induce fat accumulation prior to IR insult, in order to reproduce the condition of hepatic steatosis found in the transplant setting. This *in vitro* system offers a greater understanding of the individual contributions of hepatocytes to the injury associated with fatty liver disease and IR by describing changes in the protein and gene expression of HO-1 and MMP-9, known to play important roles in the progression of injury and disease. It also provides an isolated environment in which to apply gene therapies and assess their effects on specific hepatic cell populations.

The model was initially applied to assess the viability of normal and steatotic populations of hepatocytes in response to ischemic insult and subsequent reperfusion. While viability was affected by varying lengths of ischemia, it did not show correlation with length of ischemic time (Table 1 and Figure 8). This is not in agreement with reports of decreasing viability of HepG2 cells when subjected to increasing lengths of time in hypoxia [117, 118], however the significant death reported after 72 h of hypoxia was performed at 37°C in a nutrient-rich culture medium, and not in a preservation solution. As HTK is designed for organ preservation, it is possible that our results reflect the protective properties of this solution. Furthermore, it has been reported that the rat cell

line, McA-RH7777 is more resistant to cell necrosis than primary hepatocytes during anoxia at 37°C [122], indicating that our *in vitro* IR may not adequately model the cellular injury caused during rat tissue ischemia *in vivo*.

Fatty acid accumulation did not appear to have a detrimental effect on hepatocyte viability (Table 1 and Figure 8). This is not congruent with the observation that FA accumulation can impair cellular functions after ischemia and reperfusion [67], or with the observation that an accumulation of palmitate in cultured hepatocytes leads to cell death [31, 32]. However, it is in agreement with the observation that incubation of cultured cells in a relatively high concentration (2 mM) of combined oleic and palmitic acid (2:1) induces apoptosis, while exposure to a lower concentration (1 mM) of the combined FAs, which was the concentration used in our model, did not significantly affect viability [30]. In addition, hepatocytes in rat livers are resistant to injury even after 96 h of cold ischemia, maintaining approximately 90 % viability when assessed shortly after the initiation of reperfusion [46]. Our observation that cell death did not correlate well with increasing ischemic time is likely due to the fact that in the liver, Kupffer cells are the primary mediators of cell injury immediately following reperfusion [59], and infiltrating neutrophils contribute to hepatocyte injury and death observed at later times following reperfusion [57, 58]; our *in vitro* IR did not include the addition of either of these cell types.

We were not successful in examining PPAR $\alpha$  or SREBP-1 expression in hepatocytes subjected to the cold preservation or cold ischemia models, as Western

analysis was unreliable in our cell populations (data not shown). Furthermore, while hepatocytes are able to induce the expression of both HO-1 [76] and MMP-9 [98] during injury or stress, they are not the predominant producers of these proteins in the liver. Upon histological examination of normal human livers, Kupffer cells chiefly express HO-1 [76, 84], and are seen to upregulate its expression in response to IR [76]. Additionally, as Kupffer cells are liver macrophages, they are, most likely, the principal source of hepatic hydrolytic enzymes [59], such as MMP-9, whose activity is involved in the progression of IR injury. We were unsuccessful in isolating the Kupffer cell population from rat livers for use in culture; however it was observed that a murine macrophage cell line (J774), at rest, produced significantly more MMP-9 gelatinase activity than did the HepG2 or McA-RH7777 hepatocyte cell lines even after *in vitro* IR (Figure 23C). Kupffer cells subjected to this model of *in vitro* IR are, therefore, more likely to result in increased expression of HO-1 and MMP-9, and would be expected to be the superior target cell population for gene therapies.

Although intracellular gelatinase activity was not detectable in hepatocytes subjected to cold preservation (Figure 23B), or cold ischemia (Figure 23C), there was activity detected in supernatants collected from HepG2 cells following 6 h CI + 30 min WI + 3 h R (Figure 23D). While this appeared to indicate a cellular response to IR, it was later determined that the serum component of the complete medium used to mimic reperfusion had intrinsic gelatinase activity detectable at a similar size to that of MMP-9 (data not shown). Moreover, during reperfusion, the substitution of medium which lacked serum appeared to abrogate the increase in hepatocyte MMP-9 protein levels in response

to IR (data not shown). It is possible that upregulation is occurring at the level of gene expression, as indicated by the preliminary RT-PCR examinations of MMP-9 mRNA from human hepatoma cells following *in vitro* IR (Figures 14 and 15). MMP-9 therefore, remains a potential target for gene therapies that could suppress its expression during IR.

Finally, our attempt to use lentiviral gene therapy in human HepG2 cells was unsuccessful, as protein expression of both MMP-9 (Figure 20A, B) and the housekeeping gene GAPDH (Figure 20B, C) were not affected by transduction with the vectors encoding shRNA targeted to these genes. Henry *et al.* designed lentiviral vectors that resulted in target knock down (examined using luciferase assay and real-time RT-PCR) in hepatocellular carcinoma cells that were transduced *in vitro* [120]. It is possible, therefore, that HepG2 cells are unable to fully process shRNAmir constructs delivered by the lentiviral plasmids we purchased and result in protein knock down; several different attempts were made to alter the transduction conditions to result in effective knock down. It is also possible that the serum within the culture medium affected the levels of MMP-9 collected during lysate collection. Our siRNA gene therapy did not cause decreased levels of MMP-9 protein in HepG2 cells (Figure 21), as both tubulin and MMP-9 expression was inconsistent between experiments, and levels of MMP-9 mRNA were not reduced following siRNA treatment (Figure 22). This may also be a result of the serum component of the complete medium which was supplied to cells following transfection, or may be a consequence of inefficient duplex uptake or association with RISC in HepG2 cells. In addition, lentiviral vectors that were designed for overexpression of HO-1 in human (Figure 16) and rat (Figure 17) cells were not employed in this study, as the

addition of exogenous HO-1 became the focus of another body of work, however, plasmids will be stored so that they are available for future work. Moreover, while the vector encoding human HO-1 was successfully cloned, (DNA sequence analysis has yet to be performed), the vector encoding rat HO-1 was not successfully cloned from the TOPO vector into the final pLEX-MCS backbone (clones frequently integrated the R-HO-1 sequence in an incorrect orientation) and HO-1 overexpression without the use of virions was explored in the other project.

#### **4.5 Future Directions**

The cellular mediators of IR injury include TNF- $\alpha$ , ROS and hydrolytic enzymes, and are produced mainly by the resident macrophages of the liver, the Kupffer cells, which creates an inflammatory environment that leads to hepatocyte injury and death. Our *in vitro* model of IR could, therefore, also be tested by using KC, in order to assess changes in their gene expression, protein expression and gelatinase activity in response to the cold anoxia, rewarming, and reperfusion. Furthermore, hepatocyte co-culture experiments with KC or neutrophils could provide information regarding the extent of injury sustained by liver cells burdened with fatty build-up in comparison to normal cells following IR. As TNF- $\alpha$  expression is increased in livers with extensive steatosis [33, 34] and KC phagocytic activity is enhanced in livers having significant fatty infiltration [68], co-culture experiments may indicate that steatotic hepatocytes are more susceptible to injury following *in vitro* IR.

The size of MMP-9 protein detected in hepatocytes was predominantly 92 kDa (Figures 10, 12, 13 and 20A, B), indicating the presence of the full-length enzyme while the smaller, 82 kDa active enzyme is faintly visible below some of the bands, but appears much more visible in other HepG2 lysates (Figure 20A). This indicates that both forms of the enzyme are present, in different amounts, in human hepatoma cells. As MMP-9 is usually secreted as a pro-enzyme and activated after cleavage, co-culture experiments could result in significant conversion of the enzyme being produced by hepatocytes. Additionally, further assessments of the effectiveness of MMP-9-targeted gene therapy could result in the detection of successful knockdown of expression in hepatocytes, or more likely in the resident macrophages, the KC. These cells expressing reduced levels of MMP-9 could be subjected to *in vitro* IR, with or without additional cell types, and would be assessed for changes to their viability or gene and protein expression. However, future research into long-term silencing of MMP-9 should consider that reduced MMP-9 activity may inhibit liver regeneration and hepatocyte proliferation; broad inhibition of MMPs following hepatectomy decreases MMP-9 activity and inhibits liver regeneration [113] while hepatocyte proliferation *in vitro*, in response to cytokine stimulation, is dependent on MMP-9 expression [114]. Transient suppression of MMP-9 activity may therefore prove sufficient to reduce injury from IR without affecting repair and regeneration after transplantation.

The current use of the model, however, could provide insight into the possible production of proinflammatory cytokines by normal and fatty hepatocytes following IR. As these cytokines influence the production of neutrophil-attractant chemokines at early



times post-transplant [54], their possible production by the parenchymal cells would have significance regarding the progression of inflammation. Supernatants were collected from normal and steatotic liver cells after *in vitro* IR and could be analyzed for enhanced cytokine production as a result of either IR, or the accumulation of intracellular fat.

The ultimate goal of this study was to develop an *in vitro* model which would permit us to examine the protein production of hepatic cells in response to IR, and to allow the application of gene therapies to these cells *in vitro*. This model was designed in order to assist in the development of gene therapies which can be applied during organ preservation and would influence the progression of IR injury.

#### **4.6 Conclusions**

Current methods for studying *in vitro* ischemia and reperfusion may not include the various phases of injury that are known to be involved. I have presented a model of *in vitro* IR that allows for the inclusion of pre-preservation injury, cold preservation injury, rewarming injury and reperfusion injury for cultured cells. In addition, I have examined the expression profile of HO-1 and MMP-9 in hepatocytes following treatment with this model, and determined that ischemia upregulates HO-1 expression, and may upregulate MMP-9 expression. However, the observed induction of HO-1 was not sufficient to prevent cell death following IR, therefore the addition of exogenous HO-1 may be of potential therapeutic benefit to the cultured cells in this model. As well, MMP-9 remains a promising target for RNA interference therapies aimed at impeding the progression of IR injury.

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