Liver Regeneration after Resection and Transplantation:

Mechanisms and Therapeutic Strategies

Suomi M.G. Fouraschen

The studies presented in this thesis were performed at the Department of Surgery, Laboratory of Experimental Transplantation and Intestinal Surgery, Erasmus MC-University Medical Center, Rotterdam, The Netherlands, at the Department of Surgery, Penn Transplant Institute, University of Pennsylvania, Philadelphia, PA, United States and at the Department of Molecular and Experimental Medicine, The Scripps Research Institute, La Jolla, CA, United States.

The research was financially supported by the ASTS-ESOT Exchange Grant, The Astellas Trans(p)la(n)t(at)ional Research Prize, the National Institutes of Health through the National Institute of Diabetes and Digestive and Kidney Diseases (grants U01-DK-062494 and RO1 DK073192-02; K. Olthoff PI) and the Foundation for Liver and Gastrointestinal Research (SLO).

The publication of this thesis was financially supported by the Dutch Society for Hepatology (Nederlandse Vereniging voor Hepatology, NVH) and the Dutch Transplant Society (Nederlandse Transplantatie Vereniging, NTV).

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Cover design: Suomi M.G. Fouraschen with the permission of and special thanks to Scott Eaton, designer of the digital sculpture of Prometheus (The London Studio, London, United Kingdom, www.scott-eaton.com) and Dave Ganley, designer of the wooden eagle sculpture (Intricate Wood Carvings, Whitefish, MT, United States, www.intocarving.com).

Layout: Suomi M.G. Fouraschen

Printed by Wöhrmann Print Service, Zutphen

ISBN: 97894-6203-445-7

Liver Regeneration after Resection and Transplantation:

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Leverregeneratie na resectie en transplantatie:

mechanismen en therapeutische strategieën

Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de
rector magnificus

Prof.dr. H.G. Schmidt

en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op
26 september 2013 om 15.30 uur

door

Suomi M.G. Fouraschen

geboren te

Maastricht

Promotiecommissie

Promotor: Prof.dr. H.W. Tilanus

Overige leden: Prof.dr. H.J. Metselaar
Prof.dr. K.M. Olthoff
Prof.dr. G. Kazemier

Copromotoren: Dr. L.J.W. van der Laan
Dr. J. de Jonge

Contents

Chapter 1 General introduction & Thesis outline 9

I *Mechanisms*

Chapter 2 Immediate early gene expression profiles of living donor livers show a shift in key cellular functions related to the extent of regeneration 27
(Submitted)

Chapter 3 Genetic profiles and predictors of early allograft dysfunction after human liver transplantation 59
(*Submitted)*

Chapter 4 mTOR signaling in liver regeneration: rapamycin combined with growth factor treatment 81
(*World Journal of Transplantation, 2013)*

II *Therapeutic Strategies*

Chapter 5 Mobilization of hepatic mesenchymal stem cells from human liver grafts 103
(Liver Transplantation, 2011)

Chapter 6 Detection of spontaneous tumorigenic transformation during culture expansion of human mesenchymal stromal cells 125
(Experimental Biology and Medicine, 2013)

Chapter 7 Studying paracrine effects of mesenchymal stromal/stem cell-derived factors in vivo on liver injury and regeneration 147
(Methods in Molecular Biology, book chapter, conditionally accepted)

Chapter 8 Secreted factors of human liver-derived mesenchymal stem cells promote liver regeneration early after partial hepatectomy 165
(Stem Cells and Development, 2012)

Chapter 9	Mesenchymal stromal cell-derived factors promote tissue repair in a small-for-size ischemic liver model, but do not protect against early effects of ischemia and reperfusion injury *(Manuscript in preparation)*	183
Chapter 10	Summary, General discussion & Future perspectives	201
Chapter 11	Nederlandse samenvatting/Dutch summary	213
Chapter 12	Appendix:	221
	Dankwoord/Acknowledgements	223
	PhD portfolio	227
	List of publications	231
	Curriculum vitae auctoris	232

Chapter 1

General introduction & Thesis outline

Prometheus bound

Prometheus, the Greek titan who tricked Zeus and stole fire from the gods to give it to mankind, was punished by being chained to a rock, having his liver eaten out every day by an eagle. This story about Prometheus' liver growing back overnight has captured the imagination of many involved in regeneration research. Did the ancient Greek know about the liver's fascinating ability to repair itself? The first scientific documentation of this phenomenon was not presented until the 19th century, and while scientists since then revealed numerous molecules and pathways involved in this process, the exact underlying mechanisms of Prometheus' regenerating liver are still not fully unraveled.

Prometheus Bound
Peter Paul Rubens and Frans Snyders
Completed 1618
Philadelphia Museum of Art

The liver is an essential organ, with a wide range of vital functions, including detoxification, protein synthesis and production of biochemicals necessary for digestion and absorption of nutrients. The liver thereby regulates metabolism and maintains homeostasis. Loss of functional liver cells by injury or disease activates the regenerative machinery of the liver in order to compensate for lost or damaged tissue. However, several factors like a patient's age, life style, nutritional status, disease condition, degree of injury and medication, but probably also genetic predisposition, can interfere with and limit the process of regeneration, resulting in impaired liver function and compromised homeostasis. Understanding the underlying mechanisms of liver regeneration is of major clinical relevance to prevent liver dysfunction in case of severe injury or compromised patients. Furthermore, extensive knowledge on the factors and pathways involved in this remarkable process

contributes to potential new therapeutic strategies to stimulate liver regeneration and improve recovery of a patient.

Liver resection and transplantation

In healthy individuals, the liver is able to compensate an acute loss of up to 70-75% of its total mass.[1-3] Clinical settings in which this extensive regenerative capacity can be used to benefit patients with (end-stage) liver disease are oncologic liver resections and living donor cq. split liver transplantation. In case of oncologic resections, however, regeneration can be compromised due to neo-adjuvant chemotherapy, poor nutritional status and increasing age of the patient population.[4-7]

Living donor and split liver transplantation, on the other hand, was introduced to help overcome donor organ scarcity and reduce mortality on the liver transplant waitlist. In the setting of living donor liver transplantation, 40-60% of the donor’s liver volume is resected and transplanted into a recipient with end-stage liver disease. Both donor and recipient thus end up with a small-for-size liver, which requires robust regeneration and is associated with significant morbidity and mortality.[2, 8, 9] The use of smaller grafts, in an attempt to reduce donor morbidity, is limited by the risk for the recipient to develop small-for-size syndrome.[10-12]

Besides receiving a graft relatively small to cope with their urgent metabolic needs, recipients are treated with immunosuppressant medication, which is essential to prevent graft rejection, but can also affect regeneration. Especially the use of the mTOR inhibitor rapamycin has raised concerns, as mTOR (mammalian target of rapamycin) is involved in the control of protein synthesis, cell size and proliferation. Multiple studies have reported detrimental effects of rapamycin on hepatocyte proliferation and liver mass reconstitution.[13-16] Steroids, on the other hand, are known to inhibit the expression of specific cytokines, among which the regeneration-initiating cytokines tumor necrosis factor alpha (TNF-α) and interleukin 6 (IL-6). Steroid treatment in the setting of liver transplantation has been described to result in inhibited hepatocyte proliferation as well as cellular hypertrophy.[17-19] In contrast, calcineurin inhibitors are suggested to improve hepatocyte proliferation, though the mechanism leading to this effect is largely unclear.[20-22] Treatment with calcineurin inhibitors, however, is associated with a 20% incidence of chronic kidney dysfunction and carries a cumulative risk for de novo malignancy of up to 55% at 15 years after liver transplantation.[23-27] Potential therapeutic strategies to improve liver regeneration and stimulate recovery after surgical injury and transplantation are therefore most welcome.

Mechanisms of liver regeneration

Liver function reflects a continuous balance between metabolic homeostasis and cellular proliferation.[28-31] In a normal setting, nearly all hepatocytes reside in the resting (G0) phase of the cell cycle and focus on their metabolic activities. Disturbance of this quies-

cent phase by surgical, toxic or infectious injury leads to activation of regenerative mechanisms. In healthy individuals, regeneration of functional liver mass is largely established by the rapid proliferative response of remaining hepatocytes. After massive toxic or chronic liver injury, however, hepatocytic regeneration can be compromised due to extensive destruction of hepatocytes. In this situation, stem and progenitor cells respond and mediate regeneration.[29, 32, 33]

In the first situation, loss of liver mass activates cell proliferation by the release of mitogenic factors. Hepatocytes are the first cells that enter the cell cycle, followed by the replication of ductal and non-epithelial cell types.[34] The role of several cytokines, growth factors and hormones in this process has been extensively studied in rodent models.[29, 31, 35-39] A widely used experimental model is the 70% partial hepatectomy model, first described by Higgins and Anderson in 1931. Studies on this experimental model have identified liver regeneration as a multi-step process.

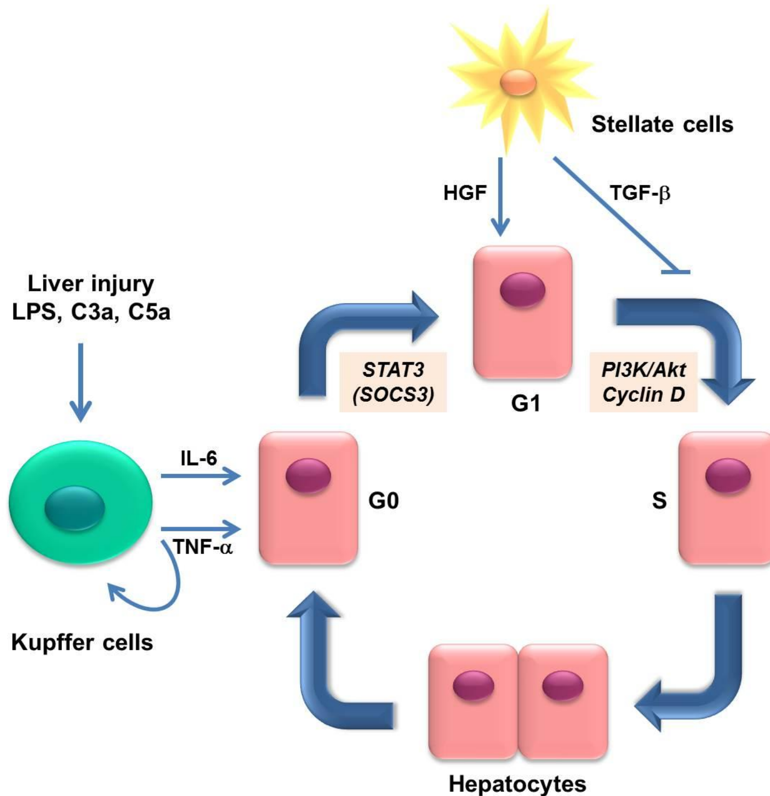

Figure 1. Hepatocyte proliferation triggered by liver injury

The release of mitogenic factors after liver injury activates hepatic non-parenchymal cells (including Kupffer cells and stellate cells) and thereby cytokine- and growth factor-dependent signaling pathways. Upregulation of the cytokines TNF-α and IL-6 primes hepatocytes to enter the G1 phase of the cell cycle after which growth factors like HGF initiate proliferation of primed hepatocytes. These cooperative signals allow hepatocytes to pass through cell cycle checkpoints, enter the DNA synthesis (S) phase and proliferate.

During the initial ('priming') phase, nuclear factor-kappa B (NF-κB) in Kupffer cells is activated by TNF-α, lipopolysaccharides (LPS) and complement components (Figure 1).[28, 40-43] Upon this activation, Kupffer cells release IL-6 which binds to its receptor on the cell surface of hepatocytes, thereby activating signal transducer and activator of transcription 3 (STAT3).[40, 44, 45] This priming phase stimulates resting hepatocytes to enter the G1 phase of the cell cycle.

Concomitant expression of immediate early genes causes transcription factor activation which is followed by the expression of cell-cycle related genes.[46] This process results in the production and activation of growth factors, including hepatocyte growth factor (HGF) secretion by hepatic stellate cells. HGF interacts with the c-met receptor on hepatocytes and thereby initiates replication of primed hepatocytes by activating the phosphoinositide-3 kinase(PI3K)/Akt signal transduction pathway.[47-49] PI3K/Akt in turn interacts with mTOR, which is involved in the control of protein synthesis, cell size and proliferation.[50, 51]

Both cascades lead to activation of a variety of signaling pathways, including upregulation of downstream cyclins like cyclin D1, which is associated with the G1-S phase transition of hepatocytes.[40, 44, 47, 52, 53] After passing through the G1 restriction point, hepatocytes are irreversibly committed to replicate.

When the regenerated liver mass is sufficient to meet the metabolic needs of the patient, the process of regeneration is terminated. Negative feedback mechanisms of cell proliferation are poorly understood, but appear to be mainly regulated by the activation of suppressor of cytokine signaling 3 (SOCS3), which inhibits STAT3 signaling, and the production of tissue growth factor beta (TGF-β) by hepatic stellate cells, which inhibits DNA synthesis and cyclin signaling.[54-56]

If this mechanism of hepatocyte proliferation appears insufficient, stem/progenitor cells contribute to the process of liver regeneration. Stem/progenitor cells represent a population of cells with the ability to replicate indefinitely and differentiate into multiple distinct specialized cells. Several subtypes have been identified throughout the last decades, dependent on their origin, differentiation potential and cell surface markers.[57, 58] Within varying study setups, stem cells have been described to contribute to liver regeneration by 1) transdifferentiation into hepatocytes and cholangiocytes[33, 59, 60], 2) cell fusion resulting in liver cells that express both donor and host genes[61-63] and 3) secretion of various trophic factors that support endogenous regeneration pathways[64-66] (Figure 2). However, there is an ongoing discussion on the exact route by which stem/progenitor cells contribute to liver regeneration and further research is needed to address this issue.

Ischemia and reperfusion injury

In the setting of living donor or split liver transplantation, grafts are not only subjected to loss of liver mass, but also to ischemia and reperfusion injury (IR injury).[67] IR injury starts with the lack of blood flow and oxygen supply, leading to anaerobic respiration and

deficiency of adenosine triphosphate (ATP) production in hepatocytes, Kupffer cells and sinusoidal endothelial cells.[68, 69] As a result, cells enter a situation marked with intracellular ionic disturbance and acidosis, cellular swelling and narrowing of the sinusoids.

Additional damage is caused by enhanced levels of reactive oxygen species (ROS), occurring shortly after reperfusion of the graft with oxygenated blood.[70-72] ROS lead to oxidative damage and induction of apoptosis and necrosis of hepatocytes and endothelial cells.[73, 74] Concomitant release of pro-inflammatory mediators, including interleukin 1 beta (IL-1β) and TNF-α, by activated Kupffer cells stimulates migration of neutrophils and CD4+ T-lymphocytes into the liver.[75, 76] Influx of these inflammatory cells results in continuous activation and stimulation of the different cell subtypes with subsequent on-going inflammatory responses and destruction of hepatocytes and sinusoidal endothelial cells.[76-78]

Impaired regeneration and liver function

As previously mentioned, factors like age, nutritional status, pre-operative clinical condition, degree of tissue injury and certain medication can influence regeneration of the liver after surgery. Severe impact of these internal and external factors can result in impaired liver function or even hepatic failure. Liver failure is clinically manifested by high transaminases, persistent cholestasis and prolonged coagulopathy, and can result in encephalopathy or even death.

Shortly after transplantation, approximately one quarter of liver recipients display evidence of such severe hepatocellular damage and functional impairment.[79-81] This condition, termed early allograft dysfunction (EAD), is associated with significantly decreased graft and patient survival.[79, 80] EAD is thought to be caused by donor and recipient characteristics combined with surgical factors and associated with oxidative stress, immune activation and severe inflammatory responses resulting in acute cellular damage and cell death.[79, 82-85] However, there is still a lack of mechanistic insight in the pathways associated with graft dysfunction and clinical outcome. A possible mechanism could be an excessively triggered inflammatory response, prohibiting the liver to maintain necessary metabolic processes and thereby leading to the symptoms of dysfunction seen in EAD patients. Elucidation of these pathways could identify specific donor or recipient risk factors leading to this condition and determine biomarkers for the early detection or even prediction of allograft dysfunction.

Therapeutic strategies to improve liver regeneration

Identification of factors involved in liver regeneration has allowed development of recombinant cytokines and growth factors to promote liver regeneration. Successful effects have been reported for many factors, including TNF, IL-6, HGF, vascular endothelial growth factor (VEGF) and their receptors.[86-89] However, these proteins often have a short half-life, necessitating repeated or continuous administration and thereby limiting the application of this therapeutic strategy.[90-92]

To overcome this problem, gene transfer technologies were used to induce intrinsic production of growth factor proteins.[47, 93, 94] Besides disappointing transduction rates into hepatocytes in vivo, major disadvantages have been reported on the use of viral vectors for transfection, including the risk of insertional mutagenesis by random integration into the host chromosome as well as serious inflammatory responses and potentially fatal toxicity.[95] The use of non-viral vectors for in vivo liver gene therapy, including various liposome preparations, nanoparticles and naked or complexed DNA, showed that expression is often low and transient because of instabililty of the DNA in cells.[96, 97]

Recently, microRNAs (miRNAs) have emerged as a promising treatment strategy. MiRNAs are endogenous small non-coding RNAs (approximately 22 nucleotides) with a posttranscriptional regulatory function by binding to target messenger RNAs (mRNAs).[98] One miRNA can bind to multiple target mRNAs, leading to inhibition of their translation or inducing their degradation. Several publications describe miRNAs as potential biomarkers for hepatic injury and liver graft dysfunction.[99-102] Furthermore, miRNA gene transfer technologies as well as the development of anti-miRs (miRNA inhibitors) for specific miRNAs have brought forward therapeutic opportunities to stimulate liver regeneration.[103-105] Despite these promising results, additional mechanistic studies are essential to address the lack of knowledge on how miRNAs control gene and protein expression in tissues.

Probably the most investigated potential therapeutic interventions are stem/progenitor cell-based strategies. As previously described, stem/progenitor cells are cells that have the ability to divide and renew themselves as well as to differentiate into specialized cell types. They have been described to contribute to liver regeneration by transdifferentiation, cell fusion and paracrine effects of their trophic factors (Figure 2). Different types of stem and progenitor cells, including embryonic stem cells, induced pluripotent stem cells, hematopoietic stem cells, mesenchymal stromal/stem cells and oval cells, have been reported to promote liver regeneration.[57, 106, 107] Currently, the first stem cell-based studies in humans suffering from liver disease are being conducted. So far, results have shown that stem cell therapy improves liver function by decreasing serum bilirubin and transaminase levels as well as markers associated with fibrosis, normalizing albumin, total protein and INR levels and reduction or disappearance of ascites.[108-111] Though promising, further investigation is needed to fully evaluate the therapeutic potential of stem cells as well as raised safety issues, like the risk of disease transmission or malignant transformation.

Mesenchymal stromal/stem cells and their trophic factors

Initially, mesenchymal stromal/stem cells (MSCs) were identified as a heterogeneous population of stromal cells in the bone marrow, providing a supportive niche for hematopoietic stem cells. More recently, MSCs have been found in multiple tissue compartments, including lung, liver and adipose tissue.[112, 113] MSCs have multipotent stem cell properties and can give rise to many mesodermal tissues such as bone, cartilage and adipose

tissue.[114-116] The first report that MSCs can also differentiate into hepatocyte-like cells was published in 2005.[117] Since then, they have been suggested to be the most potent stem cell subtype for liver regeneration, providing pleiotropic effects in response to tissue injury.[113, 116-118]

Multiple studies describe the potential role of MSCs to promote liver regeneration after toxic injury and protect against fulminant hepatic failure.[57, 117, 119-122] After transplantation of MSCs, however, very low engraftment and transdifferentiation percentages were reported, suggesting contribution of mechanisms other than direct differentiation into liver cells.

A promising mechanism under investigation is the paracrine support by MSC-derived trophic factors. Beneficial effects of MSC-secreted factors have been reported in the setting of toxic liver injury and hepatic failure.[64] The use of MSC-derived factors in a clinical setting may have major advantages over the use of MSCs, since there is no risk of rejection or possible malignant transformation and the factors can be produced in large clinical grade quantities. Limitations however could be lower efficacy, more systemic or diluted effects and limited duration of therapeutic benefit.

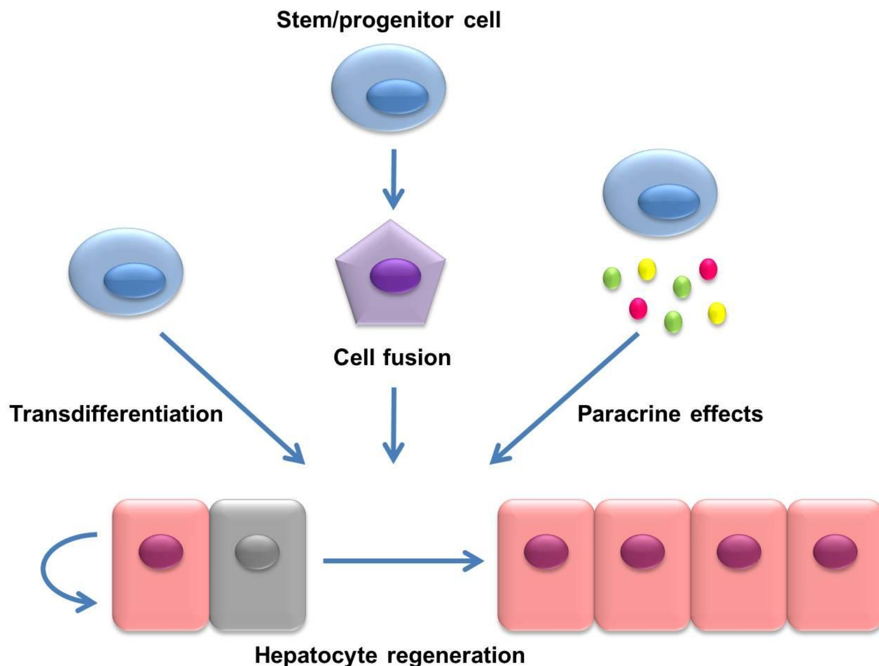

Figure 2. Contribution of stem/progenitor cells to liver regeneration

Stem/progenitor cells have been described to contribute to liver regeneration by 1) transdifferentiation into functional liver cells, 2) cell fusion with resident liver cells resulting in expression of both donor and host genes in the same cell and 3) paracrine effects on regeneration pathways by secreted trophic factors.

Aim and outline of the present thesis

The aim of this thesis is to further investigate the mechanisms involved in the process of liver regeneration as well as to explore potential therapeutic strategies to modulate and accelerate regeneration of the liver after surgical injury. Throughout this thesis several aspects that influence regeneration after liver resection and transplantation are described.

The first part highlights underlying molecular mechanisms and functional pathways involved in liver regeneration after resection, as well as genetic profiles and biomarkers of liver grafts that show signs of dysfunction early after transplantation. In **chapter 2** early gene expression profiles in regenerating living donor livers are identified by microarray analyses. The marked differences in genomic profiles between donors with successful and incomplete regeneration suggest a possible inhibition or delay in initiation of regenerative pathways in the poorly regenerating livers. Similar, in **chapter 3**, underlying molecular pathways and networks involved in the development of EAD are analyzed, showing downregulation of metabolic capabilities and upregulation of pro-inflammatory molecules. We furthermore defined a validated diagnostic gene expression signature to detect liver grafts prone to develop EAD. In **chapter 4** the effects of mTOR inhibition on liver regeneration after partial hepatectomy are investigated. We report that mTOR inhibition by the immunosuppressant rapamycin severely impairs liver regeneration and increases autophagy after liver resection in mice. Furthermore, we show that this impaired regeneration can be partly reversed by exogenous growth factor treatment.

In the second part of this thesis various characteristics of liver-derived mesenchymal stromal/stem cells are outlined and a promising new stem cell-based treatment strategy to stimulate liver regeneration after surgical injury is described. **Chapter 5** provides evidence for the presence of MSCs in the adult human liver. These cells have phenotypic and functional characteristics similar to those of bone marrow (BM-)MSCs and migrate from liver grafts at time of transplantation. In **chapter 6** MSC cultures derived from bone marrow and liver tissue were evaluated for the presence of aberrant cells, showing that spontaneous transformation of MSCs resulting in tumorigenesis is rare and only occurs after long-term culture. **Chapter 7** gives a detailed description of the methods used in our lab to isolate and culture MSCs as well as to concentrate their secreted factors. Furthermore, the surgical techniques of animal models to investigate liver regeneration after partial hepatectomy and/or IR injury are outlined. Hence, **chapter 8 and 9** describe the effects of MSC-derived factors on liver regeneration after surgical resection and/or IR injury. These data show that treatment with concentrated MSC-conditioned culture medium promotes hepatocyte proliferation and regenerative responses after surgical resection, but does not protect against early effects of IR injury. Finally, in **chapter 10**, the results presented in this thesis are summarized and discussed.

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Part I

Mechanisms

Chapter 2

Immediate early gene expression profiles of living donor livers show a shift in key cellular functions related to the extent of regeneration

Suomi M.G. Fouraschen[1, 2], Sunil M. Kurian[3], Joshua Wolf[1], Jean C. Emond[4], Jeroen de Jonge[2], Luc J.W. van der Laan[2], Daniel R. Salomon[3], Abraham Shaked[1] and Kim M. Olthoff[1]

[1]Department of Surgery, Penn Transplant Institute, University of Pennsylvania, Philadelphia, PA, United States; [2]Department of Surgery, Laboratory of Experimental Transplantation and Intestinal Surgery, Erasmus MC-University Medical Center, Rotterdam, The Netherlands; [3]Department of Molecular and Experimental Medicine, The Scripps Research Institute, La Jolla, CA, United States; [4]Department of Surgery, Columbia University, New York, NY, United States

(Submitted)

ABSTRACT

In the setting of living donor liver transplantation healthy donors undergo resection of 40-60% of their liver volume, which is associated with significant incidence of post-operative complications and a small but present risk of liver failure or even death. A better understanding of factors influencing liver regeneration may provide targets for intervention, minimizing morbidity and mortality. The aim of this study is to identify differences in early hepatic gene expression profiles between donors with successful and incomplete regeneration of their remnant liver mass.

Global hepatic gene expression profiles of 23 right lobe donors were investigated at baseline and immediately post resection using microarrays. Expression levels were correlated with the regenerated liver volumes at three months after donation. Immediate early changes in gene expression revealed a functional shift away from metabolic functions and resulting in activation of acute phase response, cell death and proliferation related pathways. Significant differences were found between expression patterns of donors with successful and limited regeneration of their remnant liver mass.

Conclusion: Living donor livers show differential expression of a high number of genes immediately post-resection compared to baseline. Marked differences between donors with successful and incomplete liver regeneration suggest a possible inhibition or delay in initiation of recovery and regeneration related molecular pathways in the poorly regenerating livers, and may identify potential areas for intervention.

INTRODUCTION

In the setting of adult-to-adult living donor liver transplantation (LDLT), healthy donors undergo resection of 40-60% of their entire liver volume, after which robust regeneration of their remnant liver is required to restore homeostasis and sustain metabolic support. While the majority of donors do well after surgery, there remains significant morbidity and mortality associated with the procedure. Most donors show incomplete regeneration in the first 3-6 months after donation, with a significant incidence of post-operative complications and a small but present risk of liver failure or even death.[1-6] Recent clinical data from the National Institutes of Health (NIH) multicenter Adult-to-Adult Living Donor Liver Transplant (A2ALL) consortium have highlighted that there is an incidence of 30-40% post-operative complications[7], regeneration is highly variable[8] and some long-term laboratory abnormalities persist.[9] With the concern over the morbidity and mortality of the donors, annual numbers of LDLT have declined, where now only approximately 250 adult LDLTs are performed per year in the United States.[10] Furthermore, a number of transplant centers are moving toward the use of smaller, left lobe grafts in an attempt to decrease donor morbidity [11, 12], but this is limited by small graft volumes and the concern for development of small-for-size syndrome (SFSS) in the recipient.[4, 13, 14]

By determining the best donor biologic parameters and identifying potential interventions that enhance recovery and regeneration, it may be possible to expand the living donor pool, minimize donor risk and increase the numbers of LDLT resulting in fewer deaths on the waitlist. A better understanding of cellular and molecular mechanisms influencing liver regeneration may provide possible targets for intervention to enhance regeneration and minimize subsequent morbidity and mortality.

At any time, liver function reflects a complex balance of cellular proliferation and metabolic homeostasis.[15-18] The role of cytokines, growth factors and hormones in liver regeneration has previously been described in rodent models of partial hepatectomy.[16, 18] Genomic analyses demonstrated an early shift from genes involved in lipid biosynthesis to genes supporting cell proliferation.[19-23] While liver regeneration has been extensively studied in animal models, it has been difficult to do so in humans. Only few studies have evaluated human genomic liver expression following liver resection.[24-27] The A2ALL consortium provides a unique opportunity to study human liver regeneration on a clinical and molecular level, and has collected liver biopsies from baseline living donor livers prior to hepatectomy as well as from remnant livers following resection. These samples allow assessment of peri-operative changes in hepatic gene expression and correlation with the extent of liver regeneration.

In this study, we profiled the effects of liver resection on hepatic gene expression in healthy living liver donors and investigated differences in expression profiles between donors with successful regeneration of their remnant liver versus those with less robust regeneration.

MATERIALS AND METHODS

Donor characteristics

Using the A2ALL cohort study database, we identified 23 right lobe donors with complete volumetric data and per-operatively collected liver biopsies. All donors underwent surgery between 2007 and 2010 at either the Hospital of the University of Pennsylvania or the New York-Presbyterian Hospital/Columbia University Medical Center and provided written informed consent to this study. Data on donor demographics, clinical variables and lab values were collected peri-operatively as well as three months post-donation. All study protocols and consent procedures were approved by the Institutional Review Boards and Privacy Boards of the University of Michigan Data Coordinating Center and each of the participating transplant centers.

Liver volumetric data

All 23 donors had preoperative volumetric imaging by magnetic resonance imaging (MRI) or computed tomography (CT). Their right lobe liver graft was weighed post-resection in the operating room after which their remnant left lobe volume was calculated by subtracting the graft weight from the total liver volume (TLV) on preoperative imaging.

Three months after donation donors had volumetric imaging of their regenerated liver mass. Regeneration outcome measures included absolute growth, percent volume increase and percent reconstitution (Table 1). Absolute growth was defined as the absolute increase in liver volume of the remnant lobe from time of donation to three months post-donation. As the absolute growth could very well be affected by the size of the remnant liver, the extent of regeneration was also defined by the volume increase as percent of the remnant liver volume as well as the reconstituted liver mass three months post-donation relative to the preoperative total liver volume.

Table 1. Measures of liver regeneration

	Absolute growth (cc)	Volume increase (%)	Volume reconstitution (%)
Definition	Change in volume of the remnant lobe from resection to 3 months post-donation	Percent increase in volume of the remnant lobe by 3 months post-donation	Percent of pre-operative total liver volume achieved by 3 months post-donation
Calculation	3-month liver volume (cc) – remnant liver volume (cc)	Absolute growth (cc) / remnant liver volume (cc)	3-month liver volume (cc) / TLV (cc)

Liver biopsies

Two core liver biopsies were obtained from each donor. The first biopsy was taken prior to resection from the baseline liver (PRE) and a subsequent biopsie was taken within an hour after resection from the remnant liver (POST). Samples were collected in RNAlater (Qiagen, CA) and stored at 4°C overnight after which they were transferred to -80°C until further processing.

RNA-extraction and gene expression arrays

Total RNA was extracted from the biopsies using Trizol (Invitrogen, CA), after which the RNA was further purified using the RNeasy kit (Qiagen, CA), according to the manufacturer's instructions. RNA analyzed on an Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA) with RNA Integrity Numbers (RINs) above 7 was considered for further analysis. Biotinylated cRNA was prepared with the Ambion MessageAmp Biotin II kit (Ambion, TX) after which labeled cRNA was hybridized to Affymetrix Human Gene 1.0 ST Array GeneChips using standard Affymetrix protocols.

Microarray analyses

To determine the effect of liver resection as well as the early regenerative responses at a genomic level, hepatic gene expression levels in all 23 POST biopsies were compared to expression levels in all 23 PRE biopsies.

Differences in gene expression profiles between donors with successful and limited regeneration of their remnant liver mass were investigated by dividing donors into two groups: a REG+ group with regeneration parameters above the mean and a REG- group with regeneration parameters below the mean. This was done for all three regeneration categories, i.e. absolute growth, percent volume increase and percent reconstitution. Gene expression patterns between REG+ and REG- donors were compared for baseline liver biopsies (REG+ PRE vs. REG- PRE), remnant left lobe biopsies (REG+ POST vs. REG- POST) as well as gene expression changes between both time points (REG+ POST-PRE vs. REG- POST-PRE). Because of the extensive amount of data, we chose to mainly describe the results found in the reconstitution category. Results found in the other regeneration categories can be found as supplemental information (not shown in this thesis).

In a sub-analysis, the six most successful regenerated donors were compared to the six least regenerated donors, i.e. the upper (QTL REG+) and lower (QTL REG-) quartile for the combined regeneration measures, to investigate the extremes of regeneration in this cohort.

Statistical analysis

Donor characteristics and clinical data are shown as mean ± SD, unless described otherwise (Table 2). Clinical data were compared using the Mann-Whitney test and p-values <0.05 were considered significant.

Signal intensities from Affymetrix GeneChips were analyzed using BRB-ArrayTools software. Samples with GAPDH and/or Actin 3' to 5' ratios below 8 and outliers by Principal Components Analysis (PCA) clustering were excluded. Normalized signals were generated using RMA after which class comparisons were performed using a paired t-test with random variance and p<0.005 as cut-off for significance. No probeset filtering was done based on signal intensities. Functional analyses of the differentially expressed genes were performed using Ingenuity Pathway Analysis (IPA).

Table 2. Donor characteristics

	All donors	Absolute growth		Volume increase		Reconstitution		Quartiles	
	(N=23)	REG+ (N=10)	REG- (N=13)	REG+ (N=9)	REG- (N=14)	REG+ (N=11)	REG- (N=12)	QTL REG+ (N=6)	QTL REG- (N=6)
Donor characteristics									
Male/Female	11/12	7/3	4/9	4/5	7/7	5/6	6/6	3/3	2/4
Age (y; min-max; median)	24.0-59.8 (31.9)	24.8-59.8 (29.2)	24.0-51.0 (38.0)	24.1-59.8 (26.2)	24.0-56.5 (40.8)	24.1-59.8 (28.4)	24.0-56.5 (44.3)	25.0-59.8 (28.5)	24.0-51.0 (41.5)
BMI (min-max; median)	19.4-34.3 (23.9)	20.8-32.4 (24.1)	19.4-34.3 (23.9)	20.8-31.3 (23.5)	19.4-34.3 (24.3)	20.8-31.3 (24.6)	19.4-34.3 (23.9)	20.8-31.3 (22.9)	19.4-34.3 (23.4)
Liver volumes (mean ± SD; range)									
Total liver volume (cc)	1572±330 (1142-2476)	1700±371 (1155-2476)	1474±268 (1142-2115)	1477±307 (1142-1936)	1633±340 (1202-2476)	1521±301 (1142-1936)	1619±360 (1202-2476)	1562±314 (1155-1936)	1423±183 (1202-1743)
Graft weight (g)	803±219 (550-1452)	892±255 (575-1452)	734±166 (550-1175)	804±195 (575-1100)	802±241 (550-1452)	770±170 (575-1100)	833±261 (550-1452)	839±203 (575-1100)	662±101 (550-800)
Remnant weight (cc)	769±185 (367-1122)	807±233 (367-1122)	740±141 (475-943)	673±199 (367-986)*	832±151 (652-1122)*	751±232 (367-1122)	786±137 (650-1024)	723±226 (367-986)	761±110 (652-943)
Remnant weight/TLV (%)	49.0±7.5 (27.1-60.6)	47.4±9.8 (27.1-60.6)	50.3±5.1 (41.6-59.5)	45.4±8.6 (27.1-54.9)	51.4±5.8 (41.4-60.6)	48.8±9.1 (27.1-60.6)	49.2±6.0 (40.5-59.5)	46.0±10.3 (27.1-54.9)	53.5±3.7 (49.3-59.5)
Remnant weight/Donor weight (%)	1.1±0.3 (0.5-1.6)	1.0±0.3 (0.5-1.5)	1.1±0.3 (0.6-1.6)	1.0±0.3 (0.5-1.5)	1.1±0.3 (0.7-1.6)	1.1±0.3 (0.5-1.5)	1.0±0.3 (0.7-1.6)	1.0±0.3 (0.5-1.5)	1.1±0.3 (0.8-1.6)
Regenerated liver volume (cc)	1273±297 (917-1873)	1500±287 (1136-1873)#	1099±157 (917-1420)#	1359±363 (957-1873)	1218±246 (917-1690)	1411±338 (957-1873)	1147±192 (917-1528)	1510±350 (1136-1873)#	1014±85 (917-1156)#
Liver function POD 7 (mean ± SD; range)									
Total serum bilirubin (mg/dL)	1.2±0.6 (0.6-2.5)	1.2±0.6 (0.6-2.4)	1.3±0.6 (0.7-2.5)	0.9±0.3 (0.6-1.4)	1.4±0.7 (0.7-2.5)	1.0±0.4 (0.6-2.0)	1.4±0.7 (0.7-2.5)	0.9±0.3 (0.6-1.4)	1.3±0.7 (0.7-2.5)
INR	1.12±0.14 (0.90-1.40)	1.10±0.15 (0.95-1.40)	1.14±0.13 (0.90-1.40)	1.13±0.19 (0.95-1.40)	1.11±0.10 (0.90-1.30)	1.12±0.17 (0.95-1.40)	1.12±0.11 (0.90-1.30)	1.10±0.20 (0.95-1.40)	1.07±0.11 (0.90-1.20)
Regeneration (mean ± SD; range)									
Absolute growth (cc)	504±224 (73-976)	693±181 (504-976)$	359±122 (73-482)$	686±208 (425-976)#	387±143 (73-585)#	660±197 (425-976)$	361±135 (73-545)$	787±176 (574-976)#	253±97 (73-334)#
Volume increase (%)	70.6±41.0 (8.6-209.5)	95.5±46.8 (49.2-209.5)#	51.4±23.0 (8.6-101.5)#	107.3±41.5 (71.7-209.5)$	46.9±15.8 (8.6-70.4)$	95.9±43.9 (50.6-209.5)$	47.4±19.6 (8.6-83.9)$	118.2±47.3 (81.0-209.5)#	34.7±15.6 (8.6-48.8)#
Reconstitution (%)	81.5±12.7 (61.7-112.6)	89.7±14.0 (61.7-112.6)#	75.3±7.3 (64.6-86.8)#	91.6±11.1 (74.6-112.6)#	75.1±9.2 (61.7-91.3)#	92.3±8.6 (83.8-112.6)$	71.7±5.8 (61.7-78.7)$	96.5±9.6 (84.0-112.6)#	71.7±5.5 (64.6-77.8)#

* P<0.05; # P<0.01; $ P<0.001

RESULTS

Donor characteristics

As shown in Table 2, in total 23 donors were included in this study, 11 male and 12 female donors with a median age of 31.9 years (range 24.0-59.8 years) and BMI of 23.9 (range 19.4-34.3). Mean pre-operative total liver volume (TLV) was 1572±330 cc and resected right lobe graft weight was 803±219 g. This resulted in a left lobe remnant of 769±185 cc, which made up 49.0±7.5% of the TLV and 1.1±0.3% of the total body weight of the donor).

None of the donors showed signs of liver dysfunction one week after surgery, as determined by serum bilirubin levels and INR. Three months after donation, mean regenerated liver volume was 1273±297 cc, defining 504±224 cc absolute growth and 70.6±41.0% volume increase of the remnant lobe, and resulting in 81.5±12.7% reconstitution of the pre-operative liver volume.

Besides significant differences for all three regeneration outcome measures, comparison between the REG+ and REG- groups revealed a significantly lower remnant left lobe weight in the volume increase category (REG+ 673±199 vs. REG- 832±151 cc, p=0.044) as well as a significant difference in regenerated liver volume in the absolute growth category (REG+ 1500±287 vs. REG- 1099±157 cc, p=0.001).

In the quartile sub-analysis, comparison between the QTL REG+ and QTL REG- donors revealed a mean absolute growth of 787±176 cc versus 253±97 cc (p=0.002), percent volume increase of 118.2±47.3% versus 34.7±15.6% (p=0.002) and percent reconstitution of 96.5±9.6% versus 71.7±5.5% (p=0.002) respectively.

Liver resection activates cell death and proliferation and inhibits lipid and carbohydrate metabolism

Figure 1 shows the differences in gene expression levels between all POST and all PRE biopsies. A total of 9095 array probesets related to 6370 genes (3156 upregulated and 3214 downregulated) were significantly differentially expressed in the remnant liver lobes compared to the pre-resection livers. Functional analysis showed that most of these genes are related to molecules important for cell death and survival (1565 molecules), cellular growth and proliferation (1517 molecules) as well as cellular development (1176 molecules; Table 3A). More specific, pathways related to oxidative stress and acute phase responses as well as the PPARα, mTOR and PI3K/AKT signaling pathways were among the most significantly regulated pathways (Table 3B). Similar results were seen when specifically looking at the topmost upregulated genes, as these included SAA1 (28.5-fold), SAA2 (15.1-fold), TGFB3 (6.9-fold), CRP (6.8-fold) and CASP4 (4.8-fold): genes known to be important in acute phase response, apoptosis and proliferation (Table 3C).

Effects on metabolic functions mainly involved lipid and carbohydrate metabolism (631 cq. 340 molecules), both showing inhibitory effects on their molecular pathways, like the FXR/RXR activation pathway (7 genes up, 27 genes down), fatty acid β-oxidation I

pathway (4 up, 11 down), bile acid biosynthesis pathway (1 up, 7 down) and glycogen biosynthesis II pathway (0 up, 4 down). Topmost downregulated genes were also related to lipid metabolism as well as cell adhesion and signal transduction, and included THRSP (-6.7-fold), CELSR3 (-5.9-fold) and PER3 (-4.2-fold; Table 3C).

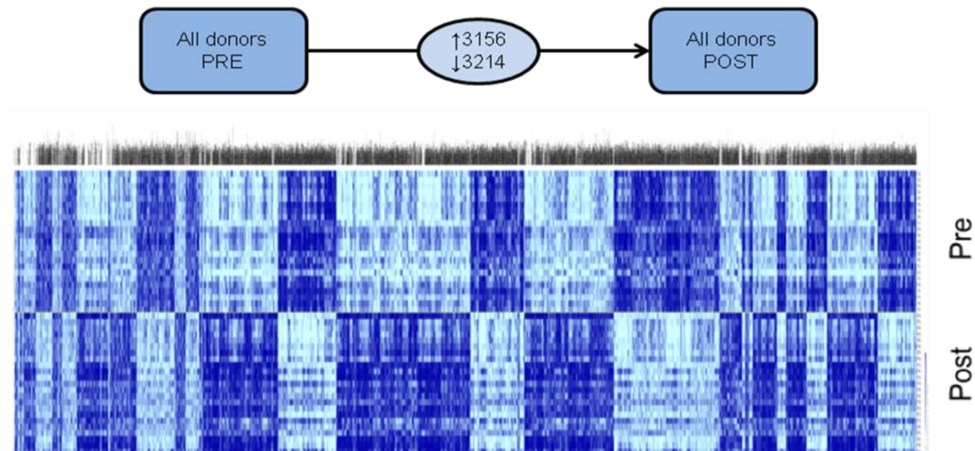

Figure 1. Differential gene expression between all PRE and POST samples

Number of differentially expressed probesets with visualization of the intensity of gene expression in all PRE and all POST biopsies, showing clear differences in up- and downregulation of genes between the two time points. PRE baseline liver biopsies taken prior to resection; POST remnant liver biopsies taken within 1 hour after resection.

Table 3A. Functions all POST versus PRE

Molecular and cellular functions	Molecules	P-value
Gene Expression	1127	3.18E-27 - 1.04E-03
Cell Death and Survival	1565	1.88E-21 - 9.24E-04
Cellular Growth and Proliferation	1517	1.17E-18 - 8.78E-04
Cellular Assembly and Organization	879	1.01E-17 - 1.06E-03
Cellular Function and Maintenance	926	1.01E-17 - 1.06E-03
RNA Post-Transcriptional Modification	147	2.12E-14 - 3.99E-04
Cell Cycle	708	2.19E-13 - 1.05E-03
Post-Translational Modification	483	2.10E-12 - 5.32E-04
Protein Synthesis	675	4.27E-12 - 1.04E-03
Molecular Transport	819	7.99E-12 - 1.02E-03
Protein Trafficking	187	7.99E-12 - 1.02E-03
Cellular Development	1176	7.26E-11 - 1.06E-03
Cellular Movement	914	1.36E-09 - 7.94E-04
Lipid Metabolism	631	1.18E-08 - 9.45E-04
Small Molecule Biochemistry	847	1.18E-08 - 9.54E-04
Cell Morphology	889	2.84E-08 - 8.32E-04
Nucleic Acid Metabolism	251	3.10E-08 - 5.69E-04
Carbohydrate Metabolism	340	8.01E-08 - 1.03E-03
Cell Signaling	253	8.53E-08 - 1.83E-04
Protein Folding	42	1.04E-07 - 1.04E-07

Table 3B. Pathways all POST versus PRE

Canonical pathways	P-value	Genes up	Genes down
Protein Ubiquitination Pathway	5.31E-10	79/268 (29%)	41/268 (15%)
Acute Phase Response Signaling	3.54E-09	54/178 (30%)	30/178 (17%)
NRF2-mediated Oxidative Stress Response	1.05E-08	40/192 (21%)	47/192 (24%)
PPARα/RXRα Activation	4.98E-07	39/191 (20%)	43/191 (23%)
Chronic Myeloid Leukemia Signaling	1.05E-06	33/105 (31%)	16/105 (15%)
EIF2 Signaling	1.61E-06	52/200 (26%)	31/200 (16%)
Germ Cell-Sertoli Cell Junction Signaling	3.05E-06	34/164 (21%)	38/164 (23%)
mTOR Signaling	4.93E-06	41/210 (20%)	44/210 (21%)
PI3K/AKT Signaling	6.64E-06	36/144 (25%)	24/144 (17%)
Role of NFAT in Regulation of the Immune Response	9.17E-06	37/198 (19%)	40/198 (20%)

Table 3C. Genes up- and downregulated all POST versus PRE

Genes upregulated			
Symbol	**Name**	**Fold Change**	**P-value**
SAA1	serum amyloid A1	28.530	< 1.00E-07
SAA2	serum amyloid A2	15.070	< 1.00E-07
CHI3L1	chitinase 3-like 1	12.750	< 1.00E-07
TGFB3	transforming growth factor, beta 3	6.860	< 1.00E-07
CRP	C-reactive protein, pentraxin-related	6.810	< 1.00E-07
ODC1	ornithine decarboxylase 1	6.660	< 1.00E-07
ADAMTS1	ADAM metallopeptidase with thrombospondin type 1 motif, 1	5.770	< 1.00E-07
TGM2	transglutaminase 2	5.600	< 1.00E-07
CASP4	caspase 4, apoptosis-related cysteine peptidase	4.770	< 1.00E-07
OSMR	oncostatin M receptor	4.720	< 1.00E-07
Genes downregulated			
Symbol	**Name**	**Fold Change**	**P-value**
CPS1-IT1	CPS1 intronic transcript 1	-10.526	< 1.00E-07
THRSP	thyroid hormone responsive	-6.667	< 1.00E-07
HNF1A-AS1	HNF1A antisense RNA 1	-6.250	< 1.00E-07
CELSR3	cadherin, EGF LAG seven-pass G-type receptor 3	-5.882	< 1.00E-07
SOWAHC	sosondowah ankyrin repeat domain family member C	-5.263	< 1.00E-07
VSNL1	visinin-like 1	-5.000	< 1.00E-07
IFIT1	interferon-induced protein with tetratricopeptide repeats 1	-4.545	< 1.00E-07
PER3	period homolog 3	-4.167	< 1.00E-07
GOLGA6C	golgin A6 family, member C	-4.000	< 1.00E-07
NR1D2	nuclear receptor subfamily 1, group D, member 2	-4.000	< 1.00E-07

Successful regenerating donors display lower immune response activity prior to resection

In the reconstitution category, comparison of the PRE biopsies between the REG+ and REG- donors revealed only 222 probesets related to 147 significantly differentially expressed genes (28 up, 119 down). Functional analysis showed that these genes are mainly related to molecules important for cell morphology (27 molecules), cellular assembly and organization (25 molecules) as well as cell death and survival (25 molecules; Table 4). Similar results were seen in the absolute growth and volume increase category (Supplemental Table 1 and 2). Interestingly, in all three regeneration categories, approximately 80% of

Table 4. Functions REG+ versus REG- PRE and POST

Molecular and cellular functions	Molecules	P-value
REG+ versus REG- PRE		
Molecular and cellular functions	**Molecules**	**P-value**
Cellular Assembly and Organization	25	1.55E-04-4.96E-02
Lipid Metabolism	16	6.68E-04-4.96E-02
Small Molecule Biochemistry	23	6.68E-04-4.96E-02
Cellular Development	24	1.06E-03-4.96E-02
Cell Morphology	27	1.07E-03-4.96E-02
Cellular Function and Maintenance	23	1.41E-03-4.96E-02
Drug Metabolism	7	2.21E-03-3.57E-02
Protein Synthesis	19	2.21E-03-4.10E-02
Protein Degradation	11	2.90E-03-4.10E-02
Carbohydrate Metabolism	12	4.47E-03-4.96E-02
Cell Cycle	12	5.13E-03-4.96E-02
Molecular Transport	19	6.42E-03-4.96E-02
Cell Death and Survival	25	7.25E-03-4.96E-02
Cell-To-Cell Signaling and Interaction	16	7.25E-03-4.96E-02
Cellular Compromise	6	7.25E-03-4.27E-02
Cellular Growth and Proliferation	20	7.25E-03-4.96E-02
Cellular Movement	7	7.25E-03-4.27E-02
DNA Replication, Recombination, and Repair	7	7.25E-03-4.96E-02
Post-Translational Modification	9	7.25E-03-4.10E-02
Protein Trafficking	1	7.25E-03-7.25E-03
REG+ versus REG- POST		
Molecular and cellular functions	**Molecules**	**P-value**
Cell-To-Cell Signaling and Interaction	22	5.09E-04-4.50E-02
Cellular Assembly and Organization	37	5.09E-04-3.89E-02
Cellular Function and Maintenance	44	5.09E-04-4.82E-02
Cellular Development	25	1.67E-03-4.82E-02
Cell Morphology	22	2.48E-03-4.00E-02
Cellular Movement	19	2.48E-03-4.53E-02
Carbohydrate Metabolism	6	5.80E-03-3.89E-02
Nucleic Acid Metabolism	6	5.80E-03-3.89E-02
Small Molecule Biochemistry	14	5.80E-03-3.89E-02
Cellular Compromise	9	7.62E-03-3.89E-02
Cellular Growth and Proliferation	25	7.62E-03-4.79E-02
Molecular Transport	11	7.62E-03-3.89E-02
Cell Cycle	3	1.31E-02-3.89E-02
Cell Death and Survival	14	1.31E-02-3.89E-02
Cell Signaling	1	1.31E-02-1.31E-02
DNA Replication, Recombination, and Repair	4	1.31E-02-3.91E-02
Drug Metabolism	1	1.31E-02-1.31E-02
Gene Expression	8	1.31E-02-3.89E-02
Lipid Metabolism	5	1.31E-02-3.89E-02
Post-Translational Modification	2	1.31E-02-2.61E-02

the differentially expressed genes showed a lower expression level in the baseline liver biopsies of REG+ donors compared to REG- donors. Many of these genes appeared to be related to immune response pathways, like the antigen presentation pathway and OX40 signaling pathway (Supplemental Table 3).

Post-resection differences between donors with successful and limited regeneration are related to cellular function and maintenance as well as development and proliferation

A similar comparison was performed between the POST biopsies of the REG+ and REG- donors. In the reconstitution category this analysis revealed 360 probesets related to 246 genes (108 up, 138 down). Most differentially expressed genes appeared to be related to molecules important for cellular function and maintenance (44 molecules), cellular assembly and organization (37 molecules), cellular development (25 molecules) as well as cellular growth and proliferation (25 molecules; Table 4). Data for the other regeneration categories showed comparable results and are shown in Supplemental Table 1 and 2. Highly regulated pathways in all three regeneration categories included the EIF2, ILK, PI3K/AKT, mTOR and DNA methylation and transcriptional repression signaling pathways, all related to cellular growth, proliferation and development (Supplemental Table 3).

Analysis of immediate early gene expression changes

Immediate early gene expression changes between the POST and PRE biopsies were analyzed and compared between REG+ and REG- donors. In the reconstitution category, the REG+ POST vs. PRE analysis revealed 5822 probesets related to 4249 genes (2457 up, 1792 down) whereas the REG- POST vs. PRE analysis revealed 6988 probesets related to 5222 genes (2382 up, 2840 down). The Venn diagram in Figure 2 shows that of these significantly differentially expressed genes 3617 genes are shared between REG+ and REG- donors, whereas 632 genes (436 up, 196 down) are unique to the REG+ group and 1605 genes (439 up, 1166 down) are unique to the REG- group. Similar results, including the contrasting ratios of up- and downregulated genes between the REG+ and REG- groups, are seen in the absolute growth and volume increase category (Supplemental Table 1).

Regenerating livers share common pathways of cell injury, proliferation and metabolism in response to resection, regardless of quantity of regeneration

Among the 3617 genes shared between the REG+ and REG- POST vs. PRE analyses, many genes are related to molecules important for cell death and survival (936 molecules), cellular development (500 molecules), cellular growth and proliferation (477 molecules) as well as cell cycle (415 molecules; Table 5A; see Supplemental Table 2 for results from the other categories).

In all three regeneration categories, pathways related to oxidative stress and acute phase responses as well as the cell cycle related RAN signaling and G1/S checkpoint regulation pathways were among the most significantly regulated shared pathways (Table 5B

and Supplemental Table 3). Related results were found for the topmost upregulated genes, which include SAA1 (>20-fold), SAA2 (>13-fold), CRP (>5-fold) TGFB3 (>5-fold) and CASP4 (>4 -fold): genes important in acute phase response, apoptosis and proliferation (Table 5C and Supplemental Table 4).

These pathways are mainly activated, as shown by the upregulation of many of their genes. In contrast, shared pathways that are silenced after liver resection are nearly all metabolic pathways, like the PXR/RXR activation pathway (11-20% of genes downregulated versus 3-5% upregulated, dependent on the regeneration category), the ethanol degradation pathway (19-26% down, 2% up) and the α-tocopherol degradation pathway (20-30% down, 0% up). This is also reflected in the top 10 downregulated genes, which include THRSP (<-5-fold), CELSR3 (<-4-fold) and PER3 (<-3-fold): genes important in lipid metabolism, cell adhesion and signal transduction (Table 5C and Supplemental Table 4). Moreover, lipid metabolism was also found among the topmost affected functions after resection (319 molecules). More specifically, the PPARα/RXRα and TR/RXR activation pathways were among the top regulated pathways shared between REG+ and REG- donors.

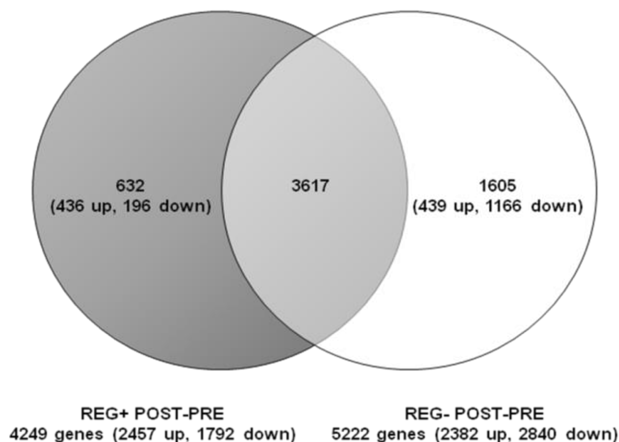

Figure 2. Venn diagram between REG+ and REG- POST-PRE

Overlap and differences in significantly differentially expressed probesets between the REG+ and REG- POST versus PRE class comparisons, revealing probesets shared between both analyses as well as probesets unique to the REG+ or REG- analysis. REG+/REG- donors with regeneration parameters above/below the mean; PRE baseline liver biopsies taken prior to resection; POST remnant liver biopsies taken within 1 hour after resection.

Expression profiles related to successful regeneration involve activation of stress response and cell cycle related signaling

Besides common functional activities, distinct differences in early gene expression changes exist between REG+ and REG- donors. Genes unique to REG+ donors appeared mostly upregulated (69% in the reconstitution category) and related to molecules important for cell death and survival (148 molecules) as well as cell cycle (48 molecules; Ta-

ble 6A; see Supplemental Table 2 for results from the other categories). The topmost significantly regulated pathways, found in all three regeneration categories, are indeed related to cellular growth, proliferation and cell cycle regulation, like the PI3K/AKT, JAK/Stat and regulation of eIF4 and p70S6K signaling pathways (Table 6B and Supplemental Table 3). Among the highest upregulated genes unique to REG+ donors were PON2 (1.8-fold), ASB1 (1.6-fold), ZNF814 (1.9-fold) and HSPA4L (1.7-fold); genes involved in stress response, intracellular signal transduction and transcription pathways (Table 6C and Supplemental Table 4). The topmost downregulated genes, on the other hand, included ASF1B (-1.9-fold), SESN1 (-1.6-fold), CITED2 (-1.6-fold) and DDIT4 (-1.5-fold); genes involved in apoptosis and the response to hypoxia as well as cell cycle arrest and (negative regulation of) cell proliferation.

Table 5A. Functions shared between REG+ and REG- POST-PRE

Molecular and cellular functions	Molecules	P-value
RNA Post-Transcriptional Modification	112	7.72E-09-1.27E-02
Cell Death and Survival	936	4.83E-08-1.45E-02
Molecular Transport	353	3.22E-07-1.53E-02
Protein Synthesis	284	6.07E-07-1.15E-02
Cellular Compromise	76	3.53E-05-1.54E-02
Cellular Function and Maintenance	200	3.53E-05-1.54E-02
RNA Trafficking	26	5.20E-05-8.06E-03
Gene Expression	665	5.64E-05-1.48E-02
Cellular Assembly and Organization	176	1.05E-04-1.48E-02
Post-Translational Modification	113	1.06E-04-1.45E-02
Nucleic Acid Metabolism	119	1.51E-04-1.27E-02
Small Molecule Biochemistry	451	1.51E-04-1.53E-02
Cellular Development	500	1.94E-04-1.52E-02
Cellular Growth and Proliferation	477	1.94E-04-1.34E-02
Cell Cycle	415	2.15E-04-1.53E-02
Lipid Metabolism	319	2.58E-04-1.45E-02
Protein Folding	35	3.15E-04-1.45E-02
Cell Morphology	155	3.43E-04-1.54E-02
DNA Replication, Recombination, and Repair	101	3.43E-04-1.27E-02
Free Radical Scavenging	136	3.55E-04-1.06E-02

Table 5B. Pathways shared between REG+ and REG- POST-PRE

Canonical pathways	P-value	REG+ genes up	REG+ genes down	REG- genes up	REG- genes down
RAN Signaling	5.03E-05	10/24 (42%)	1/24 (4%)	9/24 (38%)	2/24 (8%)
Chronic Myeloid Leukemia Signaling	2.89E-04	26/105 (25%)	8/105 (8%)	25/105 (24%)	9/105 (9%)
tRNA Charging	1.59E-04	15/81 (19%)	3/81 (4%)	14/81 (17%)	4/81 (5%)
Acute Phase Response Signaling	1.03E-03	40/178 (22%)	12/178 (7%)	39/178 (22%)	13/178 (7%)
NRF2-mediated Oxidative Stress Response	1.25E-05	29/192 (15%)	32/192 (17%)	28/192 (15%)	33/192 (17%)
PPARα/RXRα Activation	1.01E-03	28/191 (15%)	23/191 (12%)	28/191 (15%)	23/191 (12%)
Cell Cycle: G1/S Checkpoint Regulation	1.15E-03	19/66 (29%)	4/66 (6%)	18/66 (27%)	5/66 (8%)
Aryl Hydrocarbon Receptor Signaling	4.04E-04	22/161 (14%)	22/161 (14%)	21/161 (13%)	23/161 (14%)
Pyridoxal 5'-phosphate Salvage Pathway	2.49E-03	13/72 (18%)	10/72 (14%)	12/72 (17%)	11/72 (15%)
TR/RXR Activation	2.64E-03	13/96 (14%)	16/96 (17%)	13/96 (14%)	16/96 (17%)

Table 5C. Genes up- and downregulated shared between REG+ and REG- POST-PRE

Genes upregulated					
Symbol	**Name**	**REG+ Fold Change**	**REG+ P-value**	**REG- Fold Change**	**REG- P-value**
SAA1	serum amyloid A1	20.190	< 1.00E-07	39.180	< 1.00E-07
CHI3L1	chitinase 3-like 1	10.890	< 1.00E-07	14.740	< 1.00E-07
SAA2	serum amyloid A2	13.110	3.00E-06	17.120	< 1.00E-07
ODC1	ornithine decarboxylase 1	6.010	< 1.00E-07	7.310	< 1.00E-07
CRP	C-reactive protein, pentraxin-related	5.560	1.70E-06	8.210	< 1.00E-07
TGM2	transglutaminase 2	5.220	< 1.00E-07	5.970	< 1.00E-07
TGFB3	transforming growth factor, beta 3	5.590	3.29E-05	8.280	< 1.00E-07
CASP4	caspase 4, apoptosis-related cysteine peptidase	4.620	< 1.00E-07	4.920	< 1.00E-07
DDX21	DEAD (Asp-Glu-Ala-Asp) box helicase 21	4.480	< 1.00E-07	4.920	< 1.00E-07
ADAMTS1	ADAM metallopeptidase with thrombospondin type 1 motif, 1	4.940	2.44E-05	6.660	< 1.00E-07
Genes downregulated					
Symbol	**Name**	**REG+ Fold Change**	**REG+ P-value**	**REG- Fold Change**	**REG- P-value**
CPS1-IT1	CPS1 intronic transcript 1	-8.333	2.90E-06	-12.821	1.30E-06
THRSP	thyroid hormone responsive	-5.556	3.60E-06	-7.692	< 1.00E-07
HNF1A-AS1	HNF1A antisense RNA 1	-5.263	1.31E-04	-7.692	< 1.00E-07
VSNL1	visinin-like 1	-4.348	< 1.00E-07	-5.882	< 1.00E-07
CELSR3	cadherin, EGF LAG seven-pass G-type receptor 3	-4.545	9.02E-04	-7.143	4.50E-06
SOWAHC	sosondowah ankyrin repeat domain family member C	-4.545	2.00E-07	-6.250	< 1.00E-07
DSG1	desmoglein 1	-3.571	< 1.00E-07	-4.348	< 1.00E-07
PER3	period homolog 3	-3.704	9.00E-07	-4.762	< 1.00E-07
NR1D2	nuclear receptor subfamily 1, group D, member 2	-4.000	5.00E-07	-4.000	< 1.00E-07
IFIT1	interferon-induced protein with tetratricopeptide repeats 1	-3.846	8.00E-07	-5.263	< 1.00E-07

Expression profiles related to limited regeneration involve silencing of important metabolic processes

In contrast to successful regenerating donors, genes unique to REG- donors were mainly downregulated (73%) and related to molecules important for cellular assembly and organization (218 molecules), cellular function and maintenance (201 molecules), cellular movement (112 molecules) and lipid metabolism (95 molecules; Table 6A; see Supplemental Table 2 for data on the other regeneration categories).

Remarkably, many other metabolic functions were highly affected in the REG- donors, like vitamin and mineral metabolism (46 molecules), amino acid metabolism (44 molecules) and carbohydrate metabolism (35 molecules). More specific, the valine degradation pathway, leucine degradation pathway, fatty acid β-oxidation pathway and PPARα/RXRα activation pathway were among the most significantly regulated pathways, all showing downregulation of their related genes (Table 6B and Supplemental Table 3). These results were also reflected in the topmost downregulated genes, which included IGFBP1 (-2.3-

fold), NTRK2 (-1.9-fold), ACADSB (-1.7-fold) and AKTIP (-1.7-fold): genes involved in glucose and lipid metabolic processes, tissue regeneration and (positive regulation of) cell growth (Table 6C and Supplemental Table 4). In contrast, the topmost upregulated genes included STEAP4 (4.0-fold), EMP1 (3.2-fold), CAND1 (2.6-fold) and UBE2D3 (2.0-fold): genes related to cell differentiation, development and protein ubiquitination.

Table 6A. Functions unique to REG+ and REG- POST-PRE

Unique REG+ POST-PRE		
Molecular and cellular functions	**Molecules**	**P-value**
RNA Damage and Repair	8	8.43E-06-3.29E-02
Cell Death and Survival	148	8.54E-04-3.29E-02
Drug Metabolism	7	1.08E-03-3.29E-02
Vitamin and Mineral Metabolism	6	1.08E-03-3.29E-02
RNA Post-Transcriptional Modification	21	1.20E-03-3.29E-02
Cell Morphology	40	1.99E-03-3.29E-02
Cellular Function and Maintenance	36	1.99E-03-3.29E-02
Cell Cycle	48	2.46E-03-3.29E-02
Cell-To-Cell Signaling and Interaction	18	2.56E-03-3.29E-02
Amino Acid Metabolism	4	3.17E-03-3.29E-02
Post-Translational Modification	10	3.17E-03-3.29E-02
Small Molecule Biochemistry	46	3.17E-03-3.29E-02
Cellular Growth and Proliferation	35	4.78E-03-3.29E-02
Carbohydrate Metabolism	18	6.19E-03-3.29E-02
Cellular Compromise	14	6.19E-03-3.29E-02
DNA Replication, Recombination, and Repair	32	6.19E-03-3.29E-02
Gene Expression	8	6.19E-03-3.29E-02
Lipid Metabolism	30	6.19E-03-3.29E-02
Nucleic Acid Metabolism	6	6.19E-03-3.29E-02
Energy Production	9	7.71E-03-3.29E-02
Unique REG- POST-PRE		
Molecular and cellular functions	**Molecules**	**P-value**
Cell Morphology	99	1.87E-04-4.21E-02
Cell-To-Cell Signaling and Interaction	95	1.87E-04-4.18E-02
Lipid Metabolism	95	6.24E-04-4.04E-02
Small Molecule Biochemistry	159	6.24E-04-4.04E-02
Carbohydrate Metabolism	35	7.04E-04-3.93E-02
Cellular Assembly and Organization	218	7.04E-04-3.93E-02
Cellular Development	52	7.04E-04-3.41E-02
Cellular Function and Maintenance	201	7.04E-04-4.07E-02
Cellular Growth and Proliferation	52	8.09E-04-4.07E-02
Amino Acid Metabolism	44	9.03E-04-3.93E-02
Molecular Transport	84	1.13E-03-4.16E-02
Cell Cycle	35	2.63E-03-3.93E-02
Cellular Compromise	40	2.63E-03-2.86E-02
Drug Metabolism	12	2.63E-03-3.93E-02
Cell Death and Survival	61	3.26E-03-4.18E-02
Vitamin and Mineral Metabolism	46	3.81E-03-4.04E-02
Energy Production	27	3.88E-03-2.91E-02
Cellular Movement	112	5.19E-03-4.07E-02
Nucleic Acid Metabolism	18	5.60E-03-2.23E-02
Post-Translational Modification	53	6.15E-03-3.85E-02

Table 6B. Pathways unique to REG+ and REG- POST-PRE

Unique REG+ POST-PRE			
Canonical pathways	**P-value**	**Genes up**	**Genes down**
CNTF Signaling	9.07E-04	5/55 (9%)	2/55 (4%)
PI3K/AKT Signaling	4.43E-02	7/144 (5%)	1/144 (1%)
Regulation of Cellular Mechanics by Calpain Protease	2.96E-02	4/72 (6%)	1/72 (1%)
Regulation of eIF4 and p70S6K Signaling	1.83E-01	6/174 (3%)	1/174 (1%)
Oncostatin M Signaling	9.99E-02	2/35 (6%)	1/35 (3%)
VEGF Signaling	3.36E-01	3/103 (3%)	1/103 (1%)
CDK5 Signaling	1.58E-01	4/94 (4%)	1/94 (1%)
FAK Signaling	1.43E-01	4/101 (4%)	1/101 (1%)
Melanocyte Development and Pigmentation Signaling	2.98E-01	3/91 (3%)	1/91 (1%)
JAK/Stat Signaling	1.78E-01	3/70 (4%)	1/70 (1%)
Unique REG- POST-PRE			
Canonical pathways	**P-value**	**Genes up**	**Genes down**
Valine Degradation I	3.57E-03	0/35 (0%)	6/35 (17%)
Leucine Degradation I	5.46E-03	0/26 (0%)	4/26 (15%)
TCA Cycle II (Eukaryotic)	8.80E-05	2/41 (5%)	7/41 (17%)
Molybdenum Cofactor Biosynthesis	2.63E-03	0/15 (0%)	3/15 (20%)
Fatty Acid β-oxidation I	3.03E-03	2/45 (4%)	6/45 (13%)
Extrinsic Prothrombin Activation Pathway	1.05E-02	0/20 (0%)	5/20 (25%)
β-alanine Degradation I	7.92E-03	0/10 (0%)	2/10 (20%)
L-DOPA Degradation	7.92E-03	0/13 (0%)	2/13 (15%)
Inhibition of Angiogenesis by TSP1	2.38E-02	0/39 (0%)	7/39 (18%)
PPARα/RXRα Activation	3.01E-02	5/191 (3%)	17/191 (9%)

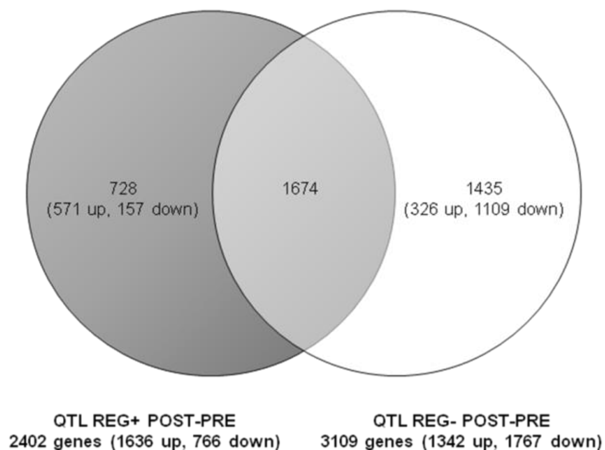

Figure 3. Venn diagram between QTL REG+ and QTL REG- POST-PRE

Overlap and differences in significantly differentially expressed probesets between the QTL REG+ and QTL REG-POST versus PRE class comparisons, revealing probesets shared between both analyses as well as probesets unique to the QTL REG+ or QTL REG- analysis. QTL REG+/QTL REG- donors with regeneration parameters in the upper/lower regeneration quartile. PRE baseline liver biopsies taken prior to resection; POST remnant liver biopsies taken within 1 hour after resection.

Table 6C. Genes up- and downregulated unique to REG+ and REG- POST-PRE

Symbol	Name	Fold Change	P-value
Unique REG+ POST-PRE - Genes upregulated			
Symbol	**Name**	**Fold Change**	**P-value**
PON2	paraoxonase 2	1.780	3.33E-03
XRRA1	X-ray radiation resistance associated 1	1.760	7.21E-05
ASB1	ankyrin repeat and SOCS box containing 1	1.550	7.36E-05
ZNF814	zinc finger protein 814	1.940	1.62E-04
HSPA4L	heat shock 70kDa protein 4-like	1.710	4.94E-04
Unique REG+ POST-PRE - Genes downregulated			
Symbol	**Name**	**Fold Change**	**P-value**
ASF1B	ASF1 anti-silencing function 1 homolog B	-1.852	2.48E-03
SESN1	sestrin 1	-1.587	2.86E-03
CITED2	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2	-1.613	5.40E-04
MSANTD4	Myb/SANT-like DNA-binding domain containing 4 with coiled-coils	-1.667	1.70E-04
DDIT4	DNA-damage-inducible transcript 4	-1.515	1.20E-04
Unique REG- POST-PRE – Genes upregulated			
Symbol	**Name**	**Fold Change**	**P-value**
STEAP4	STEAP family member 4	3.990	2.04E-05
EMP1	epithelial membrane protein 1	3.180	2.80E-04
CAND1	cullin-associated and neddylation-dissociated 1	2.620	5.80E-06
RND1	Rho family GTPase 1	2.140	5.93E-04
UBE2D3	ubiquitin-conjugating enzyme E2D 3	1.960	4.67E-04
Unique REG- POST-PRE - Genes downregulated			
Symbol	**Name**	**Fold Change**	**P-value**
IGFBP1	insulin-like growth factor binding protein 1	-2.326	3.47E-03
SLC26A4	solute carrier family 26, member 4	-2.000	1.14E-04
NTRK2	neurotrophic tyrosine kinase, receptor, type 2	-1.852	7.40E-06
ACADSB	acyl-CoA dehydrogenase, short/branched chain	-1.724	1.22E-04
AKTIP	AKT interacting protein	-1.695	2.04E-05

Analysis of the extremes of regeneration in living donors

Comparison between the most successful and most limited regenerating donors was independent of the three regeneration measures. Immediate early gene expression changes between the POST and PRE biopsies of the QTL REG+ donors provided 3207 probesets related to 2402 genes (1636 up, 766 down), whereas the REG- POST vs. PRE analysis provided 3863 probesets related to 3109 genes (1342 up, 1767 down). The Venn diagram in Figure 3 shows that of these significantly differentially expressed genes 1674 genes are shared between the QTL REG+ and QTL REG- donors, whereas 728 genes (571 up, 157 down) are unique to the QTL REG+ group and 1435 genes (326 up, 1109 down) are unique to the QTL REG- group.

Independent of regeneration rate, acute phase responses and proliferation are activated after liver resection whereas metabolic functions are suppressed

Similar to the REG+ versus REG- analyses, many genes shared between the QTL REG+ and QTL REG- donors appeared to be related to cell death and survival (469 molecules),

cellular growth and proliferation (453 molecules) and cellular development (246 molecules; Supplemental Table 2). This was reflected in the topmost significantly regulated pathways, which again included activation of the acute phase response signaling pathway, but also of pathways related to iNOS, IL-6, IL-10 and EIF2 signaling (Supplemental Table 3). Topmost upregulated genes also again included the acute phase response, apoptosis and proliferation related genes SAA1 (>20-fold), SAA2 (>13-fold), CRP (>6-fold), TGFB3 (>5-fold), and CASP4 (>4-fold; Supplemental Table 4).

Topmost downregulated genes were also similar to the shared REG+ and REG- analysis, including THRSP (<-5-fold), CELSR3 (<-4-fold) and PER3 (<-3-fold), and thus were involved in lipid metabolism, cell adhesion and signal transduction. This reflects the overall results on metabolic functions in the shared analysis: downregulation of genes related to metabolism, especially lipid metabolism, as shown for the bile acid biosynthesis pathway (7% down 0% up), the α–tocopherol degradation pathway (20% down, 0% up), L-cysteine degradation pathway (20% down, 0% up), cholesterol biosynthesis pathways (8% down, 0% up) and fatty acid biosynthesis pathway (14% down, 0% up).

Very successful regenerating donors show more activation of oxidative stress and proliferation responses as well as lipid metabolism

Even more than in the unique REG+ analysis, the largest part of genes unique to the QTL REG+ donors were upregulated (78%) and related to cell death and survival (107 molecules) as well as cell cycle (61 molecules; Table 7A). Highly regulated pathways included the protein ubiquitination and NRF2-mediated oxidative stress response pathways (Table 7B). Besides these pathways, many of the topmost significantly regulated pathways play a role in metabolism, especially lipid metabolism, like the triacylglycerol degradation, diphthamide biosynthesis and retinol biosynthesis pathways. In these very successful regenerating donors, the top upregulated genes included CNPY2 (2.7-fold), TES (2.4-fold) and XDH (2.4-fold), which are involved in regulation of gene expression and proliferation as well as metabolic processes (Table 7C). The topmost downregulated genes, on the other hand, included ARRDC4 (-2.0-fold), PRKCE (-1.9-fold) and SESN1 (-1.9-fold), which are involved in signal transduction and cell cycle regulation.

Very limited regenerating donors show more silencing of metabolic activity

Also in the QTL REG- donors, results similar to the unique REG- analysis were found, with most genes downregulated (77%) and related to cell death and survival (282 molecules), small molecule biochemistry (215 molecules) and lipid metabolism (176 molecules; Table 7A). This was clearly reflected in the top regulated pathways, which included nicotine degradation, FXR/RXR activation, PPARα/RXRα activation and oxidative stress response pathways (Table 7B). In these very limited regenerating donors, the topmost upregulated genes included STEAP4 (4.0-fold), EMP1 (3.1-fold) and S100A8 (2.7-fold), which are involved in fat cell differentiation, inflammatory response and cell proliferation

(Table 7C). The topmost downregulated genes included TNRC6C (-5.3-fold), NR0B2 (-3.2-fold) and G6PC (-2.9-fold), which are involved in gene expression, transcription, lipid and carbohydrate metabolism.

Table 7A. Functions unique to QTL REG+ and QTL REG- POST-PRE

Unique QTL REG+ POST-PRE		
Molecular and cellular functions	**Molecules**	**P-value**
RNA Post-Transcriptional Modification	29	1.79E-05-4.13E-02
Cellular Assembly and Organization	42	6.99E-05-4.13E-02
Lipid Metabolism	26	1.15E-04-4.13E-02
Small Molecule Biochemistry	45	1.15E-04-4.13E-02
Cellular Compromise	32	1.38E-04-4.13E-02
Cellular Function and Maintenance	50	1.38E-04-4.13E-02
Cell Cycle	61	7.51E-04-4.13E-02
Cell Death and Survival	107	1.00E-03-4.13E-02
Molecular Transport	19	1.52E-03-4.13E-02
Carbohydrate Metabolism	14	1.70E-03-4.13E-02
Cell Morphology	45	1.70E-03-4.13E-02
Drug Metabolism	4	1.70E-03-4.13E-02
Nucleic Acid Metabolism	15	1.70E-03-4.13E-02
Protein Degradation	34	2.21E-03-1.81E-02
Protein Synthesis	64	2.21E-03-3.48E-02
DNA Replication, Recombination, and Repair	28	2.56E-03-4.13E-02
Cell-To-Cell Signaling and Interaction	30	4.87E-03-4.13E-02
Cellular Development	46	4.96E-03-4.13E-02
Cellular Growth and Proliferation	38	4.96E-03-4.13E-02
Cellular Movement	6	4.96E-03-4.13E-02
Unique QTL REG- POST-PRE		
Molecular and cellular functions	**Molecules**	**P-value**
Amino Acid Metabolism	42	4.35E-06-2.89E-02
Small Molecule Biochemistry	215	4.35E-06-3.56E-02
Energy Production	40	8.80E-06-3.56E-02
Lipid Metabolism	176	8.80E-06-3.56E-02
Molecular Transport	146	2.75E-05-3.56E-02
Vitamin and Mineral Metabolism	41	2.19E-04-1.96E-02
Cell-To-Cell Signaling and Interaction	48	3.00E-04-3.56E-02
Cellular Development	66	4.04E-04-3.56E-02
Cellular Growth and Proliferation	58	4.04E-04-3.56E-02
Carbohydrate Metabolism	87	5.39E-04-3.12E-02
Drug Metabolism	13	5.39E-04-3.56E-02
Cellular Compromise	28	5.75E-04-3.56E-02
Cellular Movement	74	7.06E-04-3.56E-02
Cell Death and Survival	282	7.59E-04-3.56E-02
Cell Morphology	116	7.59E-04-3.56E-02
Cellular Function and Maintenance	113	7.59E-04-3.56E-02
Protein Degradation	57	1.18E-03-4.01E-03
Protein Synthesis	133	1.18E-03-2.90E-02
Cell Cycle	55	3.31E-03-3.56E-02
Gene Expression	5	3.82E-03-3.82E-03

Table 7B. Pathways unique to QTL REG+ and QTL REG- POST-PRE

Canonical pathways	P-value	Genes up	Genes down
Unique QTL REG+ POST-PRE			
Protein Ubiquitination Pathway	2.78E-04	22/268 (8%)	1/268 (0%)
Triacylglycerol Degradation	3.42E-04	5/32 (16%)	1/32 (3%)
Role of NFAT in Cardiac Hypertrophy	8.65E-04	15/207 (7%)	2/207 (1%)
NRF2-mediated Oxidative Stress Response	9.22E-04	15/192 (8%)	2/192 (1%)
Chronic Myeloid Leukemia Signaling	1.39E-03	10/105 (10%)	1/105 (1%)
Prostate Cancer Signaling	1.62E-03	9/98 (9%)	1/98 (1%)
Diphthamide Biosynthesis	1.70E-03	2/13 (15%)	0/13 (0%)
GDP-L-fucose Biosynthesis I (from GDP-D-mannose)	1.70E-03	2/7 (29%)	0/7 (0%)
Retinol Biosynthesis	2.06E-03	3/58 (5%)	3/58 (5%)
Colanic Acid Building Blocks Biosynthesis	2.06E-03	4/36 (11%)	0/36 (0%)
Unique QTL REG- POST-PRE			
Canonical pathways	**P-value**	**Genes up**	**Genes down**
Nicotine Degradation II	8.92E-05	1/83 (1%)	11/83 (13%)
Serotonin Degradation	3.21E-04	0/75 (0%)	11/75 (15%)
Superpathway of Melatonin Degradation	3.21E-04	1/78 (1%)	10/78 (13%)
Nicotine Degradation III	3.43E-04	1/71 (1%)	9/71 (13%)
Melatonin Degradation I	5.61E-04	1/63 (2%)	9/63 (14%)
FXR/RXR Activation	8.29E-04	1/101 (1%)	15/101 (15%)
Estrogen Biosynthesis	1.92E-03	1/49 (2%)	8/49 (16%)
NRF2-mediated Oxidative Stress Response	1.99E-03	7/192 (4%)	19/192 (10%)
PPARα/RXRα Activation	3.02E-03	6/191 (3%)	18/191 (9%)
LPS/IL-1 Mediated Inhibition of RXR Function	3.31E-03	5/239 (2%)	23/239 (10%)

DISCUSSION

The majority of living liver donors do well after surgery, however, many show incomplete regeneration in the first 3-6 months after donation, with a significant incidence of post-operative complications and a small but present risk of liver failure or even death.[1-6] A better understanding of the underlying functional molecular mechanisms influencing liver regeneration may provide possible targets for intervention to enhance regeneration and minimize subsequent morbidity and mortality. In this study, the effects of liver resection on hepatic gene expression in healthy living liver donors were investigated using microarray analysis. We furthermore analyzed the differences in gene expression profiles between donors with successful regeneration of their remnant liver versus those with less robust regeneration.

Overall, liver resection activated pathways involved in acute phase and oxidative stress responses, triggered cell proliferation related signaling and silenced metabolic functions. This is in line with previous findings on gene expression in rodent models of liver resection as well as in liver grafts for adult human transplantation.[17, 19, 20, 22-27] However, after comparing donors with successful and limited regeneration of their remnant liver mass, we found significant differences in their gene expression profiles.

Table 7C. Genes up- and downregulated unique to QTL REG+ and QTL REG- POST-PRE

Symbol	Name	Fold Change	P-value
Unique QTL REG+ POST-PRE - Genes upregulated			
Symbol	**Name**	**Fold Change**	**P-value**
VNN3	vanin 3	3.200	4.19E-05
CNPY2	canopy 2 homolog (zebrafish)	2.710	1.21E-05
TES	testis derived transcript (3 LIM domains)	2.430	5.20E-04
XDH	xanthine dehydrogenase	2.400	1.22E-05
SNORD12C	small nucleolar RNA, C/D box 12C	2.400	4.38E-04
Unique QTL REG+ POST-PRE - Genes downregulated			
Symbol	**Name**	**Fold Change**	**P-value**
ARRDC4	arrestin domain containing 4	-2.041	3.53E-03
WDR74	WD repeat domain 74	-2.041	3.27E-03
GTPBP6	GTP binding protein 6 (putative)	-2.000	1.76E-03
PRKCE	protein kinase C, epsilon	-1.887	1.64E-03
SESN1	sestrin 1	-1.887	1.08E-03
Unique QTL REG- POST-PRE - Genes upregulated			
Symbol	**Name**	**Fold Change**	**P-value**
STEAP4	STEAP family member 4	3.970	2.47E-03
EMP1	epithelial membrane protein 1	3.110	4.58E-03
KIAA0040	KIAA0040	3.000	4.71E-05
S100A8	S100 calcium binding protein A8	2.660	4.26E-04
C19orf42	chromosome 19 open reading frame 42	2.590	4.09E-03
Unique QTL REG- POST-PRE - Genes downregulated			
Symbol	**Name**	**Fold Change**	**P-value**
TNRC6C	trinucleotide repeat containing 6C	-5.263	6.00E-07
NR0B2	nuclear receptor subfamily 0, group B, member 2	-3.226	2.49E-05
SNRPN	small nuclear ribonucleoprotein polypeptide N	-3.030	6.00E-07
ELFN1	extracellular leucine-rich repeat and fibronectin type III domain containing 1	-3.030	1.76E-03
G6PC	glucose-6-phosphatase, catalytic subunit	-2.857	1.58E-05

One of the interesting findings is the contrasting ratio of up- and downregulated genes between donors with successful and limited regeneration after liver resection: the more successful their regeneration, the higher the percentage of upregulated genes and vice versa.

Furthermore, some pathways appeared to be important in the response to surgical tissue injury, regardless of the quantity of regeneration. One of these pathways was the acute phase response signaling pathway. This pathway was shared between successful and limited regenerating donors, even when comparing donors in the upper and lower regeneration quartiles. The acute phase response pathway is known to be involved in the rapid inflammatory response activated at time of tissue injury to provide protection against trauma, surgery, micro-organisms or immunological disorders.[28-31] This protective response is accompanied by an increase in inflammatory factors, like pro-inflammatory cytokines, as well as a change in plasma concentration of acute phase proteins, described to be largely caused by an altered metabolic activity of the liver. During the acute phase response, positive acute phase response proteins are upregulated, whereas negative acute phase response proteins are downregulated. In the current study most genes relat-

ed to the acute phase response signaling pathway were upregulated, with several genes encoding positive acute phase response proteins (like SAA1, SAA2 and CRP) listed among the topmost upregulated genes. These results display a clear activation of the acute phase response at time of liver resection, independent of the quantity of regeneration.

Figure 4A

NRF2-mediated Oxidative Stress Response

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Figure 4B

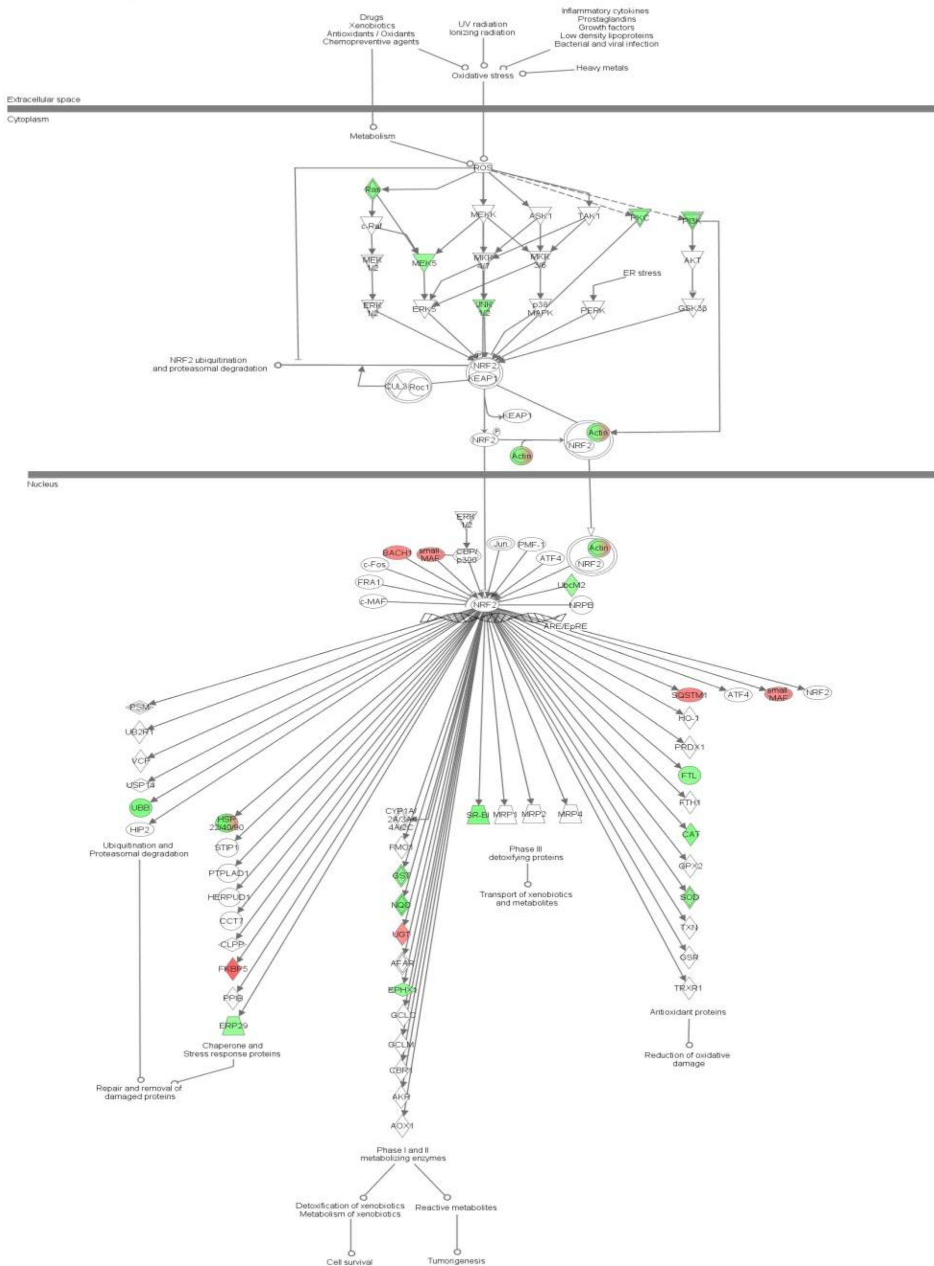

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Figure 4. NRF2-mediated oxidative stress pathway

Gene expression levels **A.** unique to QTL REG+ POST-PRE and **B.** unique to QTL REG- POST-PRE.

Another pathway shared between successful and limited regenerating donors is the NRF2-mediated oxidative stress response pathway. Oxidative stress is caused by an imbalance between the production of reactive oxygen species and the detoxification of reactive intermediates. These reactive intermediates, like peroxides and free radicals, can damage cellular components such as proteins, lipids and DNA and can even trigger cell death. As a defense response, cells activate detoxifying and antioxidant enzymes. Nuclear factor-erythroid 2-related factor 2 (Nrf2) is known to activate the transcription of these defense enzymes by binding to the antioxidant response elements (ARE) within the promoter of these enzymes.[32-34] Interestingly, in our study, this pathway appeared highly regulated in both the upper and lower regeneration quartile, but with opposite effects on its activation, showing mostly upregulated genes in the best regenerating donors and vice versa (Figure 4). These results suggest that better activation of the oxidative stress response at time of liver resection contributes to more robust regeneration.

The EIF2 signaling pathway was also shared between successful and limited regenerating donors, with most of its pathway genes being upregulated. This pathway plays a major role in the mRNA translation phase of protein synthesis and is activated by a variety of stimuli causing environmental stress.[35, 36] Our data thus point toward activation of mRNA translation and protein synthesis at time of liver resection. Interestingly, investigation of EIF2 signaling pathway related genes unique either regeneration group revealed that nearly all genes are upregulated in the most successful regenerating donors and vice versa (Figure 5). This might suggest higher initiation activation of protein synthesis in more successful regenerating donors.

The aryl hydrocarbon receptor signaling pathway is another highly regulated pathway shared between successful and limited regenerating donors. The aryl hydrocarbon receptor complex induces transcriptional activation of genes encoding xenobiotic metabolizing enzymes, phase II metabolizing enzymes as well as other growth factors and proteins involved in cell cycle progression and apoptosis.[37-41] Aryl hydrocarbon receptor is also known to interact with retinoblastoma protein (RB) and transforming growth factor-β (TGF-β), thereby contributing to the inhibition of cell cycle progression and attenuating TGF-β-mediated apoptosis. In the current study, TGM2 and TGFB3, both part of the aryl hydrocarbon receptor signaling pathway, were among the topmost upregulated genes shared between successful and limited regenerating donors. In this pathway, they play a major role in the initiation of apoptosis, suggesting that regardless of the regeneration rate, a certain amount of liver cells are triggered for apoptotic cell death at time of resection.

Furthermore, the PPARα/RXRα activation pathway is shared between both regeneration groups. However, the PPARα/RXRα activation pathway is also listed among the topmost significantly regulated pathways in the unique (QTL) REG- analyses, showing mainly downregulation of the pathway genes. In contrast to this downregulation, the unique (QTL) REG+ groups only show upregulation of their significantly differentially expressed

Figure 5A

EIF2 Signaling

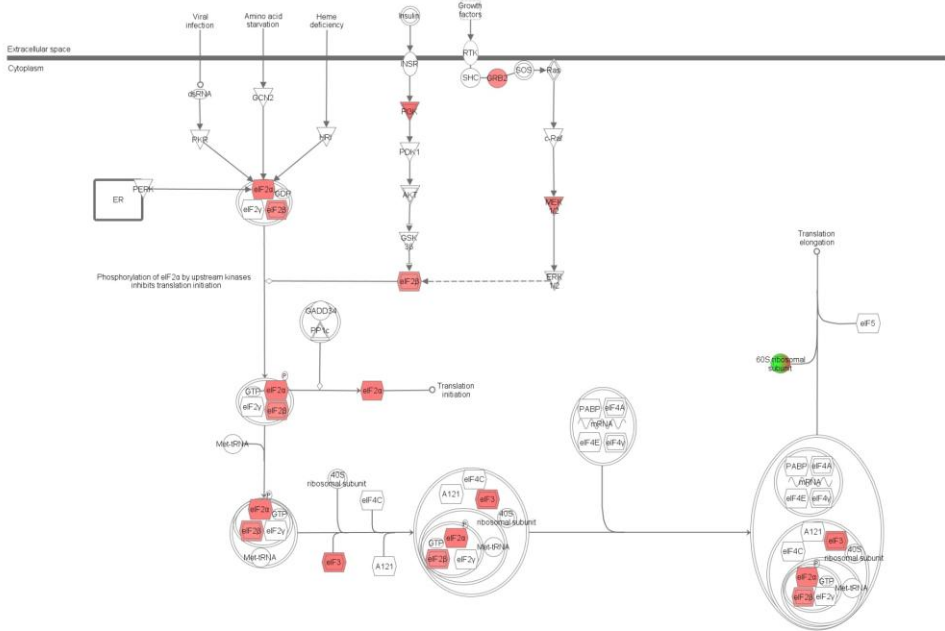

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Figure 5B

EIF2 Signaling

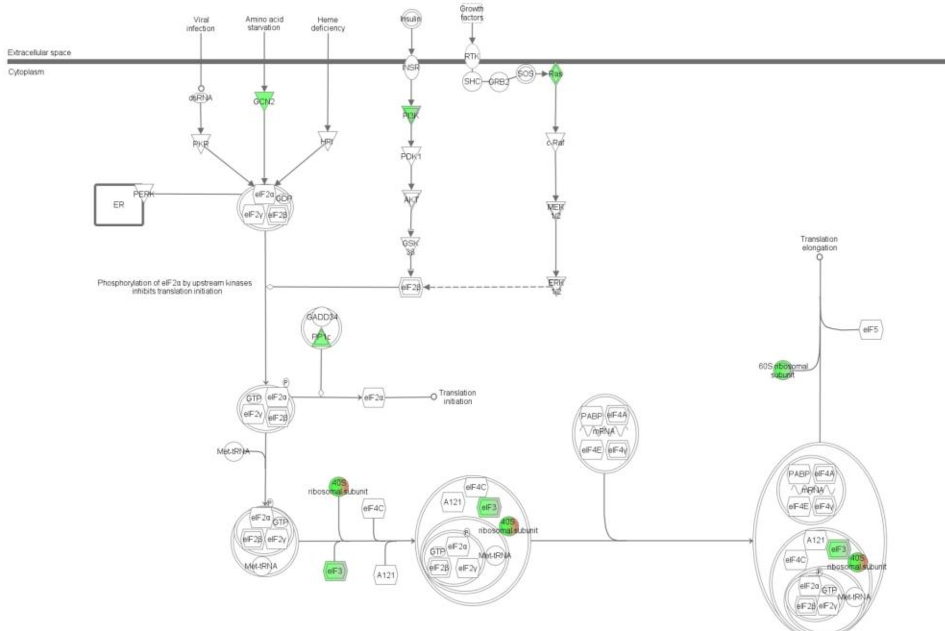

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Figure 5. EIF2 signaling pathway

Gene expression levels **A.** unique to QTL REG+ POST-PRE and **B.** unique to QTL REG- POST-PRE.

genes belonging to this pathway (Figure 6). The peroxisome proliferator activated receptor α (PPARα) is a ligand activated transcription factor that belongs to the family of nuclear receptors.[42-46] PPARα is activated by polyunsaturated fatty acids and synthetic drugs like fibrates. After heterodimerization with its partner RXRα, PPARα plays a central role in fatty acid oxidation and uptake in tissues like liver, heart, skeletal muscle and kidney. In addition, PPARα is also expressed in endothelial cells, vascular smooth muscle cells and macrophages where it exerts anti-inflammatory and anti-oxidative effects. The difference in up- and downregulation of this pathway between successful and limited regenerating donors suggests that donors with limited regeneration show less anti-inflammatory and anti-oxidant activation and/or lower fatty acid oxidation and uptake.

Especially the effects on fatty acid and lipid metabolism are prominent in this study, as lipid metabolism appears to be the metabolic activity most affected by liver resection. Moreover, the regeneration rate seems to correlate with the degree by which this metabolic function becomes affected. Interestingly, where stress response, inflammation and cell proliferation related genes become more activated after liver resection, metabolic pathways, especially lipid metabolism related pathways, are silenced. These early effects on gene expression profiles caused by liver resection support the general hypothesis that the liver balances between its two major functional mechanisms: regenerative processes to restore liver tissue after injury and metabolic processes to ensure homeostasis.

Borozan et al. reported regulation of stress response related genes after liver resection and transplantation in liver donors. Their results also showed significant regulation of lipid metabolism.[26] Our group previously performed a comparative gene expression analysis between living and deceased donor liver grafts, which revealed that living donor liver grafts show upregulation of genes encoding purine, pyrimidine and structural protein synthesis, while genes associated with metabolic liver functions such as bile acid metabolism and protein metabolism are markedly decreased.[24] Research by Ho et al. showed, similar to our data, highly upregulated expression levels of SAA1, SAA2 and CRP: genes encoding positive acute phase response proteins.[47] They also similarly showed that THRSP, a gene known to play a role in lipid metabolism, was one of the topmost downregulated genes.

This mechanistic hypothesis can clarify the similarities and differences between donors with successful regeneration of their remnant liver lobe and donors with deficient regeneration. In the shared analyses, both regeneration groups showed activation of pathways involved in cell death and survival, stress response as well as cellular development and proliferation, but also suppression of pathways involved in metabolism. In the unique analyses, successful regenerating donors showed mostly upregulated gene expression levels in their post-resection liver biopsies compared to their baseline biopsies, resulting in activation of pathways involved in stress response, cell cycle regulation and cell proliferation. In contrast, donors in the limited regenerating group mainly show downregulated gene expression levels post-resection, resulting in inhibition of pathways involved in lipid and carbohydrate metabolism.

A possible conclusion from these contrariwise genetic profiles can therefore be that donors with deficient liver regeneration show inhibited or delayed initiation of recovery and regeneration-related molecular pathways, as their livers mainly focus on suppression of metabolism. However, the causal mechanism behind this inhibition or delay needs to be further elucidated. Nevertheless, the marked differences in early gene expression profiles between successful and limited regenerating donors identify genes and pathways eligible for further investigation of potential therapeutic strategies to improve liver regeneration and recovery.

Figure 6A

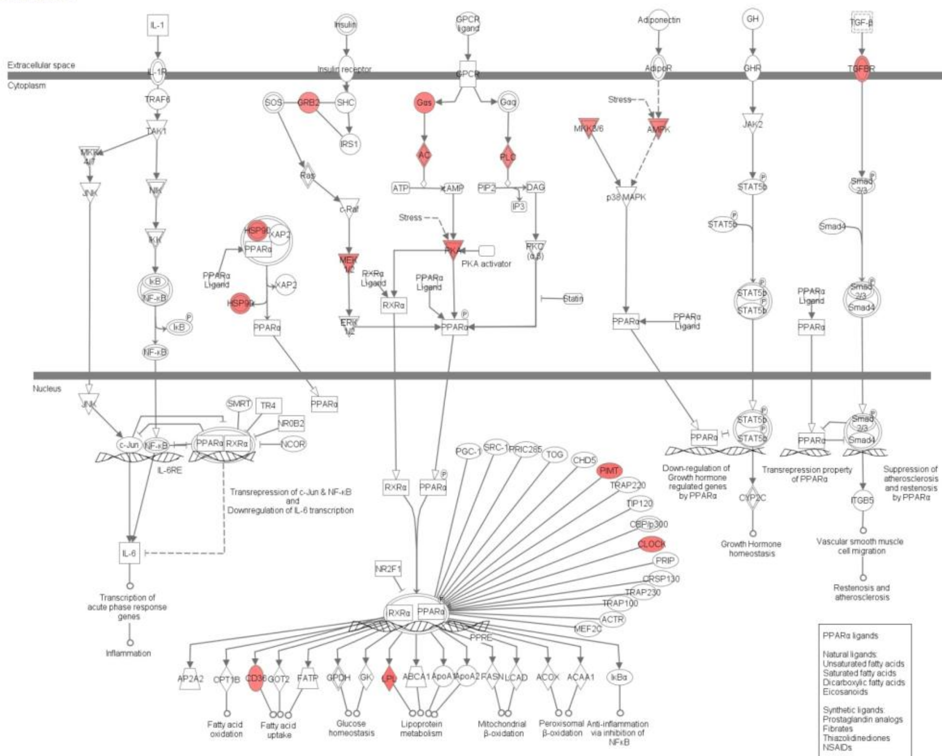

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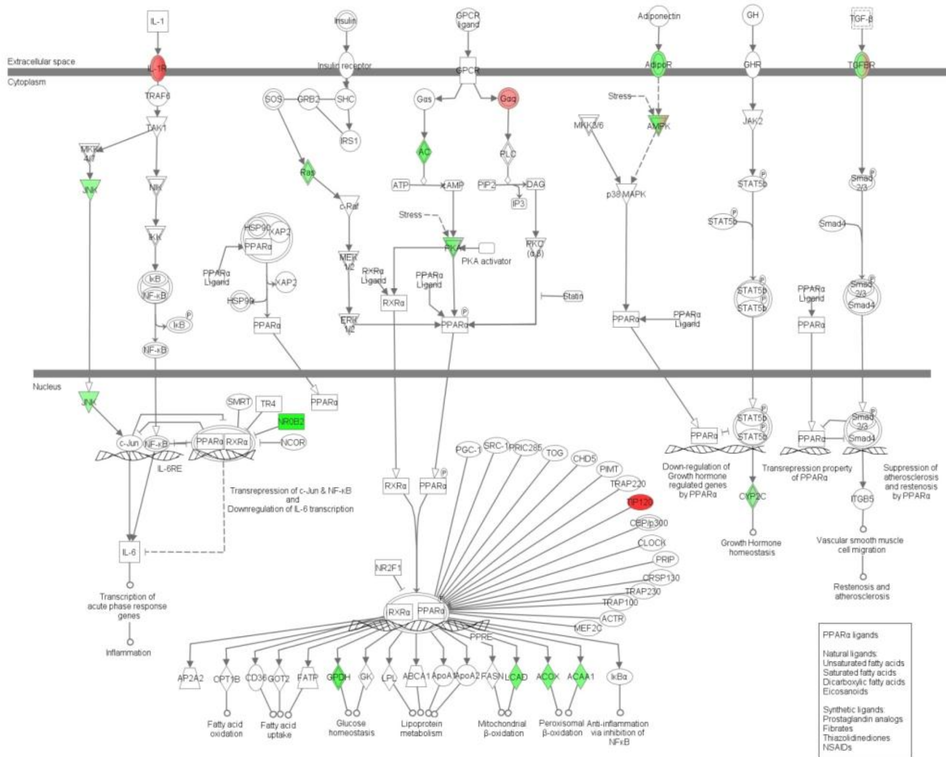
Figure 5. EIF2 signaling pathway

Gene expression levels **A.** unique to QTL REG+ POST-PRE and **B.** unique to QTL REG- POST-PRE.

Figure 6B



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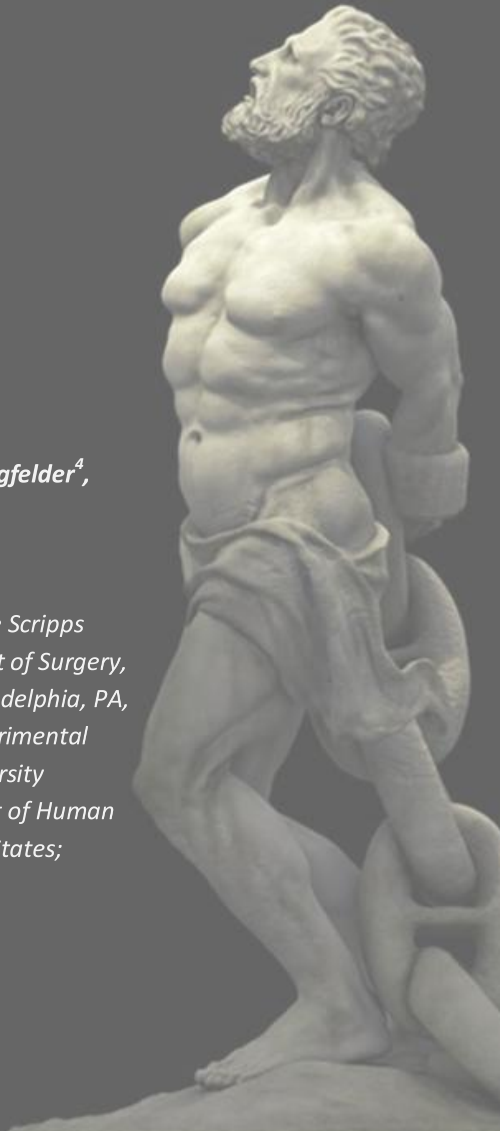
Chapter 3

Genetic profiles and predictors of early allograft dysfunction after human liver transplantation



Sunil M. Kurian[1, #], Suomi M.G. Fouraschen[2, 3, #], Peter Langfelder[4], Steve Horvath[4], Abraham Shaked[2], Daniel R. Salomon[1] and Kim M. Olthoff[2]

[1]Department of Molecular and Experimental Medicine, The Scripps Research Institute, La Jolla, CA, United States; [2]Department of Surgery, Penn Transplant Institute, University of Pennsylvania, Philadelphia, PA, United States; [3]Department of Surgery, Laboratory of Experimental Transplantation and Intestinal Surgery, Erasmus MC-University Medical Center, Rotterdam, The Netherlands; [4]Department of Human Genetics, University of California, Los Angeles, CA, United States; [#]Both authors contributed equally to this study

(Submitted)

ABSTRACT

Early hepatic allograft dysfunction (EAD) after transplantation is defined by hepatocyte injury and the inability to restore homeostasis. Clinically, EAD manifests few days to a week post-transplantation by high serum transaminases, persistent cholestasis and coagulopathy, but the biological mechanisms are poorly understood. This current translational study aims to investigate the molecular mechanisms involved in the development of EAD, and to define a diagnostic gene expression signature of EAD.

Global hepatic gene expression profiles of 40 liver transplant recipients of deceased donor grafts with EAD and 36 recipients without graft dysfunction were investigated using microarrays. Expression was analyzed at the end of the cold storage period and one hour after graft reperfusion, revealing a shift in inflammatory and metabolic responses between EAD and non-EAD. A diagnostic gene expression signature of 152 classifiers, determined using two cohorts, was validated in a third independently collected matched cohort using a complementary expression technology.

This study reports the first high-throughput human gene expression study, comparing the clinical EAD phenotype with its gene expression profiles. Our data may have both diagnostic and mechanistic implications for EAD, defining potential targets for early intervention that can change the postoperative course, reducing morbidity and/or mortality in liver graft recipients.

INTRODUCTION

Following liver transplantation, some liver allografts display evidence of severe hepatocyte injury and significant functional impairment. Termed early allograft dysfunction (EAD), this condition affects approximately one quarter of liver recipients and is associated with decreased graft and patient survival.[1, 2] EAD is usually manifested by high transaminases, persistent cholestasis, or prolonged coagulopathy.[2-6] The clinical parameters used to define EAD are commonly indices of hepatocellular damage and synthetic impairment, leaving its underlying molecular and cellular mechanisms still unclear.

EAD is often thought to be secondary to a combination of donor and recipient characteristics and surgical factors, such as length of cold ischemia and ischemia/reperfusion (IR) injury. These combined factors are associated with acute cellular damage, cell death, oxidative damage from reactive oxygen species, immune activation, and severe inflammatory responses occurring within the liver.[7-10]

While liver IR injury has been studied in great detail in animal models, there are relatively few human translational studies on mechanistic pathways of injury associated with post-transplant graft function and correlating with clinical outcomes. We have shown lower pre-operative serum levels of IL-6 and higher levels of IL-2R in EAD patients as well as post-operative upregulation of proteins of the NF-κB pathway and higher serum levels of chemokines and cytokines associated with T-cell immunity.[11] Another recent study showed that early high serum phosphorus levels were an independent predictor of EAD.[12] However, there is still a lack of mechanistic insight in signaling pathways and gene networks relevant for the development of EAD, which may be revealed through examination of proximal phenotypes, for example the hepatic transcriptome. Importantly, the clinical endpoint of EAD can be correlated with mechanistic pathways using genomic and proteomic studies in an attempt to identify specific donor or recipient risk factors. A better understanding of the underlying transcriptional changes of EAD may provide biomarkers for the early diagnosis and possibly prognosis of significant allograft dysfunction, leading to intervention and new treatment strategies and thereby reducing morbidity and mortality after liver transplantation.

In this study, we correlated the “clinical” phenotype of EAD with a “molecular” signature found in the liver graft immediately following reperfusion. We focused on validating pathways that have been previously described in animal models of IR injury (as a validation of previous literature), as well as the discovery of novel pathways expressed in human liver grafts, that could be predictive of the development of EAD and may be targets for therapeutic intervention. In tandem, we also identified a gene expression signature that was diagnostic of EAD. This signature was identified using independently collected training and test cohorts of samples and validated in another cohort of samples using a different, yet complementary gene expression technology, providing validated high value diagnostic markers of EAD.

MATERIALS AND METHODS

Patient population and clinical data

Using published clinical criteria for EAD[2, 13] we identified 40 recipients of deceased donor grafts with EAD, and compared them to 36 recipients without EAD who were matched for age, gender, donor and/or recipient HCV status and MELD score. All patients were transplanted between 2005 and 2010 at the Hospital of the University of Pennsylvania and provided written informed consent to this study. The study protocols and consent procedures were approved by the Institutional Review Board of the University of Pennsylvania.

Clinical data were collected from donor and recipient charts and from the electronic transplant clinical database, including donor and recipient demographics, intra-operative details and postoperative liver function. Cold ischemic time of the liver graft was defined as time from donor cross-clamp to removal from ice prior to placement into the recipient. Warm ischemic time was defined as time from removal from ice to the first of either arterial or portal reperfusion.

Liver biopsies

Two core liver biopsy specimens were obtained from each deceased donor graft at the time of transplantation. The first specimen was taken on the backbench, at the end of the cold storage period (COLD). A subsequent biopsy was taken approximately one hour after reperfusion, following completion of the biliary anastomosis and prior to closure of the abdomen (POST). Biopsies were collected in RNAlater (Qiagen, Valencia, CA) and stored overnight at 4°C after which they were transferred to -80°C until further processing.

Gene expression profiling

For the first analysis, gene expression profiles were analyzed from biopsies of 30 EAD and 26 non-EAD patients using Affymetrix HG-U133_Plus_2 microarrays (Affymetrix, Santa Clara, CA). Early hepatic gene expression changes were determined by comparing expression levels in the POST biopsies to the COLD biopsies.

Two strategies were used to identify specific gene expression signatures of EAD. First, we determined the expression profile related to the biology of EAD by comparing the early changes in hepatic gene expression between the EAD and non-EAD patients. Secondly, we identified a diagnostic signature for EAD with a high predictive power to distinguish EAD samples from non-EAD samples using two cohorts; a training cohort to identify the diagnostic signature (14 EAD versus 13 non-EAD patients) and a test cohort to verify the signature (16 EAD versus 13 non-EAD patients).

The ideal technology for clinical diagnostic use should be both portable (for use as a point-of-care diagnostic platform) and have a fast turnaround time. Therefore, we validated the microarray EAD signature on the Panomics Quantigene Platform (Affymetrix) that

uses Luminex bead based multiplex assays. We used 10 EAD and non-EAD patients from our original training and test cohorts, as well as a third independent cohort of 10 EAD and 10 non-EAD patients. Quantigene assays are based on a non-PCR based Luminex Bead technology, which should eliminate any amplification bias due to suboptimal RNA amplification. In addition, elimination of the PCR step makes the assay more amenable to multiplexing, with detection of up to 36 genes per well, thereby minimizing waste of clinical samples.

RNA preparation and gene expression detection

Total RNA was extracted using Trizol (Invitrogen, Carlsbad, CA) and purified using the RNeasy kit (Qiagen), according to the manufacturer's instructions. RNA analyzed on an Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA) with RNA Integrity Numbers (RINs) above 7 was considered for further analysis.

Affymetrix Human Genome U133 Plus 2.0 GeneChips using standard Affymetrix protocols were used. The Panomics Quantigene Plex assays were performed with target specific probe sets and signals were read using a Luminex 100 instrument (Luminex, Austin, TX). Signals were normalized against the geometric mean of the average expression values for three housekeeping genes: HPRT1, PPIB and ACTB.

Statistical analysis

Donor characteristics and clinical data are shown as mean ± SD, unless described otherwise (Table 1). Clinical data were compared using the Mann-Whitney test (Graphpad Prism software) and p-values <0.05 were considered significant.

Signal intensities from the microarrays were background corrected and normalized using RMAExpress.[14] Samples with GAPDH and/or Actin 3' to 5' ratios below 8 and outliers by Principal Components Analysis (PCA) clustering were excluded. BRB-ArrayTools was used for statistical analysis. CEL files with normalized signal intensities are posted in NIH Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo). No probeset filtering was done based on signal intensities.

Class comparisons were performed using an F-test (p<0.005) with random permutations of the class labels, and for each random permutation the F-tests were re-computed for each gene. Functional analyses were performed using Ingenuity Pathway Analysis (Ingenuity Systems, Redwood City, CA).

For the predictive signature, the Diagonal Linear Discriminant Analysis (DLDA) algorithm was used.[15] Receiver Operating Characteristic (ROC) curves were constructed with the JROCFIT program (http://www.rad.jhmi.edu/jeng/javarad/roc/JROCFITi.html). Clinical study parameters were tested using a multivariate logistic regression model with an adjusted (Wald test) p-value and a local false discovery rate calculation (q-value). A detailed analysis can be downloaded from http://labs.genetics.ucla.edu/horvath/htdocs/CoexpressionNetwork/tmp/LiverTransplant/UPennStudy-Results.zip.

For the Panomics assays, fold changes between normalized gene expression data of COLD and POST biopsies were determined using the $2^{-\Delta\Delta Ct}$ method.

RESULTS

Donor, recipient and transplant characteristics

Table 1 summarizes the recipient and donor characteristics as well as the surgical details of the patients. The only significant difference between the EAD and non-EAD patients was donor gender (70% male in the EAD group vs. 44% in the non-EAD group; p=0.036). There was also a trend toward an increase in graft failure in the EAD group, but this was not statistically significant (p=0.268).

The biology of EAD at a transcriptional level

Microarrays are increasingly being used for clinical research applications, including the search for diagnostic and prognostic biomarkers. Genes found to be reliable biomarkers need not necessarily be the best biological markers of the investigated process[16], but rather “innocent bystanders” or surrogate markers that happen to track well with the disease. An example is the Prostate Specific Antigen (PSA), widely used as a biomarker of prostate cancer, but biologically not directly involved in disease progression.[17]

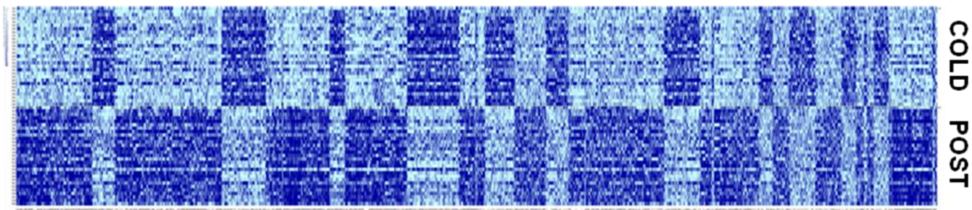
We focused first on the biology of EAD by a class comparison of all EAD POST vs. EAD COLD, and compared them to all non-EAD POST vs. non-EAD COLD (p<0.005; FDR <20%). As shown in Figure 1, there were distinct differences in gene expression levels between POST and COLD biopsies in both the EAD and non-EAD group. There were 1877 significantly differentially expressed probesets in the EAD POST versus COLD biopsies, with 1265 probesets upregulated and 612 downregulated POST (Figure 2). Similarly, in the non-EAD group 1304 significantly differentially expressed probesets were found between the POST and COLD biopsies (789 probesets upregulated and 515 downregulated). A Venn diagram of the 1877 and 1304 probesets yielded 1061 probesets (953 genes) unique to the EAD biopsies (682 upregulated and 379 downregulated POST; Figure 3). We acknowledge that this may be an underestimate of the true sharing between the EAD and non-EAD samples, but this analysis represents an attempt to discover the biology of EAD. Table 2 shows the top 10 canonical pathways related to the 953 genes, including 5 signaling networks involving PPAR, ILK, NF-κB, PI3K/AKT and IL-6.

IPA revealed that 86 genes were inflammatory of which 72 (84%) were upregulated in the EAD POST biopsies (Supplemental Table 1). There were also 50 genes involved in liver inflammation, injury and toxicity expressed in the EAD livers (Supplemental Table 2), of which 40 (80%) were upregulated in the EAD POST biopsies. IPA-assisted literature searches furthermore identified 18 EAD-related genes found in our analysis that are already targeted by commercially available drugs (Supplemental Table 3). The multivariate logistic regression on clinical variables and gene expression showed that none of the clini-

Table 1. Patient characteristics

	All patients			Patients for Affymetrix microarray analysis						Patients for Panomics validation		
				Training set			Test set					
	EAD (n=40)	Non-EAD (n=36)	p-value	EAD (n=14)	Non-EAD (n=13)	p-value	EAD (n=16)	Non-EAD (n=13)	p-value	EAD (n=15)	Non-EAD (n=15)	p-value
Recipient and donor details												
Recipient: Primary disease												
- Hepatitis B	4	-		-	-		3	-		2	-	
- Hepatitis C	23	22		7	9		9	7		11	9	
- Post-alcoholic cirrhosis	4	7		4	-		-	5		-	4	
- Primary sclerosing cholangitis	3	1		-	-		3	-		-	1	
- Cryptogenic cirrhosis	2	4		2	3		-	1		-	-	
- Nonalcoholic steatohepatitis (NASH)	2	1		1	-		1	-		-	1	
- Drug toxicity	1	-		-	-		-	-		1	-	
- Hemochromatosis/hemosiderosis	1	-		-	-		-	-		1	-	
- Autoimmune liver disease	-	1		-	1		-	-		-	-	
Recipient: Male/Female	32/8	32/4	0.355	9/5	12/1	0.165	15/1	12/1	1.000	13/2	12/3	1.000
Recipient: Age (min-max; median)	34-69 (56)	31-73 (55.5)	0.987	44-68 (58.5)	31-65 (56)	0.437	34-66 (53.5)	42-73 (54)	0.524	39-69 (55)	52-73 (56)	0.480
Recipient: MELD (min-max; median)	9-41 (24)	10-39 (22.5)	0.413	9-41 (20.5)	10-36 (25)	0.330	11-41 (25)	12-39 (18)	**0.048**	13-34 (25)	13-34 (22)	0.430
Donor: Male/Female	28/12	16/20	**0.036**	8/6	6/7	0.706	13/3	6/7	0.064	11/4	6/9	0.139
Donor: Age (min-max; median)	21-78 (51)	8-75 (52)	0.556	21-68 (47.5)	24-66 (52)	0.680	30-78 (54)	11-72 (54)	0.227	22-65 (52)	8-75 (39)	0.678
Donor: Cause of death												
- Cerebrovascular accident/intracerebral bleed	24	19		9	6		12	8		6	7	
- Anoxia secondary to cardiac arrest	5	7		2	2		1	2		2	5	
- Traumatic brain injury	11	9		3	4		3	3		7	3	
- Meningitis	0	1		-	1		-	-		-	-	
Transplant surgery details												
Total operative time (min)	329±101	320±66	0.799	335±109	303±48	0.716	303±49	299±37	0.913	347±118	354±79	0.419
Packed red blood cells (units)	6.0±7.3	4.8±4.1	0.859	6.7±7.0	4.4±3.8	0.420	4.4±4.8	4.7±4.9	0.757	5.7±9.0	5.1±3.2	0.188
Cold ischemic time (min)	370±99	335±101	0.226	391±123	352±90	0.680	334±68	350±120	0.930	361±102	308±104	0.221
Warm ischemic time (min)	59±30	53±11	0.666	68±48	59±7	0.382	54±10	49±12	0.281	54±11	51±11	0.418
Total ischemic time (min)	429±99	388±104	0.131	459±115	410±93	0.382	388±71	399±120	0.809	415±103	359±108	0.229
Length of stay in hospital days (min-max; median)	6-76 (9)	6-86 (8)	0.150	6-33 (9.5)	6-86 (8)	0.434	6-76 (9)	6-25 (8)	0.214	6-25 (8)	6-17 (7)	0.245
6 month graft failure (n)	6	2	0.268	3	1	0.596	2	1	1.000	1	-	1.000

cal parameters outlined in Table 1 significantly impacted gene expression (FDR <5%), so the gene expression differences were not further corrected for the clinical variables.



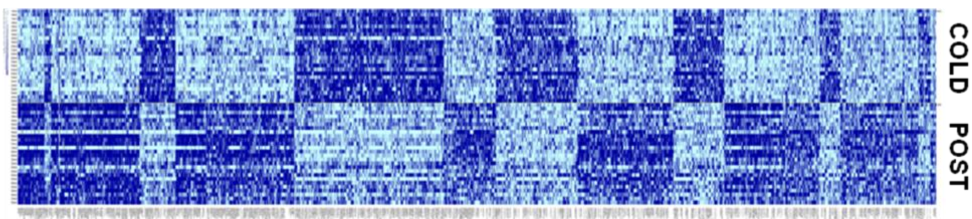


Figure 1. EAD and non-EAD heatmaps

Visualization of the intensity of gene expression in POST and COLD biopsies of EAD and non-EAD recipients, showing clear differences in up- and downregulation of genes between the two time points. EAD early allograft dysfunction; COLD liver graft biopsies taken at the end of the cold storage period; POST liver graft biopsies taken approximately 1 hour after reperfusion.

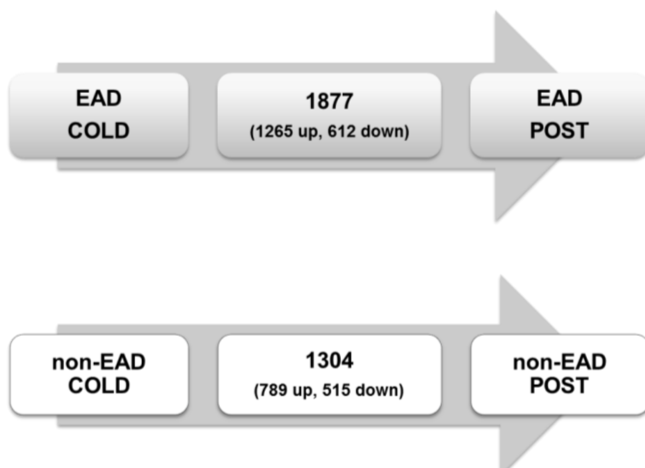


Figure 2. Differentially expressed probesets POST versus COLD

Number of array probesets showing a significant up- or downregulation in gene expression level between POST and COLD biopsies (p<0.005). EAD early allograft dysfunction; COLD liver graft biopsies taken at the end of the cold storage period; POST liver graft biopsies taken approximately 1 hour after reperfusion.

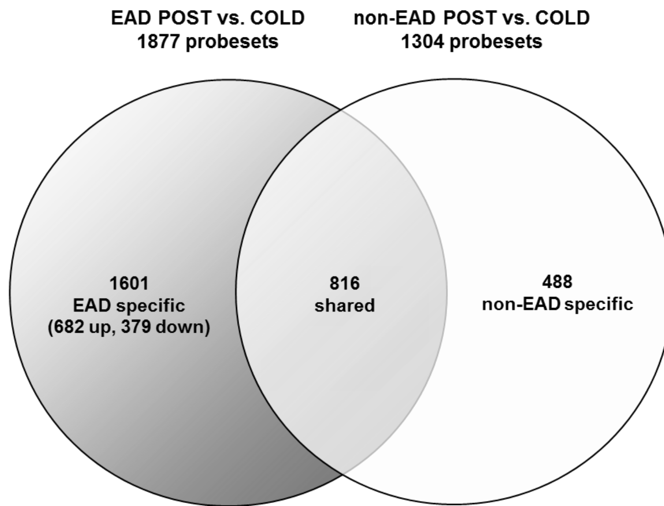


Figure 3. Venn diagram of differentially expressed probesets

Overlap and differences in significantly differentjally expressed probesets between the EAD and non-EAD POST versus COLD class comparisons, revealing probesets shared between both analyses as well as probesets unique to the EAD or non-EAD analysis. EAD early allograft dysfunction; COLD liver graft biopsies taken at the end of the cold storage period; POST liver graft biopsies taken approximately 1 hour after reperfusion.

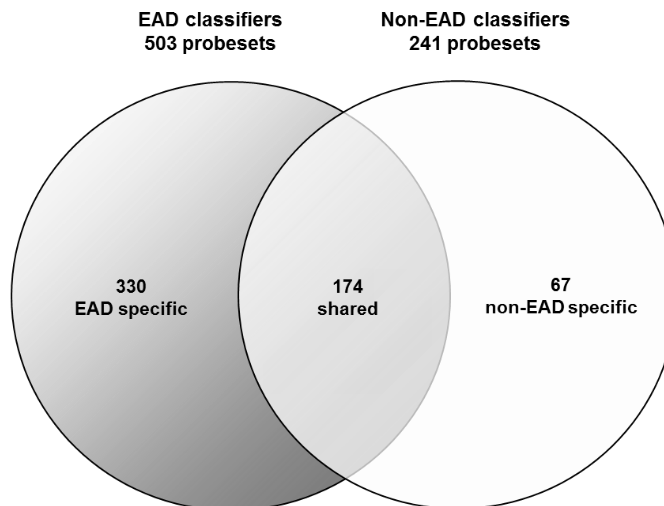


Figure 4. Venn diagram of high-predictive-value classifiers

Overlap and differences in significantly differentially expressed probesets between the EAD and non-EAD high-predictive-value classifiers, revealing probesets shared between both analyses as well as probesets unique to the EAD or non-EAD analysis. EAD early allograft dysfunction.

Table 2. Canonical pathways related to EAD

Pathways	P-value	Up	Down	Molecules
PPAR Signaling	3.97E-05	10/107 (9%)	2/107 (2%)	TRAF6,NR0B2,MAPK1,HSP90AB1,PDGFA,IL1RN,IL1RL1, HSP90AA1,INSR,TNFRSF1B,NFKBIB,CITED2
Prostate Cancer Signaling	5.51E-05	6/97 (6%)	5/97 (5%)	MTOR,MAPK1,HSP90AB1,HSP90AA1,PDPK1,CREB5,NFKBIB, CTNNB1,SIN3A,PTEN,ATM
NF-κB Signaling	7.11E-05	11/176 (6%)	5/176 (3%)	TRAF3,FLT1,FGFR1,IRAK3,MALT1,IGF2R,TRAF6,CSNK2A2, BCL10,IL1RN,BTRC,MAP3K8,INSR,NFKBIB,TNFRSF1B,ATM
ILK Signaling	7.74E-05	12/193 (6%)	5/193 (3%)	MYL6,MAPK1,PPP2R2A,VIM,PDPK1,CREB5,PTEN,MTOR, RHOB,RND3,KRT18,RHOU,CHD1,IRS2,CTNNB1,FNBP1,ATM
PI3K/AKT Signaling	1.00E-04	9/140 (6%)	4/140 (3%)	JAK1,MAPK1,PPP2R2A,PDPK1,EIF4E,PTEN,MTOR, HSP90AB1,HSP90AA1,MAP3K8,NFKBIB,CTNNB1,MCL1
IL-6 Signaling	2.69E-04	9/100 (9%)	2/100 (2%)	TRAF6,CSNK2A2,MAPK1,IL1RN,IL1RL1,SRF,MAP2K3, CEBPB (includes EG:1051),TNFRSF1B,TNFAIP6,NFKBIB
Wnt/β-catenin Signaling	3.40E-04	9/174 (5%)	6/174 (3%)	SOX4,GJA1,AXIN2,PPP2R2A,KREMEN1,ACVR2B,SOX13, HNF1A,CSNK1E,CSNK2A2,TLE3,BTRC,FZD5,UBC,CTNNB1
TGF-β Signaling	4.00E-04	8/89 (9%)	2/89 (2%)	TRAF6,RUNX3,MAPK1,MAP2K3,SMURF2,ACVR2B,SMAD5, INHBB,TGIF1,SMURF1
AMPK Signaling	4.04E-04	6/168 (4%)	7/168 (4%)	PFKFB3,GNAS,MAPK1,PPP2R2A,PRKAR2A,PDPK1,CFTR, MTOR,ACACA,IRS2,MAP2K3,INSR,ATM
IL-8 Signaling	5.66E-04	11/193 (6%)	4/193 (2%)	NAPEPLD,GNAS,MAPK1,FLT1,IRAK3,BRAF,TRAF6,MTOR, RHOB,RND3,RHOU,GNA13,NFKBIB,FNBP1,ATM

Identification of a diagnostic signature for EAD

In addition to our characterization of the biology of EAD, we wished to identify an expression signature with a high predictive power to distinguish EAD and non-EAD samples, as an early diagnostic tool. A logical way to discover these biomarkers is a direct comparison between the EAD and non-EAD samples. However, class comparison of the EAD COLD vs. non-EAD COLD samples (F-test; p<0.005) revealed only 213 differentially expressed genes with FDRs >40%. We obtained a similar result for the POST samples (F-test; p<0.005; FDR >63%). Therefore, we performed class comparisons between the POST and COLD samples in the EAD and the non-EAD groups individually and then compared them using a Venn diagram.

In the training cohort, the class comparison analysis of the 14 pairs of EAD POST versus EAD COLD samples yielded 915 differentially expressed probesets (p<0.005). All 915 probesets were then used to perform a class prediction on the test cohort (15 pairs) using DLDA. This approach trimmed down the number of classifiers to 503 probesets with 100% predictive power to distinguish EAD POST versus EAD COLD in the test set. These 503 probesets were therefore identified as 'high-predictive-value candidate classifiers' from two independent cohorts of EAD samples.

A similar analysis of the non-EAD samples yielded 542 differentially expressed probesets from the training cohort and subsequently 241 'high-predictive-value candidate classifiers' to distinguish non-EAD POST versus non-EAD COLD after class prediction on the test cohort.

The 503 EAD POST vs. COLD classifiers and the 241 non-EAD POST vs. COLD classifiers were compared to eliminate probesets from the EAD 'high-predictive-value candidate classifiers' that also had some degree of predictive value for non-EAD samples. This analy-

sis identified 330 “potentially predictive” probesets unique to EAD (Figure 4). To test if these probesets were indeed predictive of EAD POST vs. COLD, we tested their predictive accuracy using DLDA in all the EAD POST versus COLD samples, which showed 97% predictive accuracy. A class prediction with these 330 EAD-specific classifiers on the non-EAD POST vs. COLD samples revealed that there was a subset of 205 probesets that could also predict the non-EAD POST vs. COLD samples with predictive values of >90%. This was possibly due to the overestimation based on a Venn analysis and/or due to the high expression levels of most of these probesets in the non-EAD samples, leading to some degree of significant differential expression between the non-EAD POST and COLD samples, albeit at lower p-values than in the EAD samples.

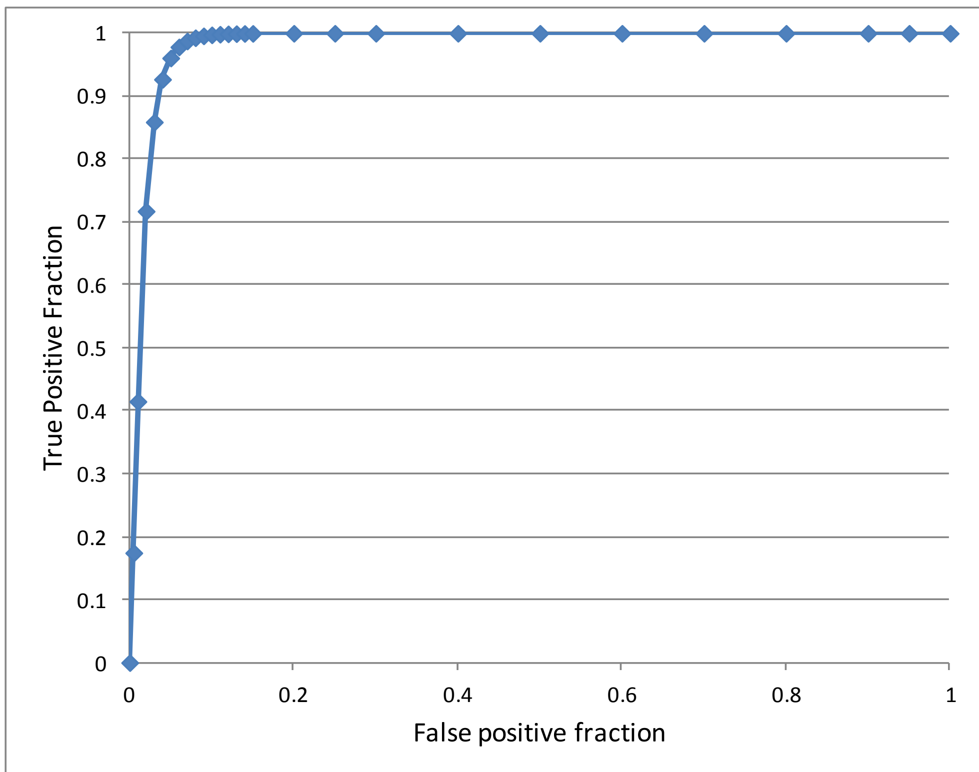


Figure 5. ROC curve of the 152 EAD-specific diagnostic probesets

Receiver Operating Characteristics (ROC) curve of the 152 EAD-specific diagnostic probesets that predict EAD POST versus COLD with a 97% predictive accuracy and an Area Under the Curve (AUC) of 0.981. EAD early allograft dysfunction.

To eliminate this potential problem of false positive results, we analyzed the potentially predictive classifiers with a sliding scale of increasing statistical p-value stringencies. At a p-value $<10^{-6}$, 152 probesets were identified that predicted all EAD POST vs. COLD

with 97% predictive accuracy (Area Under the Curve (AUC) 0.981), but failed at predicting all non-EAD POST vs. COLD samples (Figure 5). Figure 6 shows the heatmaps of the 152 classifiers in both the EAD and non-EAD samples. These 152 predictive probesets represented 119 genes (111 genes upregulated; 8 downregulated; Supplemental Table 4). Among the highest upregulated genes in the EAD POST samples were FOS (known to be involved in cell proliferation and differentiation, but also in apoptotic cell death), SERPINE1 (an inhibitor of fibrinolysis and in high concentrations associated with thrombophilia) and DUSP5 (known to negatively regulate members of the mitogen-activated protein (MAP) kinase superfamily, which in turn are associated with cellular proliferation and differentiation). Thus, a core set of classifiers was identified, that could be used as an early predictive tool to detect patients prone to develop EAD after deceased donor liver transplantation.

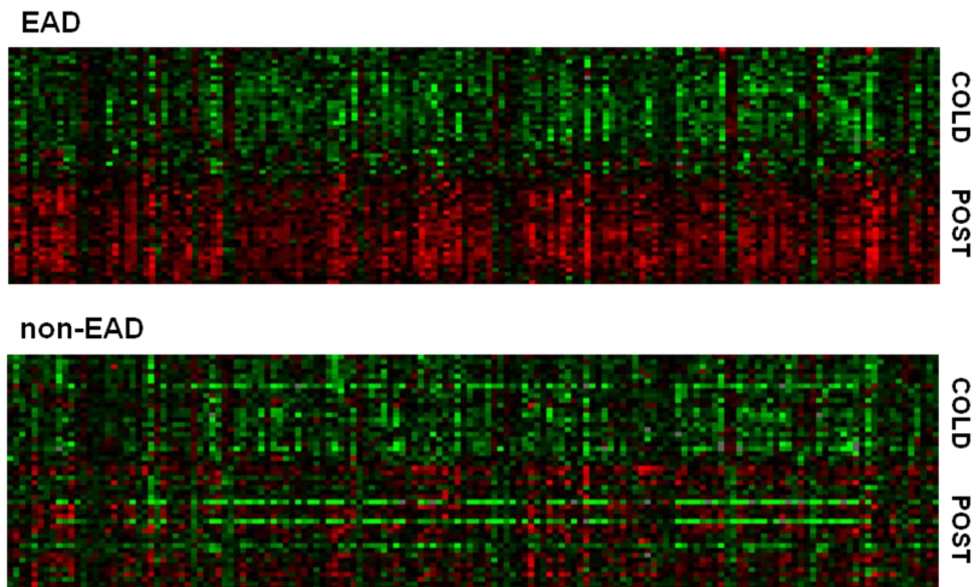


Figure 6. EAD and non-EAD heatmaps of the 152 EAD-specific diagnostic probesets

Heatmaps of the 152 EAD-specific diagnostic probesets in both the EAD and non-EAD POST and COLD biopsies, showing distinct differences in expression levels between both recipient groups. EAD early allograft dysfunction; COLD liver graft biopsies taken at the end of the cold storage period; POST liver graft biopsies taken approximately 1 hour after reperfusion.

Validation of biological pathways and diagnostic markers

To validate our findings for both the biology-related as well as the diagnostic gene expression signature for EAD, 15 EAD and 15 non-EAD samples from the original training and test cohorts as well as a third independent cohort were analyzed using Panomics multiplex assays. Twenty seven genes were chosen based on the biology of EAD, including five genes

from existing literature (i.e. ETS1, IL1RN1, IL6, NFKB1 and PPARA), and 15 genes were chosen based on their predictive value (Figure 7 and Supplemental Table 5).

Among the biology-related genes 82% of genes analyzed showed the same directionality in fold change index (gene expression ratio of EAD POST to EAD COLD samples) compared to microarray analyses. For the 5 genes chosen based on literature, multiplex expression levels revealed moderate fold changes (generally <2-fold up- or downregulation) for the EAD POST versus COLD comparison, with exception of NFKB1 (6.8-fold upregulation). As for the diagnostic genes, 93% of genes showed similar fold change indexes with the multiplex assays compared to the microarray findings. These multiplex data including a third independent test cohort of EAD and non-EAD patient samples supports the validity of our microarray findings.

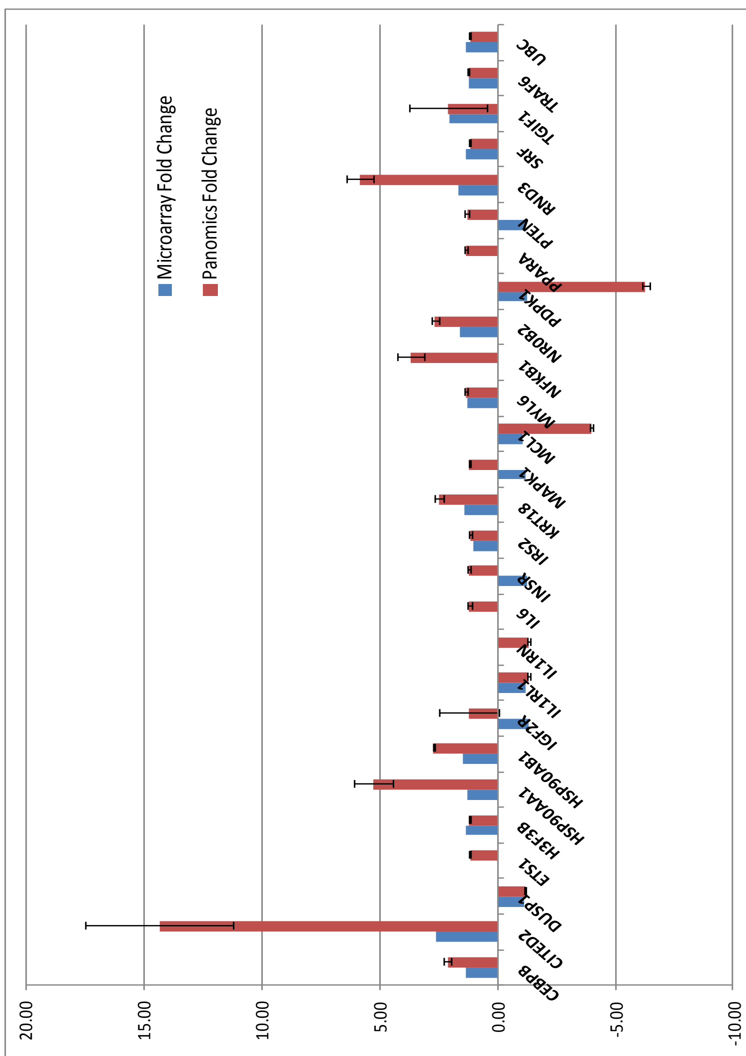


Figure 7A

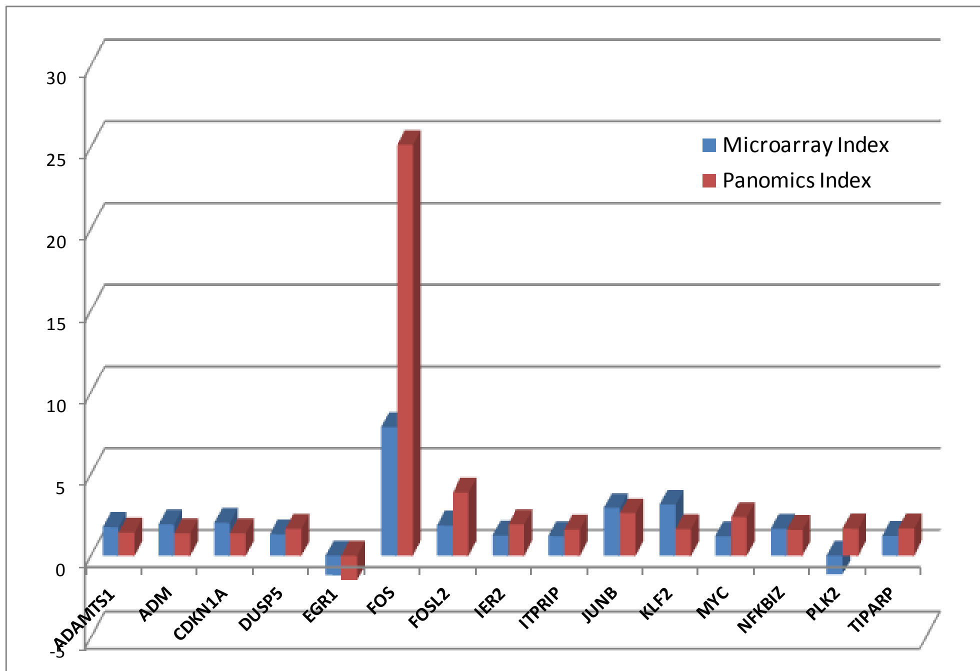


Figure 7B

Figure 7. Panomics validation of genes related to the biology and diagnosis of EAD

A. Validation of genes related to the biology of EAD, showing the overlap and differences in the fold change directionality of the tested genes; **B.** Validation of genes diagnostic for EAD, showing the overlap and differences in the fold change of the tested genes. EAD early allograft dysfunction.

DISCUSSION

Early allograft dysfunction (EAD) after liver transplantation is associated with increased incidence of graft failure and mortality, and can significantly increase morbidity. It is currently believed to be largely due to the effects of ischemia/reperfusion injury and graft quality. While the clinical picture of EAD has been defined, there have been very few publications that address the underlying biology of EAD. Moreover, the only study addressing the molecular aspects of EAD in humans is an earlier study using PCR techniques to analyze quantitative gene expression in post-perfusion biopsies after liver transplantation.[18] The authors identified five genes (CTGF, WWP2, CD274, VEGF and its receptor FLT1) showing significantly downregulated expression levels in the post-perfusion biopsies. To date there has not been any study investigating global gene expression profiles in EAD using high-throughput assays such as DNA microarrays. We have recently shown, using Luminex protein assays on 25 cytokines, chemokines and immunoreceptors, that upregulation of the proteins of the NF-κB pathway and higher serum levels of chemokines and cytokines associated with T-cell immunity, including MIG (CXCL9), IP-10 (CXCL10) and

IL-2R, were correlated with EAD in the first month after transplantation.[11] High-throughput global gene expression profiling analyses studies, however, will contribute to our knowledge of the biology of EAD and thereby provide new intervention strategies to prevent graft loss and/or patient morbidity and mortality.

In our study the specific "clinical" phenotype of EAD was correlated with the "molecular" signature or gene expression pattern found in the liver graft immediately following reperfusion. The primary aim of this study was to investigate EAD-specific molecular pathways and gene networks to obtain insight into the biology of EAD. In addition, we hoped to identify a set of genes whose expression changes would be diagnostic for the development of EAD at an early stage.

Our microarray findings revealed several genes and related pathways showing significantly different expression after reperfusion when comparing EAD patients to non-EAD patients, suggesting specific genes and pathways characteristic of, and possibly causative of EAD. These genes fall into 3 general functional categories: metabolism, inflammation, and cellular proliferation.

The top candidate pathway in this analysis is the PPARα signaling pathway, known to be a key regulator of hepatic lipid metabolism[19] and whose deficiency in rodents leads to accumulation of triacylglycerols.[20] PPARα deficiency impairs hepatic glycogen repletion, despite normal insulin and glucose levels, showing that alterations in PPARα levels can severely impair lipid metabolism. In our microarray analysis the PPARα gene *per se* was not significantly differentially expressed between the EAD COLD and POST samples, but all PPARα probesets showed decreased expression levels in the EAD POST samples, suggesting a downward shift in the metabolic machinery of livers developing EAD.

Furthermore, in the PPARα signaling pathway, IL-1, its receptor IL-1R, TRAF6 and TNF were upregulated in the EAD POST biopsies. TRAF6 is an adaptor protein of the tumor necrosis factor receptor-associated factor family and modulates both tumor necrosis factor receptor (TNFR) and interleukin-1 receptor/Toll-like receptor (IL-1R/TLR) signaling. A rodent study by Liu et al., using renal proximal tubule cells in which TRAF6 was silenced by siRNA, showed inhibition of inflammatory responses and increased cell survival upon LPS challenge.[21] TRAF6, via its activation of NFκB, also plays an important role in antiviral immune responses and the production of IFNγ.[22]

NFκB signaling, another significantly expressed signaling pathway in the EAD POST biopsies, primarily signals through the IκB molecule and its interactions with TRAF6 and IL-1. This NFκB-IκB system and its tandem functionality is known to be involved in the control of inflammatory responses.[23, 24] The IκB family of genes (IKBA, IKBB and IKB) regulates the function of NFκB by trapping it in inactive cytoplasmic complexes, hence serving as a feedback mechanism to prevent excess inflammatory responses by NFκB.[25, 26]

Other key inflammation-associated molecules significantly differentially regulated between EAD POST and COLD biopsies are the heat shock proteins (HSP), including

HSP90AA1, HSP90AB1, HSPA14, HSPB8HSPA1L and HSPB11. The HSP family is associated with inflammatory responses, and the inhibition of the HSP90 family members can attenuate inflammation and prolong survival.[27-29] The HSP90 inhibitor 17-dimethylamino-ethylamino-17-demethoxygeldanamycin (17-DMAG), which binds to and thereby inactivates HSP90, was shown to resolve atherosclerotic plaques in mice.[30] Mice treated with 17-DMAG showed an increased expression of HSP70 and decreased expression of STAT and NFκB, which are all markers of reduced inflammatory responses. Similar results were observed in an *in vitro* model using bovine pulmonary arterial endothelial cells as well as in a murine model of lung injury and sepsis.[31-32]

Another marker was TNFRSF1B, one of the TNF receptors that play a major role in apoptosis. The production of this receptor in transgenic mice, at levels relevant to human disease, results in a severe inflammatory syndrome mainly involving pancreas, liver, kidney and lung.[33] Importantly, it was shown that this process was independent of the presence of TNF, lymphotoxin alpha or other TNF receptors.

Similarly CXCL1, a potent inflammatory molecule, was highly significantly upregulated in the EAD POST biopsies. It has been shown in a mouse model of Fas ligation with an anti-Fas antibody, that CXCL1 and MIP2 were strongly upregulated in the absence of Fas.[34] In our analysis, upregulation of CXCL1 was also found in the absence of differential expression of FAS. A list of these and other liver injury, inflammation and dysfunction related markers are given in Supplementary Table 2.

Molecules associated with liver regeneration and cell survival, such as beta-catenin, are also upregulated.[35] In a recent study of 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) induced hepatobiliary injury, beta-catenin conditional knockout mice showed amelioration of injury after 5 months, which coincided with extensive repopulation of beta-catenin negative livers with beta-catenin positive hepatocytes and was preceded by the appearance of beta-catenin positive hepatocyte clusters, suggesting growth and survival advantages due to the repopulation.[36]

The above-mentioned results, showing the downregulation of metabolic capabilities and the upregulation of pro-inflammatory molecules, suggest that the early events triggered during the development of EAD are a balance between response to injury and subsequent decreased ability to perform metabolic functions. We hypothesize that at any given time the liver displays a delicate energy balance between recovery and metabolic need, which may be quickly restored to equilibrium in well-functioning livers. In the setting of EAD, however, over-activation of inflammatory processes might deviate the liver's energy away from metabolic processes. The liver may therefore not be able to fully accommodate the metabolic demands of the body, thereby leading to the symptoms of dysfunction characterized by high serum transaminases, persistent cholestasis and prolonged coagulopathy.

Interventions with the potential to reverse this shift of the energy balance might possibly prevent morbidity and/or mortality in EAD patients. Potential therapeutic strategies

could therefore include strategies that attenuate the activation of inflammatory pathways or stimulate metabolic processes. In Supplemental Table 3 current agents affecting specific molecules or pathways detected in our analysis are shown, thereby listing potential therapeutics for further investigation. Future research should focus on the top relevant pathways, such as the PPARα or NF-κB pathways, as these pathways seem to play a major role in the development of EAD.

Our second aim was to identify a diagnostic gene expression signature, enabling early detection of patients prone to develop EAD. Among the genes identified in this analysis were the zinc finger family protein KLF2, which is known to be a regulator of T-cell migration to the peripheral blood circulation. Carlson et al. showed, using fetal liver chimeric cells, that KLF2-deficient (Klf2-/-) thymocytes display impaired expression of several receptors required for thymocyte emigration and peripheral trafficking.[37] In our data, the upregulation of this molecule could mean that the liver might be signaling for the trafficking of T-cells from the periphery to counter the injury it has undergone.

Another highly upregulated gene related to the diagnostic signature in EAD POST biopsies was TIPARP, which has been shown to be a mediator of the suppression of hepatic gluconeogenesis.[38] This fits our hypothesis that the metabolic function of EAD livers is shifted due to the early dysfunction after transplantation. Similarly, Adrenomedullin (ADM) and ADAMTS1 were upregulated in EAD POST biopsies. ADM is a marker of liver cirrhosis, both in plasma as well as in cirrhotic mouse livers.[39-42] ADAMTS1 has been identified as an inflammation-associated protein induced by IL-1.[43] In our data, IL-1B was also highly upregulated, supporting the hypothesis that this may be the reason for high levels of ADAMTS1 in the EAD livers.

One limitation of this study is that we did not find a diagnostic signature by simply comparing the gene expression profiles of EAD COLD versus non-EAD COLD biopsies. The diagnostic signature we developed differentiates between gene expression at the COLD and POST time points. Therefore, it is necessary to have biopsies from both these time points to successfully predict EAD, which will make this diagnostic signature more difficult to implement in clinical practice and prevent us from assessing the risk of EAD before the actual transplant event. Even though the diagnostic signature was validated using two cohorts, these cohorts were randomly chosen from the total pool of samples available to us in a single transplant center. Therefore, our diagnostic signature still needs to be validated in another independent cohort of samples collected from additional centers. The next level of validation successfully used a second technology (Quantigene) and included a third, independent set of samples. However, we acknowledge that this validation technique is also based on simple detection of mRNA transcripts. The next step would be to profile the proteins encoded by the differentially expressed genes we have identified, particularly those we propose are actually involved in the underlying biology of EAD. We recognize that the criteria for validating a diagnostic signature focus on demonstrating that the signature is sufficiently robust on the technology platform intended for measuring the

signature. In contrast, the rules for making claims about the biological significance of changing gene transcription require proof that these transcripts are represented in changing and post-translationally modified proteins, the functional units of the various signaling pathways we identified at the gene level. Until that work is done, we must depend on the existing literature defining the roles of these various pathways and make reasonable inferences to EAD.

In this study we describe the first report of global gene expression profiling of humans experiencing EAD in deceased donor liver allografts. We have uncovered several important candidate genes and signaling pathways that can be used as molecular predictors or biomarkers of EAD. These specific genes and pathways can be targeted for more in-depth study in experimental models of allograft dysfunction, and serve as a basis for interventional studies in human patients aimed at minimizing the incidence and impact of EAD after liver transplantation.

Acknowledgements

We would like to thank Terri Gelbart for technical support and Dr. Barbara Stranger and Bao-Li Chang for critical reading of the manuscript.

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Chapter 4

mTOR signaling in liver regeneration: rapamycin combined with growth factor treatment

Suomi M.G. Fouraschen[1], Petra E. de Ruiter[1], Jaap Kwekkeboom[2], Ron W.F. de Bruin[1], Geert Kazemier[1], Herold J. Metselaar[2], Hugo W. Tilanus[1], Luc J.W. van der Laan[1, #] and Jeroen de Jonge[1, #]

[1]Department of Surgery, Laboratory of Experimental Transplantation and Intestinal Surgery and [2]Department of Gastroenterology & Hepatology, Erasmus MC-University Medical Center, Rotterdam, The Netherlands; [#]Both authors contributed equally to this study

(World Journal of Transplantation, 2013 September 24;3(3):30-47)

ABSTRACT

Aim: To investigate the effects of mammalian target of rapamycin (mTOR) inhibition on liver regeneration and autophagy in a surgical resection model.

Methods: C57BL/6 mice were subjected to a 70% partial hepatectomy (PH) and treated intraperitoneally every 24 hours with a combination of the mTOR inhibitor rapamycin (2,5 mg/kg per day) and the steroid dexamethasone (2,0 mg/kg per day) in phosphate buffered saline (PBS) or with PBS alone as vehicle control. In the immunosuppressant group, part of the group was treated subcutaneously 4 hours prior to and 24 hours after PH with a combination of human recombinant interleukin 6 (IL-6; 500 μg/kg per day) and hepatocyte growth factor (HGF; 100 μg/kg per day) in PBS. Animals were sacrificed 2, 3 or 5 days after PH and liver tissue and blood were collected for further analysis. Immunohistochemical staining for 5-Bromo-2'-deoxyuridine (BrdU) was used to quantify hepatocyte proliferation. Western blotting was used to detect hepatic microtubule-associated protein 1 light chain 3 (LC3)-II protein expression as a marker for autophagy. Hepatic gene expression levels of proliferation-, inflammation- and angiogenesis-related genes were examined by real-time reverse transcription-polymerase chain reaction (RT-PCR) and serum bilirubin and transaminase levels were analyzed at the clinical chemical core facility of the Erasmus MC-University Medical Center.

Results: mTOR inhibition significantly suppressed regeneration, shown by decreased hepatocyte proliferation (2% vs. 12% BrdU positive hepatocyte nuclei at day 2, $P<0.01$; 0.8% vs. 1.4% at day 5, $P=0.02$) and liver weight reconstitution (63% vs. 76% of initial total liver weight at day 3, $P=0.04$), and furthermore increased serum transaminase levels (aspartate aminotransferase (AST) 641 U/l vs. 185 U/l at day 2, $P=0.02$). Expression of the autophagy marker LC3-II, which was reduced during normal liver regeneration, increased after mTOR inhibition (46% increase at day 2, $P=0.04$). Hepatic gene expression showed an increased inflammation-related response (tumor necrosis factor (TNF)-α 3.2-fold upregulation at day 2, $P=0.03$; interleukin 1 receptor antagonist (IL-1Ra) 6.0-fold upregulation at day 2 and 42.3-fold upregulation at day 5, $P<0.01$) and a reduced expression of cell cycle progression and angiogenesis-related factors (HGF 40% reduction at day 2; vascular endothelial growth factor receptor 2 (VEGF-R2) 50% reduction at day 2 and 5; angiopoietin (Ang)-1 60% reduction at day 2, all $P\leq0.01$). Treatment with the regeneration stimulating cytokine IL-6 and growth factor HGF could overcome the inhibitory effect on liver weight (75% of initial total liver weight at day 3, $P=0.02$ vs. immunosuppression alone and $P=0.90$ vs. controls) and partially reversed gene expression changes caused by rapamycin (TNF-α and IL-1Ra levels at day 2 were restored to control levels). However, no significant changes in hepatocyte proliferation, serum injury markers or autophagy were found.

Conclusion: mTOR inhibition severely impairs liver regeneration and increases autophagy after partial hepatectomy. These effects are partly reversed by stimulation of the IL-6 and HGF pathways.

INTRODUCTION

The liver has the remarkable ability to regenerate in order to compensate for lost or damaged liver tissue after injury and thereby restore liver function and maintain homeostasis. This process is ultimately required after living donor liver transplantation, in which a small-for-size graft is subjected to ischemia and reperfusion injury and transplanted into a recipient with urgent metabolic needs. In this situation, both loss of a substantial part of the initial liver mass as well as oxidative stress after reperfusion are central mechanisms of hepatic injury.[1, 2]

Liver resection triggers release of the cytokines tumor necrosis factor (TNF) and interleukin 6 (IL-6), crucial priming factors for the initiation of hepatocyte proliferation by activation of the janus activated kinases/signal transducer and activator of transcription (JAK/STAT) pathway.[3-5] This priming phase stimulates resting hepatocytes to enter the G1 phase of the cell cycle. Simultaneously, growth factors including hepatocyte growth factor (HGF), contribute to the passage of hepatocytes from the G1 into the S phase by activating the phosphoinositide-3 kinase(PI3K)/Akt signal transduction pathway.[6-8] PI3K/Akt interacts with the mammalian target of rapamycin (mTOR), involved in the control of protein synthesis, cell size and proliferation.[9, 10] Both cascades lead to activation of a variety of signaling pathways, including upregulation of several downstream cyclins like cyclin D1, which is associated with the G1-S phase transition of hepatocytes.[3, 4, 6, 11, 12]

Besides being a key regulator of cell growth and proliferation, mTOR was recently identified to play an important role in the control of autophagy.[13-15] Autophagy is an evolutionarily conserved lysosomal degradation pathway that plays an important protective role in case of cellular injury by mediating the elimination of damaged cellular components.[13] In non-hepatic cells, autophagy has not only been implicated as a survival response, but also as a mediator of cell death during stress conditions.[16, 17] Autophagy might therefore play a role in liver regeneration, though this has not been thoroughly studied. This is of special interest to the field of liver transplantation as mTOR inhibition, in combination with a short course of steroids, is an attractive alternative for current calcineurin inhibitor based immunosuppressive strategies. Calcineurin inhibitors are neurotoxic, associated with a 20% incidence of chronic kidney dysfunction and carry a cumulative risk for de novo malignancy of up to 55% at 15 years after liver transplantation.[18-22] mTOR inhibitors like rapamycin therefore represent an important immunosuppressive option, especially in patients with calcineurin inhibitor-induced neurotoxicity, poor renal function and possibly also in patients with hepatocellular carcinoma. However, in the initial phase after liver transplantation, the mTOR inhibitor rapamycin is rarely used, since it is reported to delay liver regeneration.[23-25]

Rapamycin inhibits mTOR complex 1 (mTORC1) by complex formation with FK506 binding protein 12 (FKBP12), thereby acting on its downstream messengers and abrogating translation initiation and protein synthesis, which results in cell cycle arrest at the G1 to S phase.[23-25] Cyclin D1 as well as p21 are shown to be important downstream messen-

gers of the rapamycin-mediated cell cycle arrest.[26-28] The exact underlying cellular and molecular mechanisms by which mTOR inhibition attenuates liver regeneration and the interplay between mTOR inhibition and autophagy in liver regeneration needs to be further characterized.

Both after kidney as well as deceased liver transplantation, mTOR inhibition in combination with steroids has proven an efficient immunosuppressive strategy. Addition of an mTOR inhibitor to steroid treatment might therefore also show beneficial effects after living donor liver transplantation, especially in patients with compromised renal function. Aim of this study is to investigate the effects of mTOR inhibition, in combination with the steroid dexamethasone, on liver regeneration and autophagy in a surgical resection model and in particular its involvement in IL-6 and HGF stimulated pathways. Besides mimicking the post-transplant treatment strategy, this combination of immunosuppressants also allowed more specific investigation of the effects of exogenous IL-6 and HGF, since steroids are multi-potent inhibitors of endogenous production of pro-inflammatory cytokines like TNF and IL-6.[29] Effects on body and liver weight, hepatocyte proliferation, autophagy and hepatic function and injury were analyzed at specific time points after surgery in a 70% partial hepatectomy (PH) model in mice.

MATERIALS AND METHODS

Animals

Male C57Bl/6 mice (age 12-15 weeks) were obtained from Charles River (Maastricht, Netherlands) and maintained in the animal facility on a 12/12 hour light/dark schedule. The animals had free access to food and drinking water and received care according to the Guide for the Care and Use of Laboratory Animals. All animal experiments were performed with approval of the institutional animal welfare committee.

Partial hepatectomy and treatments

Liver regeneration was induced in C57BL/6 mice by performing a 70% PH as first described by Higgins and Anderson in 1931. Animals were anaesthetized with isoflurane and, after a midline laparotomy, the left lateral and median lobes of the liver were ligated and resected. The peritoneum and skin were sutured separately. All procedures were performed under clean conditions. Animals were treated intraperitoneally every 24 hours, starting at time of PH, with a combination of the immunosuppressants rapamycin (2,5 mg/kg per day; sirolimus oral solution, Wyeth Pharmaceuticals, Louvain-la-Neuve, Belgium) and dexamethasone (2,0 mg/kg per day, Organon, Oss, Netherlands) in PBS (Lonza, Verviers, Belgium; total volume 0.5 ml) or with PBS alone as vehicle control. In the immunosuppressant (Rapa-Dex) group, part of the group was treated subcutaneously 4 hours prior to and 24 hours after PH with a combination of human recombinant IL-6 (500 μg/kg per day; Peprotech, Londen, UK) and HGF (100 μg/kg per day; Peprotech) in PBS. Animals (n=5-9 per group) were sacrificed 2, 3 or 5 days after PH and liver tissue and blood were

collected for further analysis. To investigate the effects of dexamethasone alone, an additional group was treated with dexamethasone alone (Dex) as described above and sacrificed at day 2 after PH.

Weight calculations

Animals were weighed daily prior to treatment and the resected liver mass was weighed after PH. The initial total liver weight was calculated as follows:

resected liver weight/70 x 100 (g)

At time of sacrifice, animals and their regenerated liver mass were weighed and the percentage of reconstitution of the liver was calculated by:

regenerated liver weight/initial total liver weight x 100 (%)

Immunohistochemistry

One hour prior to sacrifice, animals were injected intraperitoneally with 50 mg/kg BrdU (5-Bromo-2'-deoxyuridine; B5002, Sigma-Aldrich, Zwijndrecht, Netherlands). Livers were harvested and processed to 4 μm thick formalin fixed, paraffin embedded sections. Antigen retrieval was achieved by boiling the slides in 0.01 M sodium citrate; pH 6.0 (microwave 1000 Watt; 1x7 and 2x3 minutes). Endogenous peroxidase was blocked in 0.6% H_2O_2 in PBS for 30 minutes at room temperature, after which DNA was denaturated by incubation for 1 hour at 37°C in 0.1 M HCl in aqua dest. Aspecific binding was prevented by 0.5% milk powder supplemented with 0.15% glycin in PBS (blocking buffer). Slides were incubated overnight at 4°C with monoclonal mouse anti-BrdU (Bu20a; DakoCytomation, Glostrup, Denmark; 1:80 in blocking buffer). The next day slides were incubated for 30 minutes at room temperature with polyclonal rabbit anti-mouse IgG/HRP (P0161; DakoCytomation; 1:1000 in blocking buffer). After antibody incubation slides were incubated with DAB-solution and counterstained with hematoxylin. Per animal 4 high power fields (HPF; 400x) were analyzed for BrdU positive hepatocytes.

Real-time quantitative RT-PCR

At time of sacrifice, liver tissue was stored overnight at 4°C and thereafter at -80°C in Allprotect Tissue Reagent (Qiagen, Hilden, Germany) for RNA preservation. Total RNA was extracted using Trizol (Qiagen) and chloroform after mechanical disruption of the tissue. RNA was precipitated in 75% ethanol and dissolved in RNase-free water. RNA quantity and quality was analyzed using a NanoDrop ND-1000 (Thermo Fisher Scientific, Wilmington, DE, USA). One microgram of RNA was reverse-transcribed to cDNA using an iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA). PCR primers (Table 1) were synthesized by Isogen Life Science (Maarssen, Netherlands) and Biolegio (Nijmegen, Netherlands). Real-time quantitative RT-PCR was performed with a SensiMix SYBR & Fluorescein Kit (Bioline, London, United Kingdom) and MyIQ real time PCR detection system (Bio-Rad Laboratories) according to the manufacturer's instruction. Gene expression levels were

normalized using the ΔΔCT method and TATA Binding Protein (TBP) as reference gene, because it is shown to be stable during different phases of liver regeneration.[30]

Table 1. RT-PCR primer sequences

Gene	Name	Accession number	Primer (forward/reverse)
CCND1	Cyclin D1	NM_007631	GCGTACCCTGACACCAATCTC CTCCTCTTCGCACTTCTGCTC
PCNA	Proliferating cell nuclear antigen	NM_011045	CTTGGTACAGCTTACTCTGCG AGTTGCTCCACATCTAAGTCCAT
TNFA	Tumor necrosis factor alpha	NM_013693	CCCTCACACTCAGATCATCTTCT GCTACGACGTGGGCTACAG
IL1RN	Interleukin 1 receptor antagonist	NM_031167	GCTCATTGCTGGGTACTTACAA CCAGACTTGGCACAAGACAGG
IL6	Interleukin 6	NM_031168	TAGTCCTTCCTACCCCAATTTCC TTGGTCCTTAGCCACTCCTTC
HGF	Hepatocyte growth factor	NM_010427	ATGTGGGGGACCAAACTTCTG GGATGGCGACATGAAGCAG
TGFB	Transforming growth factor beta	NM_011577	CTCCCGTGGCTTCTAGTGC GCCTTAGTTTGGACAGGATCTG
KDR	Vascular endothelial growth factor receptor 2	NM_010612	TTTGGCAAATACAACCCTTCAGA GCAGAAGATACTGTCACCACC
ANGPT1	Angiopoietin 1	NM_009640	CACATAGGGTGCAGCAACCA CGTCGTGTTCTGGAAGAATGA
VEGFA	Vascular endothelial growth factor A	NM_009505	GCACATAGAGAGAATGAGCTTCC CTCCGCTCTGAACAAGGCT
FLT1	Vascular endothelial growth factor receptor 1	NM_010228	TGGCTCTACGACCTTAGACTG CAGGTTTGACTTGTCTGAGGTT
TBP	TATA binding protein	NM_013684	AGAACAATCCAGACTAGCAGCA GGGAACTTCACATCACAGCTC

Western Blotting

Liver tissue, preserved in Allprotect as described, was assessed for autophagy by investigating hepatic protein levels of microtubule-associated protein 1 light chain 3 (LC3)-II. Liver samples were homogenized in 2x Laemmli sample buffer (120 mM Tris-HCl pH 6.8, 4% SDS, 20% glycerol, 0.01% bromophenol blue and 0.1 M DTT) and heated at 95°C for 3 minutes. Proteins were separated on 15% SDS-PAGE gels and blotted on Immobilon-FL Transfer Membranes (Millipore, Billerica, USA). Blots were blocked in Odyssey blocking buffer (LI-COR Biosciences, Lincoln, USA) for 1 hour at room temperature and probed overnight at 4°C with rabbit polyclonal LC3A/B antibody (1:1000, Cell Signaling Technology, Danvers, USA) and mouse purified IgG C4/actin antibody (1:2500, BD Biosciences, Franklin Lakes, USA). Incubation with secondary antibodies (Goat-anti-mouse IgG IRDye 680 and Goat-anti-rabbit IgG IRDye 800CW, both 1:5000; LI-COR Biosciences) was performed for 1 hour at room temperature. Blots were scanned using an Odyssey Infrared Imager (LI-COR Biosciences) and the results were analyzed using Odyssey software.

Serum analysis of enzyme levels

Blood samples were collected at time of sacrifice in heparin coated microtubes. After collection, samples were centrifuged (19 minutes, 1800 rpm) to separate the serum, which

was further analyzed at the clinical chemical core facility of the Erasmus MC-University Medical Center to determine bilirubin, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels.

Statistical analysis

Data are presented as mean ± SEM. Statistical analysis was performed using the Mann–Whitney test or student t-test after checking for normal distribution. A p-value ≤0.05 was considered statistically significant.

RESULTS

Inhibition of mTOR causes progressive body weight loss after liver resection

As shown in Figure 1A, significant and progressive body weight loss was seen after PH in animals treated with Rapa-Dex compared to control treated animals (15% vs. 6% loss, P<0.01 at day 2; 11% vs. 2%, P=0.04 at day 3 and 25% vs. 7%, P<0.01 at day 5). No significant body weight loss was seen in animals treated with Dex alone (9% loss, P=0.11 at day 2; data not shown). Combined treatment with Rapa-Dex and IL-6/HGF could not overcome the progressive weight loss and showed a similar effect on body weight (14% loss, P<0.01 at day 2; 14%, P=0.06 at day 3 and 24%, P<0.01 at day 5).

Reduced liver mass reconstitution by mTOR inhibition can be overcome with exogenous IL-6 and HGF

After 70% PH in the control group, liver mass recovered to 54% of the initial total liver weight by day 2 and to 76% by day 3 (Figure 1B). Treatment with Rapa-Dex caused a significant inhibition in the reconstitution of liver mass at day 3 compared to control treatment (63% of initial total liver weight, P=0.04). A similar trend was seen at day 5, but differences did not reach statistical significance. Treatment with Dex alone did not show significant differences compared to controls (57% of initial total liver weight at day 2, P=0.30; data not shown). Combination of IL-6/HGF with Rapa-Dex completely restored liver reconstitution to control levels (75% of initial total liver weight at day 3, P=0.02 vs. Rapa-Dex and P=0.90 vs. controls).

IL-6 and HGF treatment upregulates cell cycle progression-related gene expression of cyclin D1 and PCNA, but does not restore mTOR-induced inhibition of hepatocyte proliferation

Hepatocyte proliferation, quantified by the percentage of BrdU positive hepatocyte nuclei, was significantly reduced at day 2 after PH in animals treated with Rapa-Dex compared to control treated animals (2% vs. 12%, P<0.01; Figure 2A, B). mTOR inhibition delayed hepatocyte proliferation at least until day 5 (0.8% vs. 1.4%, P=0.02). In contrast, treatment with Dex alone had no significant effect on proliferation at day 2. Addition of exogenous IL-6/HGF to Rapa-Dex treatment did not significantly stimulate hepatocyte pro-

liferation at any time point after PH, although no significant difference compared to control treatment was seen at day 3 and 5. Combined treatment of Rapa-Dex with IL-6/HGF did, however, cause a decrease in the number of hepatocytes per HPF compared to treatment with Rapa-Dex alone (170 vs. 206 cells/HPF, P=0.05; data not shown), suggesting an increase in cell size.

The inhibitory effect of mTOR inhibition on cell proliferation was also reflected in the hepatic gene expression levels of cyclin D1 and proliferating cell nuclear antigen (PCNA), known to be relevant for cell cycle progression and DNA synthesis. Compared to control treatment, Rapa-Dex treatment significantly downregulated expression of cyclin D1 (80% reduction, P<0.01; Figure 2C) and PCNA (90% reduction, P<0.01; Figure 2D) at day 2 after PH. Downregulation of cyclin D1 and PCNA gene expression after Rapa-Dex treatment continued at least until day 5 (80% and 30% reduction respectively, P<0.01). Addition of IL-6/HGF to Rapa-Dex treatment significantly upregulated both cyclin D1 (2.6-fold, P=0.04 at day 2 and 1.4-fold, P=0.03 at day 5) and PCNA (1.3-fold, P=0.03 at day 2) gene expression after PH compared to treatment with Rapa-Dex alone, but did not restore expression to control levels.

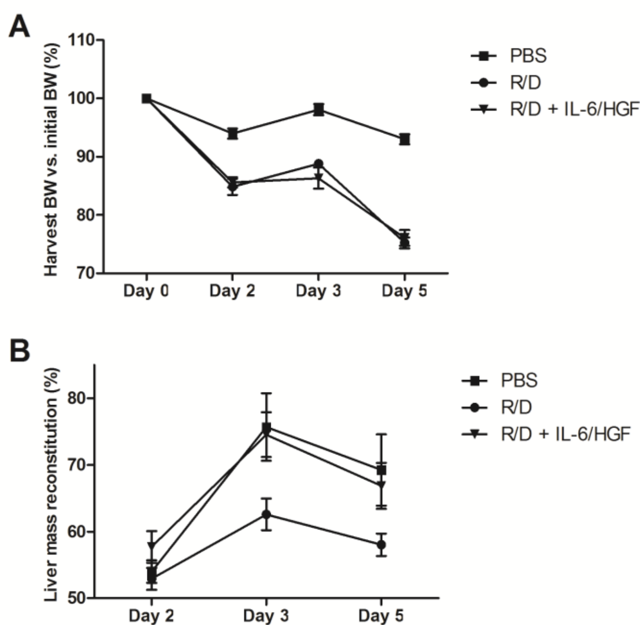

Figure 1. Effects of mTOR inhibition on body and liver weight

A. Harvest body weight at day 2, 3 and 5 after PH versus initial body weight; **B.** Harvest liver weight at day 2, 3 and 5 after PH versus total liver weight prior to PH. Data are shown as mean ± SEM. BW body weight; LW liver weight; R/D Rapa-Dex.

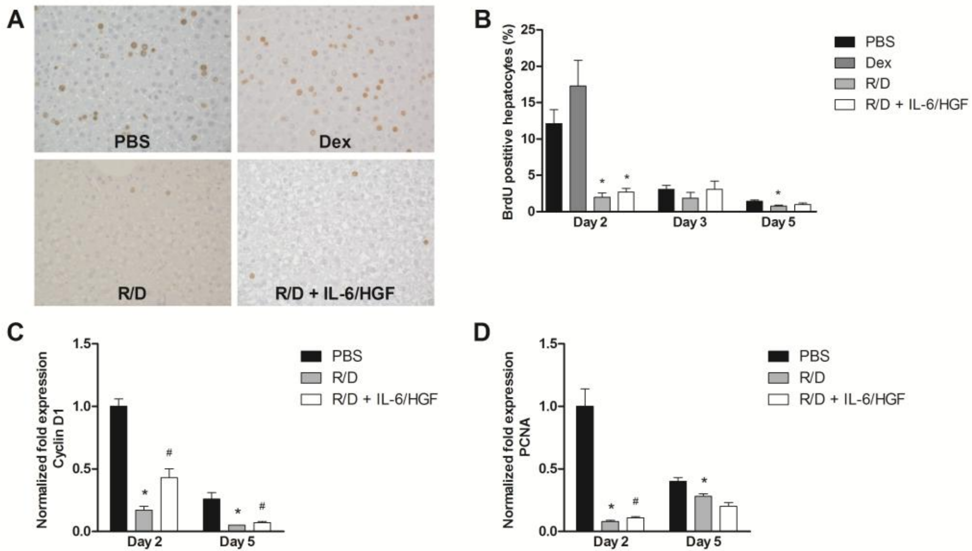

Figure 2. Effects of mTOR inhibition on hepatocyte proliferation

(A-B) Livers were processed for immunohistochemistry on BrdU to quantify hepatocyte proliferation; **A.** Representative pictures of hepatocyte proliferation at day 2 after PH; **B.** Quantification of hepatocyte proliferation at day 2, 3 and 5 after PH; **(C-D)** Hepatic gene expression levels of cyclin D1 and PCNA were determined by quantitative RT-PCR and normalized against TBP; **C.** Expression levels of cyclin D1 at day 2 and 5 after PH; **D.** Expression levels of PCNA at day 2 and 5 after PH. Data are shown as mean ± SEM; *p≤0.05 versus PBS; #p≤0.05 versus Rapa-Dex. R/D Rapa-Dex; BrdU 5-Bromo-2’-deoxyuridine; PCNA proliferating cell nuclear antigen.

Inhibition of mTOR increases autophagy and hepatocyte injury during liver regeneration

During autophagy, the cytosolic form of LC3 (LC3-I) is conjugated to phosphatidylethanolamine to form LC3-phosphatidylethanolamine conjugate (LC3-II), which is recruited to autophagosomal membranes and therefore a quantitative marker for autophagy. As shown in Figure 3A, LC3-II protein levels in control animals were significantly reduced at day 2 after PH compared to levels before resection (48% reduction, P=0.05). This finding suggests that baseline autophagy levels are reduced during liver regeneration. Compared to control treated animals, animals treated with Rapa-Dex showed a significantly higher LC3-II protein expression at day 2 (46% increase, P=0.04; Figure 3B, C). At day 5, LC3-II levels were back at pre-resection levels in control treated animals, but appeared further increased in Rapa-Dex treated animals. Treatment with Dex alone did not cause significant differences in hepatic LC3-II levels at day 2 (data not shown). Addition of exogenous IL-6/HGF to Rapa-Dex treatment had no significant effect on autophagy compared to Rapa-Dex alone, as LC3-II protein levels remained significantly elevated.

As shown in Figure 4A-C, treatment with Rapa-Dex furthermore significantly increased serum AST levels at day 2 (641 U/l vs. 185 U/l, P=0.02) and caused a non-significant increase in ALT and bilirubin levels, compared to control treatment. Treatment with Dex

alone did not cause changes in serum levels of these liver injury markers. Combined treatment with Rapa-Dex and IL-6/HGF significantly elevated levels of AST (1387 U/l, P<0.01), ALT (823 U/l vs. 67 U/L, P<0.01) as well as bilirubin (39 μmol/l vs. 18 μmol/l, P=0.04). In accordance with serum levels of these injury markers, treatment with Rapa-Dex, either with or without IL-6/HGF, caused progressive changes in liver histology with formation of necrotic areas (Figure 4D).

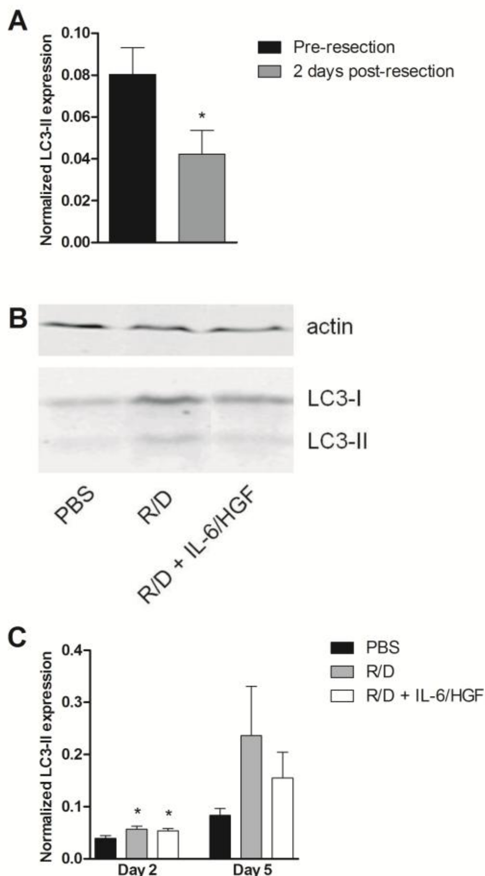

Figure 3. Effects of partial hepatectomy and mTOR inhibition on hepatic autophagy

Hepatic protein levels of the autophagy marker LC3-II were determined by Western blot analysis and normalized against actin; **A.** Effects of liver resection on autophagy at day 2 after PH; **B.** Western blot showing effects of mTOR inhibition on autophagy at day 2 after PH; **C.** Quantification of autophagy at day 2 and 5 after PH. Data are shown as mean ± SEM; *p≤0.05 versus PBS. LC3 microtubule associated protein 1 light chain 3; R/D Rapa-Dex.

mTOR inhibition alters expression of genes relevant for cell proliferation and inflammation

At day 2 after PH, treatment with Rapa-Dex significantly upregulated hepatic gene expression of the pro-inflammatory cytokine TNF-α (3.2-fold, P=0.03; Figure 5A) and the anti-inflammatory cytokine IL-1Ra (interleukin 1 receptor antagonist; 6.0-fold, P<0.01; Figure 5B) compared to control treatment. No significant effects were seen for IL-6 gene expression (Figure 5C). In contrast, gene expression of HGF was significantly downregulated (40% reduction, P<0.01; Figure 5D), whereas the observed reduced expression of TGF-β (transforming growth factor beta) was not statistically significant (Figure

5E). Addition of IL-6/HGF to Rapa-Dex treatment restored the upregulated expression of TNF-α and IL-1Ra to control levels. Combined treatment did however not reverse the downregulated expression of HGF or TGF-β. At day 5, treatment with Rapa-Dex led to progressive upregulation of IL-1Ra gene expression (42.3-fold, P<0.01) as well as upregulation of HGF gene expression (1.7-fold, P=0.03) compared to control treatment. Addition of IL-6/HGF to Rapa-Dex could not restore IL-1Ra and HGF gene expression at this time point.

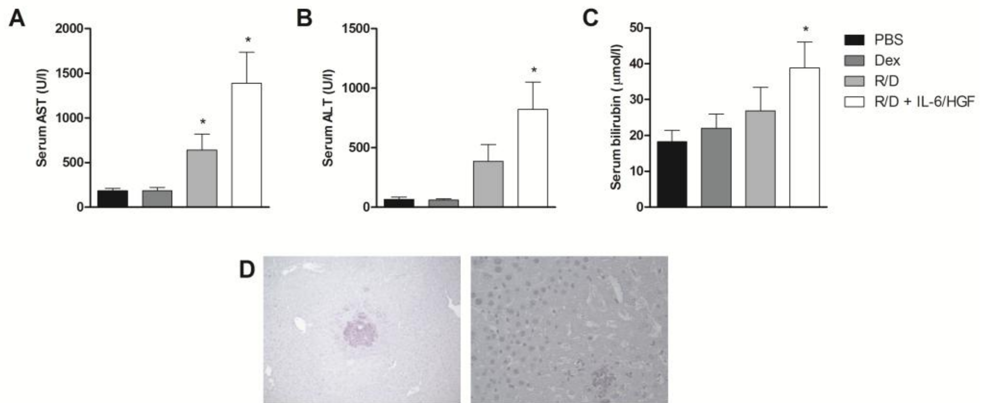

Figure 4. Effects of mTOR inhibition on hepatocyte injury

Serum levels at day 2 after PH for **A.** AST, **B.** ALT and **C.** bilirubin; **D.** Histologic changes at day 5 after PH in liver tissue from Rapa-Dex treated animals. Data are shown as mean ± SEM; *p≤0.05 versus PBS. AST aspartate aminotransferase; ALT alanine aminotransferase; R/D Rapa-Dex.

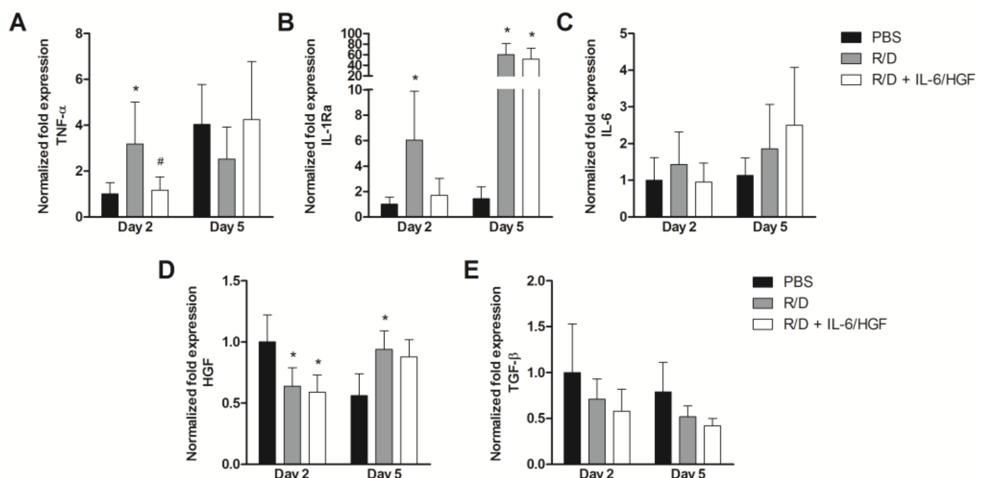

Figure 5. Effects of mTOR inhibition on inflammation and cell cycle related gene expression

Hepatic gene expression levels were determined by quantitative RT-PCR and normalized against TBP; **A.** Expression levels of TNF-α at day 2 and 5 after PH; **B.** Expression levels of IL-1Ra at day 2 and 5 after PH; **C.** Expression levels of IL-6 at day 2 and 5 after PH; **D.** Expression levels of HGF at day 2 and 5 after PH; **E.** Expression levels of TGF-β at day 2 and 5 after PH. Data are shown as mean ± SEM; *p≤0.05 versus PBS; #p≤0.05 versus Rapa-Dex. TNF-α tumor necrosis factor alpha; IL-1Ra interleukin 1 receptor antagonist; IL-6 interleukin 6; HGF hepatocyte growth factor; TGF-β transforming growth factor-beta; R/D Rapa-Dex.

As shown in Figure 6, treatment with Rapa-Dex significantly downregulated hepatic gene expression levels of VEGF-R2 (vascular endothelial growth factor receptor 2; 50% reduction, P=0.01) and Ang-1 (angiopoietin 1; 60% reduction, P<0.01) at day 2 after PH compared to control treatment. Downregulation of VEGF-R2 expression continued at least until day 5 (50% reduction, P<0.01). Addition of IL-6/HGF to Rapa-Dex treatment did not affect the downregulated expression levels of VEGF-R2 or Ang-1. Gene expression levels of VEGF-A and VEGF-R1 were not significantly reduced after Rapa-Dex treatment.

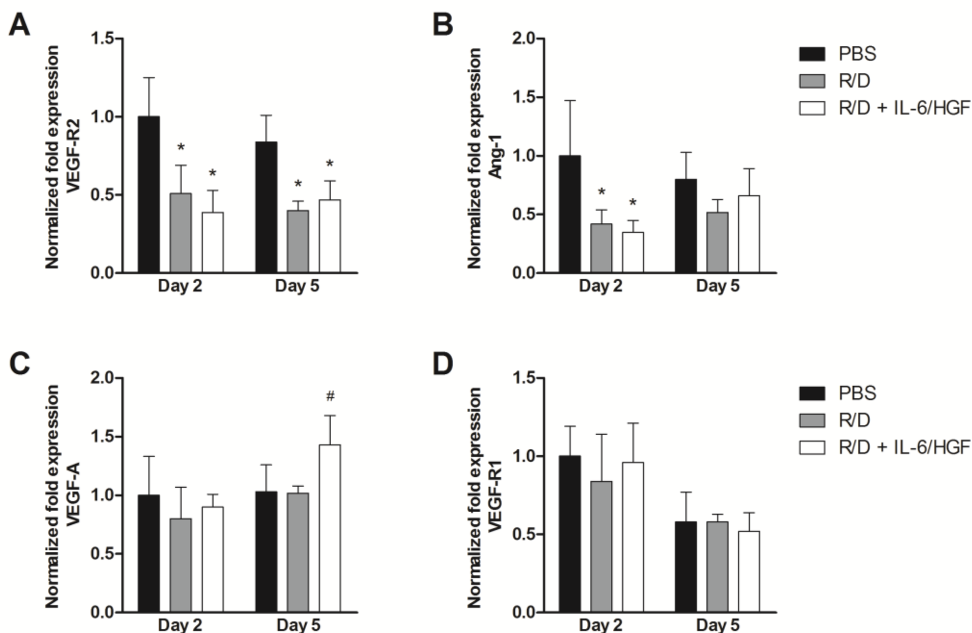

Figure 6. Effects of mTOR inhibition on angiogenic gene expression

Hepatic gene expression levels were determined by quantitative RT-PCR and normalized against TBP; **A.** Expression levels of VEGF-R2 at day 2 and 5 after PH; **B.** Expression levels of Ang-1 at day 2 and 5 after PH; **C.** Expression levels of VEGF-A at day 2 and 5 after PH; **D.** Expression levels of VEGF-R1 at day 2 and 5 after PH. Data are shown as mean ± SEM; *p≤0.05 versus PBS; $^{\#}$p≤0.05 versus Rapa-Dex. VEGF-R2 vascular endothelial growth factor receptor 2; Ang-1 angiopoietin 1; VEGF-A vascular endothelial growth factor A; VEGF-R1 vascular endothelial growth factor receptor 1; R/D Rapa-Dex.

DISCUSSION

Current immunosuppressive strategies in the first period after liver transplantation mostly involve treatment with steroids in combination with mycophenolic acid, interleukin 2 receptor antagonists or calcineurin inhibitors.[31] These regimes are however associated with chronic renal failure, with an incidence of up to 20% kidney dysfunction over time.[18] The mTOR inhibitor and immunosuppressant rapamycin, in contrast to the calcineurin

inhibitors tacrolimus and cyclosporin, does not cause nephrotoxicity and is suggested to be a good alternative in transplant patients with deteriorating renal function.[32-34]

Recently, mTOR inhibition has gained wide interest in the treatment of cancer.[35, 36] Therefore, also in patients transplanted for hepatocellular carcinoma, mTOR inhibitors are an attractive alternative with reported inhibitory effects on tumor growth and recurrence.[37-40] However, mTOR is a key regulator of cell growth and proliferation and its inhibition is reported to have detrimental effects on liver regeneration.[23-25] There may however be a more intricate relation as mTOR also regulates metabolism and inhibition of mTOR may preserve energy supplies for the remaining hepatocytes after liver resection to keep up metabolic function. This is supported by a recent publication showing excellent results in patients treated de novo with rapamycin after living donor liver transplantation as well as data from animal experiments showing no increase in mortality with rapamycin treatment, even after a 90% liver resection and despite inhibited hepatocyte proliferation.[41, 42]

Additionally, mTOR has been implicated to be of paramount importance in the control of autophagy, a general term for pathways in which cytoplasmic material, including soluble macromolecules and organelles, are delivered to lysosomes for degradation.[13, 43-45] Autophagy is thought to have evolved as a stress response mechanism that allows organisms to survive during harsh conditions, probably by regulating energy homeostasis.[16] Early histomorphologic studies showed a decrease in autophagic bodies of up to 98% at day 1 after partial hepatectomy.[46-48] This can support the hypothesis that the inhibition of intracellular autophagic degradation in regenerating liver has its biochemical equivalent, i.e. inhibited protein catabolism, and is interpreted as an important and adequate mechanism to shift from the physiological steady state to compensatory growth of the liver after partial hepatectomy. Degli Esposti et al. showed the presence of autophagy in 21% of good functioning human liver grafts 2 hours after reperfusion, without differences between normal and steatotic livers.[49] Ischemic preconditioning in this study increased autophagy only in steatotic livers, which appeared to have a protective effect on post-operative function. Wang et al. showed that autophagy is essential for hepatocyte resistance to oxidant stress and that loss of macroautophagy led to overactivation of the c-Jun N-terminal kinase signaling pathway that induced cell death.[50] Therefore we studied the interplay between liver regeneration, mTOR inhibition and autophagy in a transplant-related 70% partial hepatectomy model. In accordance with the findings of others, we found a significant decrease in proliferating hepatocytes from 12% to 2% after mTOR inhibition, with concomitant decreases in hepatic gene expression of the cell cycle genes cyclin D1 and PCNA.[25, 42, 51] This was furthermore accompanied by increased serum transaminases, suggesting increased liver injury.

Rupertus et al. recently described that rapamycin had no detrimental effects on liver regeneration, yet in their study hepatocyte proliferation was not actually measured, but only estimated from wet liver weight at 12 days after hepatectomy.[40] In our experiment, wet liver weight after mTOR inhibition was still lower at day 5 after liver resection. In the

study of Dahmen et al. BrdU incorporation decreased from 17% to less than 1% at 2 days after 90% hepatectomy, without effects on survival.[42] In the study of Palmes et al. the same effects were found, with decreased gene expression levels of TNF-α, HGF and TGF-β at day 2 after a 70% liver resection.[25] Interestingly, in our series, we found a significant upregulation of TNF-α, downregulation of HGF, but no significant changes in IL-6 and TGF-β gene expression.

Similar to the Palmes study, gene expression of the angiogenic factors VEGF-R2 and Ang-1 was downregulated in our experiments. Inhibition of angiogenesis is suggested to be one of the most relevant mechanisms by which tumor growth and recurrence is inhibited.[39, 40]

In our study, mTOR inhibition furthermore resulted in a profound upregulation of IL-1Ra gene expression, which was not reported before. IL-1Ra is an anti-inflammatory cytokine, reported to be released in response to both surgical as well as toxic liver injury and to have a protective effect after CCl4-induced toxic liver injury.[52-54]

We investigated whether the inhibition in hepatocyte proliferation could be overcome by kick-starting the priming phase of liver regeneration by pre-resection administration of IL-6 and HGF, both described to stimulate liver regeneration, especially in combined treatment.[55-57] It appeared that treatment with exogenous IL-6 and HGF partly reversed the negative effects of rapamycin by restoring TNF-α and IL-1Ra gene expression to control levels, significantly increasing gene expression of Cyclin D1 and PCNA and normalizing liver weight reconstitution. However, no significant increase in hepatocyte proliferation was found and serum transaminases were even further elevated, suggesting increased hepatocyte damage. This is in line with the findings of Haga et al., who found in their model of LPdk1KO mice that the PI3K/PDK1/Akt/mTOR pathway was regulated independent of the IL-6/JAK/STAT3 pathway.[9] An alternative explanation for the increase in liver weight could be cellular hypertrophy cq. edema, which is supported by the decreased number of hepatocytes per HPF in this treatment group.

For the first time, we describe that mTOR inhibition also significantly increased hepatic autophagy during liver regeneration after partial hepatectomy. Earlier, Kondomerkos et al. showed that mTOR inhibition by rapamycin increased autophagy in the liver and heart of newborn animals.[58] This effect may compensate for the decreased hepatocyte proliferation, as increased autophagy ameliorates oxidative stress and saves cellular energy.

Finally, the ongoing loss of body weight in mice treated with rapamycin is noteworthy. Similar effects of rapamycin on body weight have previously been reported by DiJoseph et al. and Zafar et al..[59, 60] The role of mTOR in metabolism is complicated; it has been described that chemical inhibitors of glycolysis and mitochondrial function suppress mTORC1 activity, indicating that mTORC1 senses cellular energy.[35] This is crucial, because mTORC1-driven growth processes consume a large fraction of cellular energy and thus could be deleterious to starving cells. The mTORC1 pathway indirectly senses low ATP by a mechanism that is centred on the AMP-activated protein kinase (AMPK).[61] During starvation,

mTOR must be downregulated to avoid energy expenditure in absence of nutrients. Therefore pharmacological inhibition of mTORC1 could lead to a defective energy sensing system, mimicking starvation. On the other hand, rapamycin, as mTORC1 inhibitor, may protect the regenerating liver through this mechanism by slowing down the anabolic processes and saving energy and this may account for the fact that animals survive, despite seriously hampered liver regeneration.

In summary, this study investigated the role of mTOR in liver regeneration in vivo and more specific in IL-6 and HGF stimulated signaling pathways. mTOR inhibition resulted in inhibited liver regeneration and increased hepatic autophagy. Although exogenously administered IL-6 and HGF could overcome the rapamycin-induced inhibited reconstitution of liver mass and furthermore upregulated gene expression of factors known to be downstream of mTOR, no significant beneficial effects on body weight, hepatocyte proliferation, autophagy or markers of liver injury were seen. To interpret these data on mTOR inhibition in relation to the clinical setting of living donor liver transplantation, it is important to realize that the model used is limiting in that it is purely a liver regeneration model without ischemia and reperfusion injury or alloreactivity. However, from these results, the use of mTOR inhibitors in the early post-transplant setting can currently not be recommended, despite their recently reported beneficial effects on cancer development and kidney function.

Acknowledgements

The authors would like to thank dr. Wendy van Veelen, Jasper Deuring and Fred Bonthuis for technical support.

COMMENTS

Background

The liver has a remarkable regenerative capacity to compensate for lost or damaged liver tissue after injury. This process enables living donor liver transplantation, a setting in which 40-60% of the liver of a healthy donor is transplanted into a recipient with end-stage liver disease. Treatment of the recipient with immunosuppressive medication is necessary to prevent rejection of the liver graft. Inhibition of the protein mTOR represents an important immunosuppressive strategy. In the initial phase after living donor liver transplantation, the mTOR inhibitor rapamycin is rarely used, as mTOR is a key regulator of cell growth and proliferation and concerns have been raised regarding adverse effects on liver regeneration. However, the exact mechanisms by which mTOR inhibition attenuates liver regeneration are largely unknown.

Research frontiers

The mTOR inhibitor rapamycin, in contrast to most immunosuppressive agents, does not cause nephrotoxicity and has recently gained wide interest in the treatment of cancer.

mTOR inhibitors are therefore an attractive alternative in patients with deteriorating kidney function and also in patients transplanted for hepatocellular carcinoma. Furthermore, besides being a key regulator of cell growth and proliferation, mTOR was recently identified to play an important role in the control of autophagy. Autophagy is a degradation pathway that plays a protective role in case of cellular injury. It has been implicated as a survival response as well as a mediator of cell death during stress conditions, and might therefore play a role in liver regeneration.

Innovations and breakthroughs

Previous studies have reported detrimental effects of mTOR inhibition on liver regeneration. In contrast, a recent publication shows excellent results in patients treated de novo with rapamycin after living donor liver transplantation. Here we report that mTOR inhibition severely impairs liver regeneration and increases autophagy after liver resection in mice. The most novel finding of this study is that this impaired regeneration can be partly reversed by treatment with the cytokine IL-6 and growth factor HGF, both described to stimulate liver regeneration, especially if combined.

Applications

From our results, the use of mTOR inhibitors in the early post-transplant setting can currently not be recommended, despite their recently reported beneficial effects on cancer development and kidney function. However, this study contributes to a better understanding of the role of mTOR and autophagy in liver regeneration and more specific in IL-6 and HGF stimulated signaling pathways.

Terminology

Regeneration is the process of restoration, growth and renewal that makes cells, tissues or organisms resilient to natural fluctuations or events that cause injury or loss. mTOR (mammalian target of rapamycin) is a protein kinase that regulates cell growth, proliferation and survival, as well as protein synthesis and transcription. Autophagy is the basic catabolic mechanism that involves cell degradation of unnecessary or dysfunctional cellular components through the lysosomal machinery, thereby enabling recycling of cellular components and ensuring cellular survival during starvation.

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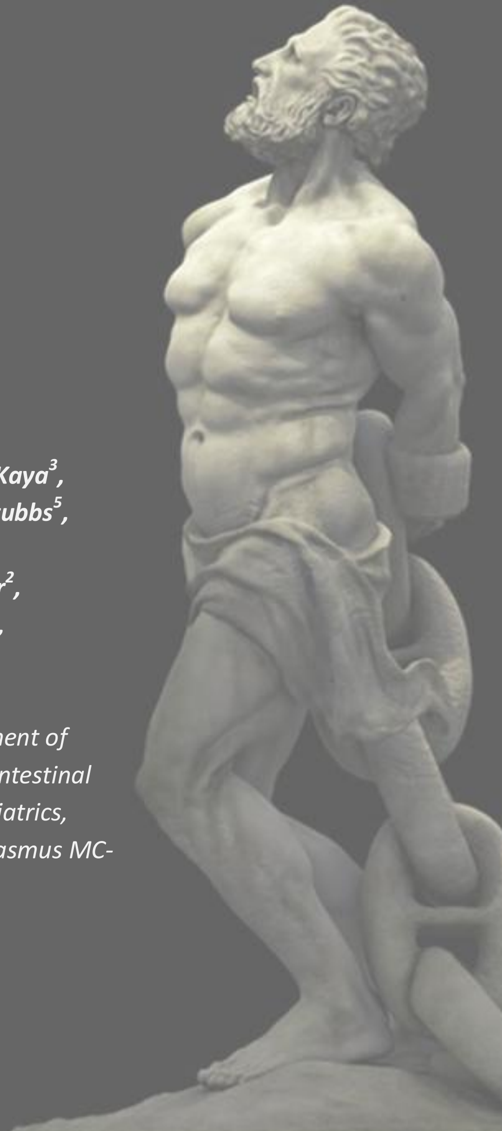
Part II

Therapeutic Strategies

Chapter 5

Mobilization of hepatic mesenchymal stem cells from human liver grafts



Qiuwei Pan[1], Suomi M.G. Fouraschen[2], Fatima S.F. Aerts Kaya[3], Monique M. Verstegen[3, 4], Mario Pescatori[5], Andrew P. Stubbs[5], Wilfred van IJcken[6], Antoine van der Sloot[2], Ron Smits[1], Jaap Kwekkeboom[1], Herold J. Metselaar[1], Geert Kazemier[2], Jeroen de Jonge[2], Hugo W. Tilanus[2], Gerard Wagemaker[3], Harry L. A. Janssen[1] and Luc J. W. van der Laan[2]

[1]Department of Gastroenterology & Hepatology, [2]Department of Surgery, Laboratory of Experimental Transplantation and Intestinal Surgery, [3]Department of Hematology, [4]Department of Pediatrics, [5]Department of Bioinformatics and [6]Center for Biomics, Erasmus MC-University Medical Center, Rotterdam, The Netherlands

(Liver Transplantation, 2011 May;17(5):596-609)

ABSTRACT

Extensive studies have demonstrated the potential applications of bone marrow-derived mesenchymal stem cells (BM-MSCs) as regenerative or immunosuppressive treatments in the setting of organ transplantation. The aims of the present study were to explore the presence and mobilization of mesenchymal stem cells (MSCs) in adult human liver grafts and to compare their functional capacities to those of BM-MSCs. The culturing of liver graft preservation fluids (perfusates) or end-stage liver disease tissues resulted in the expansion of MSCs. Liver-derived mesenchymal stem cells (L-MSCs) were equivalent to BM-MSCs in adipogenic and osteogenic differentiation and in wingless-type-stimulated proliferative responses. Moreover, the genome-wide gene expression was very similar, with a 2-fold or greater difference found in only 82 of the 32,321 genes (0.25%). L-MSC differentiation into a hepatocyte lineage was demonstrated in immunodeficient mice and in vitro by the ability to support a hepatitis C virus infection. Furthermore, a subset of engrafted MSC survived over the long term in vivo and maintained stem cell characteristics. Like BM-MSCs, L-MSCs were found to be immunosuppressive; this was shown by significant inhibition of T cell proliferation. In conclusion, the adult human liver contains an MSC population with a regenerative and immunoregulatory capacity that can potentially contribute to tissue repair and immunomodulation after liver transplantation.

INTRODUCTION

The adult liver harbors a population of facultative progenitors (oval cells in rodents and hepatic progenitor cells or hepatoblasts in humans) that respond to specific injuries and can differentiate into hepatocytes and biliary cholangiocytes.[1, 2] These liver progenitor cells are quiescent in the healthy liver but are activated when certain liver diseases impair the regenerative capacity of mature hepatocytes, cholangiocytes, or both.[3] However, oval cells/hepatic progenitors do not constitute a homogeneous population, and their precise origin and the signals governing their activation are not entirely clear.[4, 5] Previous studies have indicated the presence of a stem cell niche at the proximal biliary tree (the canals of Hering) that contains hepatic stem cells serving as precursors to hepatic progenitor cells.[6,7] Recent studies have further characterized these hepatic stem cells, which are abundant in human fetal and adult livers and have been proposed to be precursors of hepatic progenitors.[8] This population is located in ductal plates in fetal and neonatal livers and in the canals of Hering in pediatric and adult livers.[9]

The mesenchymal stem cell (MSC) is one of a few cell types on the brink of being used clinically in different areas of therapeutic application, including organ transplantation.[10] The bone marrow (BM) compartment harbors resident MSCs with multilineage differentiation potential and anti-inflammatory and immunomodulatory properties, which have been proposed to play a role in the response to liver injury.[11, 12] Encouragingly, various studies have demonstrated the therapeutic potential of bone marrow-derived mesenchymal stem cells (BM-MSCs) in different liver disease models, such as liver resection, fulminant hepatic failure and, in particular, liver transplantation.[13-15] Besides their hepatic differentiation potential, MSCs produce trophic factors that have been shown to provide paracrine support for hepatocyte proliferation, angiogenesis, tissue repair, and immunomodulation.[16-18]

In contrast to the experimental significance of BM-MSCs in liver injury responses and in contrast to the reports on the presence of MSCs in fetal human livers and on the presence of mesenchymal-like stem cells in adult rat livers[19-22], sufficient studies describing MSCs in the adult human liver are lacking. In this study, we first investigated the presence of MSCs in adult human liver tissue and their mobilization during graft cold storage at the time of liver transplantation. Secondly, phenotypic and functional analyses were performed to evaluate the biological characteristics and therapeutic potential of isolated liver-resident MSCs. Gene array analysis revealed a high degree of similarity between gene expression profiles of BM-MSCs and liver-derived mesenchymal stem cells (L-MSCs). Furthermore, Wnt responsiveness and hepatic differentiation in vitro and in mice confirm that L-MSCs represent a bona fide stem cell/progenitor population in the adult human liver.

MATERIALS AND METHODS

Isolation and culture of MSCs from liver tissue and liver perfusate solution

End-stage liver disease tissue samples were obtained from the explanted livers of liver transplant recipients. Patient and liver tissue characteristics are shown in Supporting Table 1. Liver graft preservation fluids (perfusates) were collected from human liver grafts at the time of transplantation (Supporting Table 2), as described previously.[23] Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Lonza, Verviers, Belgium) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 100 IU/ml penicillin, and 100 μg/ml streptomycin. The use of liver tissues and perfusates was approved by the medical ethics committee of the Erasmus MC-University Medical Center.

Flow cytometry

MSCs were stained for 30 minutes at 4°C with directly labeled mouse monoclonal antibodies directed against CD90 and CD105 (R&D systems, Abingdon, United Kingdom), CD34 (Miltenyi Biotec, Bergish Gladbach, Germany), CD45 (Beckman Coulter, Inc., Fullerton, CA), HLA-DR (BD Pharmingen, San Diego, CA), and CD166 (BD Pharmingen). Flow cytometry analysis was performed using FACSCalibur and CellQuest Pro software (BD Biosciences, San Jose, CA).

Adipogenic and osteogenic differentiation

For adipogenic differentiation, MSCs were cultured in DMEM supplemented with 10% fetal bovine serum, 1 μM dexamethasone, 500 μM isobutyl methyl xanthine, 5 μg/ml insulin and 60 μM indomethacin (Sigma-Aldrich) for 3 weeks. Oil Red O staining (Sigma-Aldrich) was used for detection of adipocytes. For osteogenic differentiation, cells were cultured in DMEM with 10% fetal bovine serum supplemented with 0.2 mM ascorbic acid, 100 nM dexamethasone, and 10 mM β-glycerol phosphate (Sigma-Aldrich) for 3 weeks. Alizarin Red S staining (Sigma-Aldrich) was performed to detect deposited calciumphosphates.

Cell proliferation assays

L-MSCs (5×10^3) were plated onto 96-well plates and treated with Wnt3a conditioned medium (Wnt3a-CM) and a control L-cell conditioned medium (L-CM), as described previously.[24] At the indicated times, the number of metabolically active cells was quantified by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (0.5 mg/ml).

L-MSC (5×10^4) were stained with 0.2 μM carboxyfluorescein succinimidyl ester (CFSE; Molecular Probes, Leiden, The Netherlands) for 5 min at 37°C and plated onto 6-well plates. At different time points, cells were harvested, stained with 7-aminoactinomycin D (7AAD; BD Pharmingen) and measured with flow cytometry. Generation analysis was performed with ModFit LT version 3.0 software (Verity Software House, Topsham, ME) and was gated to exclude 7AAD-positive dead cells. The proliferation index (PI), which is the

sum of the cells in all generations divided by the computed number of original parent cells, was used to indicate the extent of cell proliferation.

Gene expression profiling by microarray

Total RNA of 3 independent L-MSC cultures at passage 2 to 5 (1 culture derived from a liver tissue biopsy and 2 cultures derived from liver perfusates), 3 BM-MSC cultures at passage 2 to 5 (from different donors), and 3 hepatoma cell line (Huh7) cultures was used for genome-wide microarray analysis with the Affymetrix GeneChip HuGene 1.0 ST.v1 array (Affymetrix, Santa Clara, CA) according to the manufacturer's procedures. Transcript-level expression measures were generated with the robust multi-array average (RMA) procedure as implemented in the Affymetrix Gene Expression Console, and probeset annotations were retrieved from NetAffx with the same software. Probesets that differentially expressed among conditions were identified with the class comparison tool implemented in BRB-ArrayTools (National Institutes of Health, Bethesda, MD). Principal component analysis was performed using Partek (Partek, Inc., Saint Louis, MO). Hierarchical clustering was performed in Spotfire (Spotfire, Inc., Somerville, MA). Pathway analysis was performed using Ingenuity Pathway Analysis (Ingenuity Systems, Redwood City, CA).

In vitro hepatogenic differentiation

In vitro hepatic differentiation was performed in 3 steps as reported previously[25], but the final step was modified. For the final maturation step, cultures were incubated with infectious Japanese fulminant hepatitis 1 (JFH1)-derived hepatitis C virus (HCV) particles.[26] Hepatogenic differentiation was determined by quantitative reverse-transcription polymerase chain reaction (RT-PCR) detection of albumin and HCV internal ribosome entry site (IRES) RNA.

Real-time RT-PCR

Confluent monolayers of MSCs or liver graft tissue biopsy samples were lysed with TRIzol (Invitrogen-Gibco), and RNA was precipitated with 75% ethanol and captured with a Micro RNeasy silica column (Qiagen, Venlo, The Netherlands). RNA was quantified with a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Complementary DNA (cDNA) was prepared from 1 μg of total RNA with an iScript cDNA Synthesis Kit from Bio-Rad Laboratories (Stanford, CA). The cDNA of human and mouse albumin, CD90, CD105, cytokeratin 18 (CK18), CK19, hepatocyte growth factor (HGF), mesenchymal epithelial transition factor (c-Met), leucine-rich repeat-containing G protein-coupled receptor 5 (Lgr5), HCV IRES, cytochrome B (CyB), and glyceraldehydes 3-phosphate dehydrogenase (GAPDH) was quantified with RT-PCR (MJ Research Opticon, Hercules, CA), which was performed with SYBR Green (Sigma-Aldrich) according to manufacturer's instructions. CyB or GAPDH was used as a reference gene to normalize the gene expression, which was calculated with the delta-delta cycle threshold method.

Periodic Acid-Schiff staining

Hepatic differentiated and undifferentiated MSCs were fixed in 4% paraformaldehyde for 20 minutes, after which intracellular glycogen was stained with the periodic acid-Schiff (PAS) method. Briefly, fixed cells were oxidized in 0.5% periodic acid solution for 5 minutes. After they were rinsed in distilled water, theys were placed in the Schiff reagent for 15 minutes. Next, they were washed in lukewarm tap water for 5 minutes and counterstained in Mayer's hematoxylin for 1 minute.

MSC transplantation in mice

Immunodeficient NOD/SCID mice (Erasmus MC institutional breeding), age 6-8 weeks, were intraperitoneally injected with 100 µl/20g body weight olive oil containing 10 µl carbon tetrachloride (CCl_4). After 24 hours, 1×10^6 L-MSCs (n=5) or BM-MSCs (n=2) suspended in 0.2 ml PBS were injected into the spleen. Four weeks after engraftment, the mice were sacrificed and their livers were harvested. Untreated (n=2) or non-engrafted CCl_4-treated NOD/SCID mice (n=3) served as negative controls. Second, luciferase-labeled L-MSCs (5×10^5 cells) were subcutaneously injected into NOD/SCID mice (n=2) after which the luciferase activity was measured at different time points with an In Vivo Imaging System (IVIS) camera.

Fluorescent immunohistochemistry

Mouse liver tissue was dissected and cryoprotected in 30% sucrose to generate frozen sections. The sections were incubated with fluorescent-labeled antibody at the dilution of 1:100 for 30 minutes. After 3 washes, nuclear staining was achieved by incubating with 4,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) at the dilution of 1:50 for 5 minutes. From each condition 20-30 images were captured by confocal microscopy.

T cell proliferation/suppression assay

The effect of L-MSCs on the proliferation of T cells was determined with the mixed lymphocyte response assay. Briefly, peripheral blood mononuclear cells (PBMCs; 4×10^5) in the presence or absence of MSCs were seeded onto 96-well round-bottom plates. Irradiated allogeneic PBMCs (2×10^5) or phytohemagglutinin (PHA; Murex Biotech, United Kingdom) were used for stimulation. After 5 days, proliferation was assessed by the determination of the incorporation of 0.5 µCi (0.0185 MBq) of [^{3}H]-thymidine (Radiochemical Center, Amersham, United Kingdom) for 18 hours.

Statistical analysis

Statistical analysis was performed with either the paired nonparametric test (Wilcoxon signed-rank test) or the unpaired nonparametric test (Mann-Whitney test) with GraphPad InStat software (GraphPad Software, Inc., San Diego, CA). P-values lower than 0.05 were considered statistically significant.

RESULTS

Mobilization of hepatic MSCs from adult human liver grafts

Graft perfusion, procurement and cold-storage are associated with ischemia and tissue injury. Previously, we found that the washout of the graft preservation solution (perfusate) collected at time of liver transplantation contains high numbers of mononuclear cells which detach from the liver; these include lymphocytes, natural killer cells, antigen-presenting cells[23, 27] and hematopoietic stem cells.[28] Flow cytometry analysis of perfusate mononuclear cells revealed the presence of a small but consistent fraction of cells double-positive for the MSC surface markers CD90 and CD105 (mean 0.09% ± 0.07 SD, n=8) as well as CD90 and CD166 (0.02% ± 0.02; Figure 1A). Prospectively, fresh perfusates from 15 consecutive liver transplantations were collected and mononuclear cells were isolated and cultured for the presence of MSCs (Supporting Table 1). Fibroblast-like cells were observed in the initial cultures of all perfusates (Figure 1B). In a majority of cultures the numbers of these cells rapidly increased (Figure 1C). These cells could be expanded and passaged for several months under normal non-hypoxic culture conditions, clearly distinct from a previously described albumin^{+}CD105^{-} population of hepatic stem cells.[29] Flow cytometry analysis of expanded cells at passage 4 to 9 revealed a surface marker profile typical for MSCs (Figure 1D). A high percentage of these cells stained positive for CD90 (mean 59% ± 18 SD, n=11), CD105 (55% ± 14) and CD166 (44% ± 16), and were mostly negative for the hematopoietic stem cell marker CD34 (0.8% ± 0.7) as well as the leukocyte lineage markers CD45 (0.7% ± 0.7) and HLA-DR (1.9% ± 1). A functional analysis showed that the expanded liver-derived cells had a multi-lineage potential with a capacity for adipogenic (Figure 1E) and osteogenic differentiation (Figure 1F), similar to BM-MSCs.

To confirm the presence of MSCs in the adult human liver, tissue samples of explant livers from a variety of patients with end-stage liver disease (Supporting Table 2) were dissociated, and the unfractionated cell suspensions were cultured. After 4 to 10 days, in a majority of cultures, clusters of cells with fibroblast-like morphology were observed. Like MSCs from perfusate, these cells were highly positive for CD90, CD105 and CD166, were negative for the markers CD34, CD45 and HLA-DR, and had equivalent adipogenic and osteogenic differentiation capacity (data not shown). L-MSCs could also be expanded from disease-free liver graft tissue obtained from postmortem organ donors (data not shown). Cultures of PBMCs from end-stage liver disease patients, brain-dead multi-organ donors or healthy controls did not show any MSCs. Therefore it is unlikely that L-MSCs in perfusates are directly mobilized from the BM compartment and derived from residual donor blood.

Wnt signaling promotes L-MSC proliferation

Wnt signaling has been shown to modulate the growth of human BM-MSCs[30] and plays an important role in liver homeostasis and pathology.[31] As shown in Figure 2A, L-MSCs (both from tissue and perfusate) that were stimulated with Wnt3a exhibited a signi-

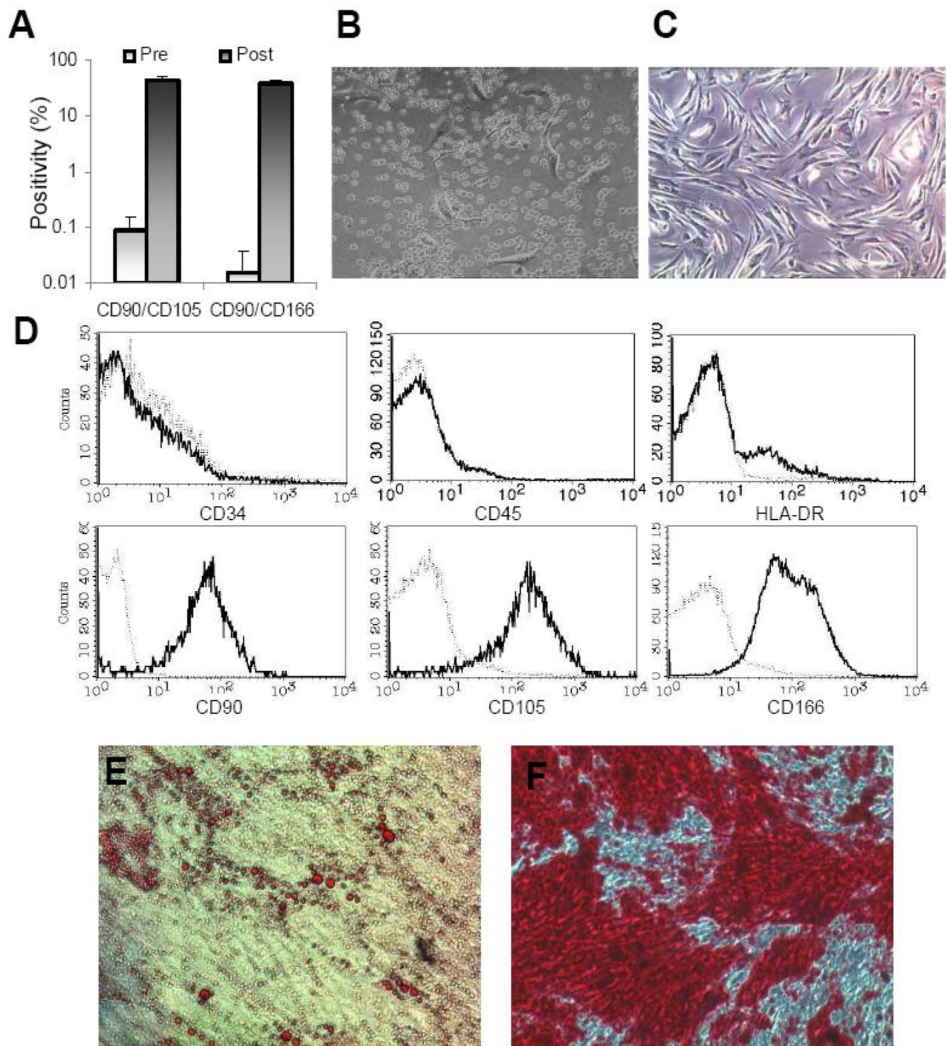


Figure 1. Characterization of MSC mobilized from human liver at time of graft cold storage

A. The percentage of cells double-positive for $CD90^{+}CD105^{+}$ (0.09% ± 0.07 SD, n=8) and $CD90^{+}CD166^{+}$ (0.02% ± 0.02 SD, n=8) was low in the liver perfusates before culturing (Pre), but the cells rapidly expanded with culturing (44% ± 8 and 37% ± 6, respectively) at passage 4 to 9 (Post; n=6, P<0.001); **B.** In the majority of the cultures, cells with a fibroblast-like morphology appeared within ten days; **C.** Fibroblast-like cells rapidly proliferated and could be subcultured and expanded for 10-20 passages; **D.** Flow cytometric analysis of surface markers showed that the expanded cells exhibited a typical MSC-like phenotype positive for CD90 (59% ± 18 SD, n=11), CD105 (55% ± 14) and CD166 (44% ± 16), and were mostly negative for the hematopoietic stem cell marker CD34 (0.8% ± 0.7) as well as the leukocyte lineage markers CD45 (0.7% ± 0.7) and HLA-DR (1.9% ± 1). The black lines in the histograms represent the specific staining and the grey line shows the background staining of isotype-matched control antibody; **E.** Adipogenic differentiation of L-MSC was detected by Oil Red O staining for lipid droplet (red); **F.** Osteogenic differentiation of these cells was evaluated through the detection of deposited calciumphosphates with Alizarin Red S staining (red). Representative stains of 4 independent cultures are shown (100×).

ficant increase in viable (metabolically active) cell numbers in comparison with control-treated MSCs as measured by the MTT assay (mean increase 44% ± 17 SD on day 6, P<0.001). In order to confirm that this increase was related to enhanced cell proliferation and not due to enhanced cell survival, a CFSE fluorescence-based proliferation assay was used. As shown in Figure 2B, Wnt3a-CM treatment accelerated CFSE-dilution of labeled L-MSCs, and this was indicative of enhanced cell proliferation. On culture day 6, the percentage of cells that underwent 8 rounds of cell division (ninth generation) was 10% for L-CM-treated cells and 50% for Wnt3a-CM-treated cells. Similar proliferative responses were seen with BM-MSCs (data not shown).

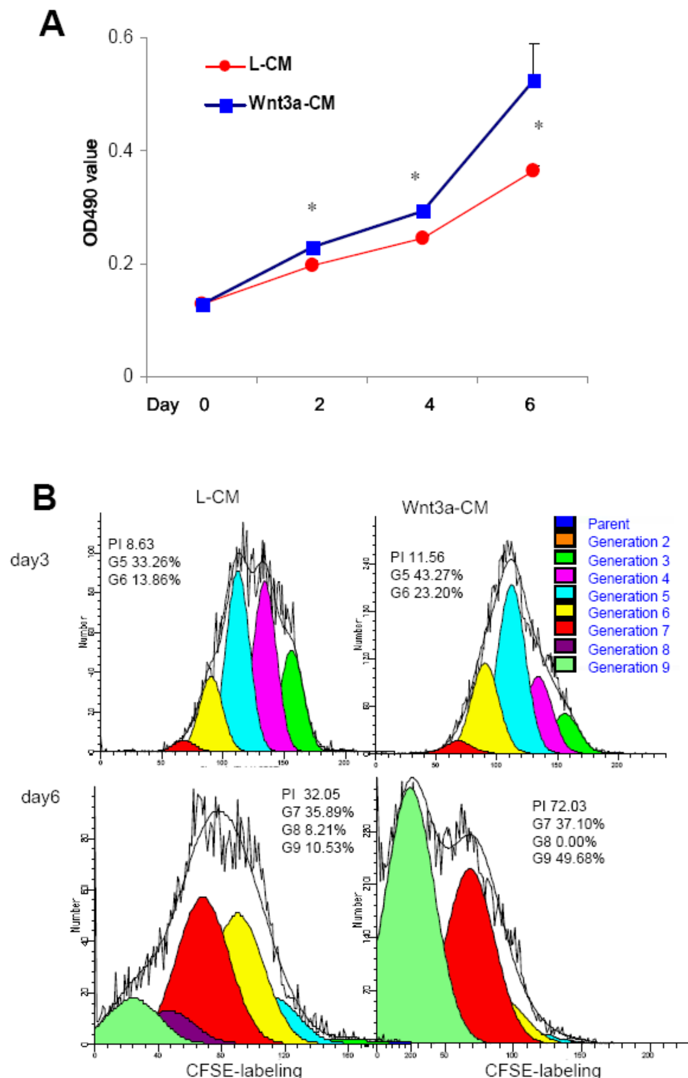


Figure 2. Wnt3a promotes L-MSC proliferation

A. The MTT assay showed that stimulation of MSCs with Wnt3a-CM significantly increased the number of cells in comparison with the control L-CM treatment on days 2 (16% ± 6 increase), 4 (20% ± 3) and 6 (44% ± 17). The means and standard deviations of 6 independent experiments are shown (*P<0.001); **B.** Increased Wnt3a-induced cell proliferation was confirmed by CFSE dilution of labeled L-MSC. A marked increase in the percentage of cells was observed in G5 and G6 on day 3 (47% for L-CM versus 66% for Wnt3a-CM) and in G8 and G9 on day 6 (19% for L-CM versus 50% for Wnt3a-CM); this was also reflected in the marked increase in the PI seen in the Wnt3a-stimulated cells at both time points (11.6 and 72.0 for Wnt3a-CM versus 8.6 and 32.1 for L-CM).

Gene expression profiles of L-MSCs and BM-MSCs

In order to gain further insight into the molecular phenotype of L-MSCs, we performed genome-wide expression profiling on early-passage MSC cultures (passage 2-5). The expression profile of these L-MSCs was compared to that of BM-MSCs (3 cultures at passage 2-5 from different donors). Cultures of Huh7 hepatoma cells (n=3) served as generic controls of a replicating cell population of hepatic origin. The principal component analysis of their genome-wide expression profiles grouped the 3 cell types into 3 separate clusters on a 3-dimensional scatter plot (Figure 3A). Notably, liver-derived Huh7 cells clustered far apart from both L-MSCs and BM-MSCs, regardless of their hepatic or extra-hepatic origin. Accordingly, in comparison with L-MSCs, more than 20% of Huh7 genes were differentially expressed. A direct comparison of gene expression of liver tissues obtained from grafts at the time of transplantation showed that L-MSCs highly expressed CK19 and HGF, whereas the expression of CK18, c-Met, and Lgr5 was lower, and albumin messenger RNA (mRNA) was not detectable (Figure 3B). A comparative analysis of L-MSCs and BM-MSCs showed comparable expression levels of most of the known MSC-associated genes (Figure 3C).[32] However, with identical analysis settings, less than 1% of the genes were differentially expressed between L-MSCs and BM-MSCs (311 of 32,321 genes, $P<0.001$; Figure 3D). Overall, the expression of only 45 genes was more than 2-fold higher in L-MSCs, and the expression of 37 genes was more than 2-fold higher in BM-MSCs (See Supporting Table 3). Among the genes differentially expressed genes in MSCs were matrix metallopeptidase 1, actin gamma 2 smooth muscle enteric, and interleukin 33 (>20-fold higher in L-MSCs), and microfibrillar associated protein 5, insulin-like growth factor binding protein 3, and retinol binding protein 4 plasma (>10-fold higher in BM-MSCs, $P<0.001$). However, no significant differences in biological or functional pathways of gene expression were observed between L-MSCs and BM-MSCs with Ingenuity Pathway Analysis (data not shown). To distinguish L-MSCs from hepatic stellate cells, a gene array analysis was performed for 10 known stellate cell-associated genes and 2 recently reported markers, CD133 and Oct4.[33, 34] The differential gene expression of these 12 markers was comparable between L-MSCs and BM-MSCs (data not shown). Together, these data indicate that in terms of transcriptome composition, L-MSCs are highly similar to BM-MSCs and, like BM-MSCs, appear distinct from hepatic stellate cells.

Long-term survival of L-MSCs in vivo

To evaluate the fate of MSCs in vivo, L-MSCs or BM-MSCs were engrafted into the livers of NOD/SCID mice subjected to CCl_4-induced liver toxicity. As shown in Figure 4A, RT-PCR analysis at 4 weeks showed the expression of the human MSC markers CD90 and CD105 in the livers of MSC-engrafted mice but not in the livers of sham-treated controls. Fluorescent immunohistochemstry confirmed the presence of human CD90-positive cells in the mouse liver tissue (Figure 4B), although these cells did not express the cell proliferation marker proliferating cell nuclear antigen (PCNA). Dissociated mouse liver tissue was

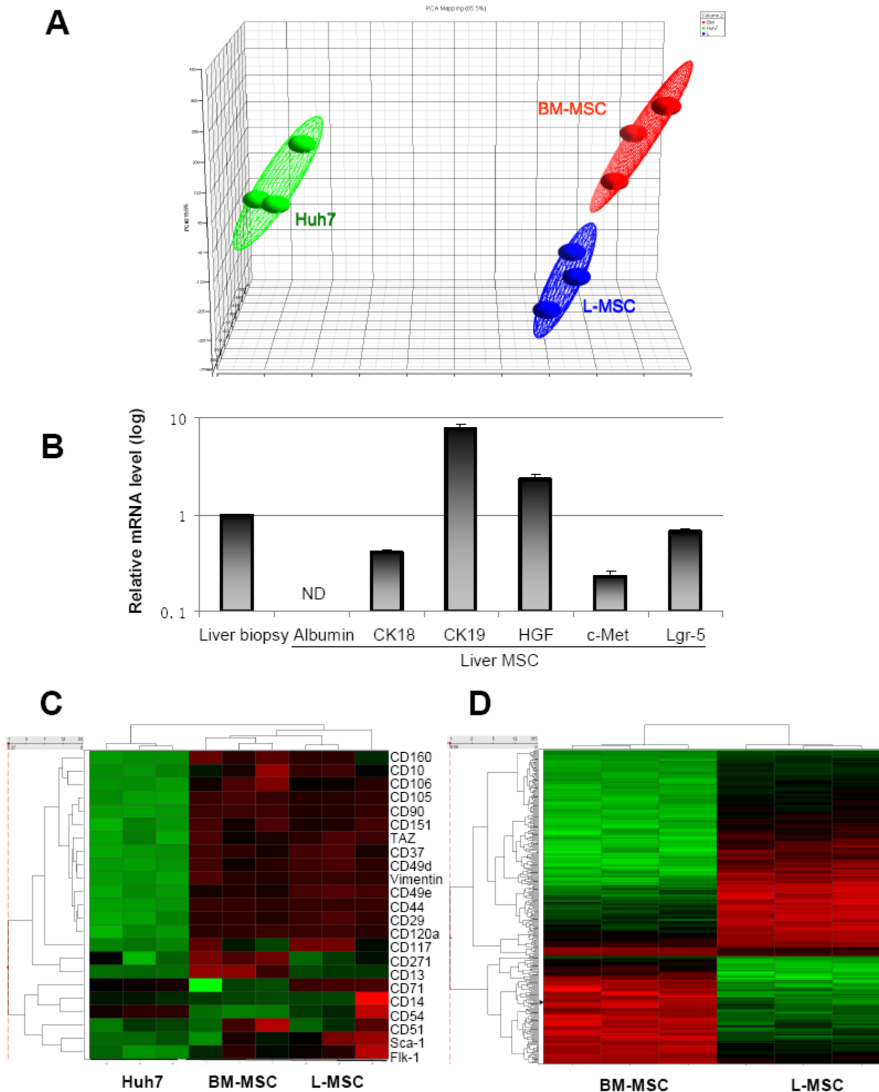


Figure 3. Gene expression profiling of L-MSCs, BM MSCs and Huh7 hepatoma cells

A. Principal component analysis of genome-wide expression profiles was used to visualize correlation relationships between samples. The 3 independent L-MSC preparations clustered into a separate grouping apart from both BM-MSCs and Huh7 cells. The short distance between the L-MSC and BM-MSC clusters in the 3-dimensional correlation space suggests that the 2 MSC populations were characterized by similar patterns of gene expression at variance with Huh7; **B.** Real-time RT-PCR analysis of the L-MSC gene expression levels relative to GAPDH and normalized to levels in donor graft liver tissue. MSCs highly expressed CK19 and HGF, whereas expression of CK18, c-Met, Lgr5, and albumin was lower or not detectable (ND). The means and standard deviations of 1 representative experiment in triplicate are shown; **C.** A gene array analysis of L-MSCs and BM-MSCs showed comparable gene expression of known MSC markers, whereas Huh7 cells generally exhibited low expression of these genes; **D.** Although the expression profiles of the 2 cell types appeared very similar, L-MSCs and BM-MSCs could be distinguished by the consistent differential expression of a small proportion of their transcriptomes (<1%, P<0.001).

cultured for 5 days. Human MSCs with typical fibroblast-like morphology rapidly expanded in culture (Figure 4C). No such cells were observed in control mice not engrafted with human MSCs (Figure 4D). Flow cytometric analysis revealed that the majority of the explanted liver cells, after culture expansion, expressed human CD90 (60.8% ± 18.2, Figure 4E) and CD105 (61.6% ± 20.8, mean ± SD, n=3; Figure 4F). This was further confirmed by RT-PCR analysis showing human specific CD90 and CD105 gene expression in cultured cells. No CD90 and CD105 mRNA was detected in cultures of sham-treated controls (data not shown). For further evaluation of the in vivo survival, luciferase labeled L-MSCs were subcutaneously engrafted into NOD/SCID mice. As shown in Figure 4G, a luciferase signal was clearly visible up to 25 days after engraftment; this confirmed longer term MSC survival in vivo, although the signal gradually declined over time.

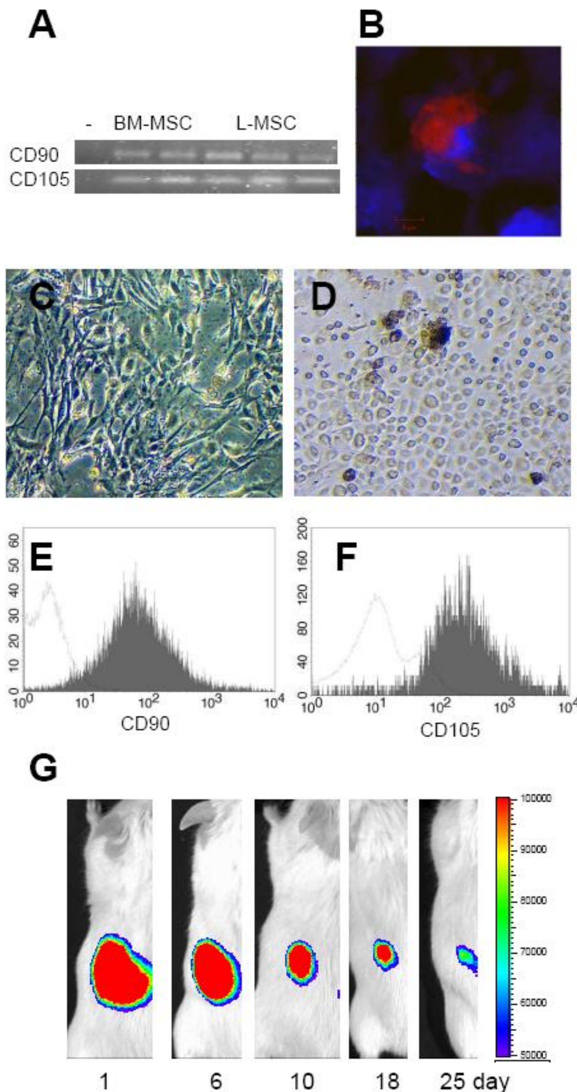


Figure 4. Human L-MSC retain stem cell characteristics after engraftment into mice

L-MSCs or BM-MSCs were engrafted into the livers of NOD/SCID mice subjected to CCl_4-induced liver injury. After 4 weeks, mouse liver tissue was analyzed for the presence of human MSCs; **A.** RT-PCR analysis showed expression of the human-specific MSC markers CD90 and CD105 in liver tissue from transplanted mice but not in controls (-); **B.** Immunofluorescent staining of mouse liver tissue showed human-specific CD90-positive cells (red) present in MSC-engrafted mice, but not in control mice (not shown). DAPI nuclear staining shown in blue; **C.** In a majority of the cultures of dissociated mouse liver cells, cells with typical fibroblast-like morphology rapidly expanded (magnification 400x); **D.** No such cells were observed in cultures of control mouse livers; **E-F.** Flow cytometric analysis of engrafted mice confirmed that a high percentage of the cells in culture were positive for human MSC surface marker CD90 (60.8% ± 18 SD) and CD105 (61.6% ± 21). Gray lines indicate isotype-matched control staining; **G.** Survival of subcutaneously engrafted L-MSCs in NOD/SCID mice. MSCs expressed the lucifease reporter gene. Luciferase signals were measured at different time points after engraftment. The signal gradually declined over time but was clearly detectable for at least 25 days; this confirmed long-term survival of viable MSCs in vivo.

Hepatic differentiation of L-MSCs in vitro and in vivo

The hepatocyte lineage differentiation potential of L-MSC was determined in vitro with an established hepatogenic culture procedure. After 30 days of culture, morphological changes of most cells were observed in all cultures (n=6). In contrast to fibroblast-like morphology of undifferentiated MSCs (Figure 5A), hepatogenic differentiation induced a polygonal morphology at 15 (Figure 5B) and 25 days of culture (Figure 5C). Fluorescent immunohistochemistry staining showed the expression of human albumin protein with differentiated MSCs (Figure 5D), but not with undifferentiated MSCs (Figure 5E). Similarly, glycogen storage was detected in differentiated MSCs (Figure 5F), but not in undifferentiated MSCs (Figure 5G). Quantitative RT-PCR analyses confirmed specific expression of albumin mRNA in differentiated BM-MSCs and L-MSCs (Figure 5H).

To further investigate the functionality of MSC-derived hepatocyte-like cells, we challenged differentiated and undifferentiated L-MSCs with infectious HCV particles (JFH-1-derived, cell culture-produced HCV); this virus has a hepatic tropism produced by Huh7.5 cells. RT-PCR analyses of HCV-specific IRES sequence clearly showed that, unlike undifferentiated MSCs, MSC-derived hepatocyte-like cells permitted HCV infection (Figure 5I). The levels of HCV RNA were approximately 100-fold lower than high-replicating HCV replicon cells.

The hepatogenic differentiation potential of L-MSCs was further evaluated in vivo in NOD/SCID mice with CCl_4-induced liver injury. Four weeks after MSC engraftment, mouse livers were harvested and RT-PCR analyses displayed detectable levels of human-specific albumin gene expression (Figure 6A). Immunohistochemical staining of mouse liver tissue showed the presence of human albumin-positive cell clusters in all MSC-engrafted mice (Figure 6B), although the frequency of these clusters was generally low. No human albumin positivity was observed in untreated or sham CCL_4-treated control mice (Figure 6C). Flow cytometry analyses of dissociated mouse livers confirmed the presence of human hepatocyte-like cells (Figure 6E), with a mean concentration of albumin-positive cells of 1.09% ± 0.39% (n=5). No positive cells were detected in livers from control mice (Figure 6F). Overall, these results indicate that L-MSCs (or a subset) have hepatogenic potential in vitro and in vivo and that MSC-derived hepatocyte-like cells can be infected with HCV.

MSCs effectively suppress T cell proliferation

It is well established that MSC populations from various tissues, including BM, spleen, heart and fat, have immunoregulatory and suppressive properties, including the inhibition of T cell proliferation.[16, 35] The effect of adult human L-MSCs on proliferation of mitogen- and alloantigen-stimulated T cells was investigated in vitro through the measurement of [^{3}H] thymidine incorporation. PBMCs or purified $CD4^+CD25^-$ T cells stimulated with mitogenic PHA or irradiated allogeneic PBMCs, were co-cultured with MSCs at different ratios. As shown in Figure 7A, significant inhibition of alloantigen-stimulated proliferation was observed with MSC/PBMC ratios of 1:2 (97% inhibition, P<0.001), 1:4 (92%, P<0.001), 1:8

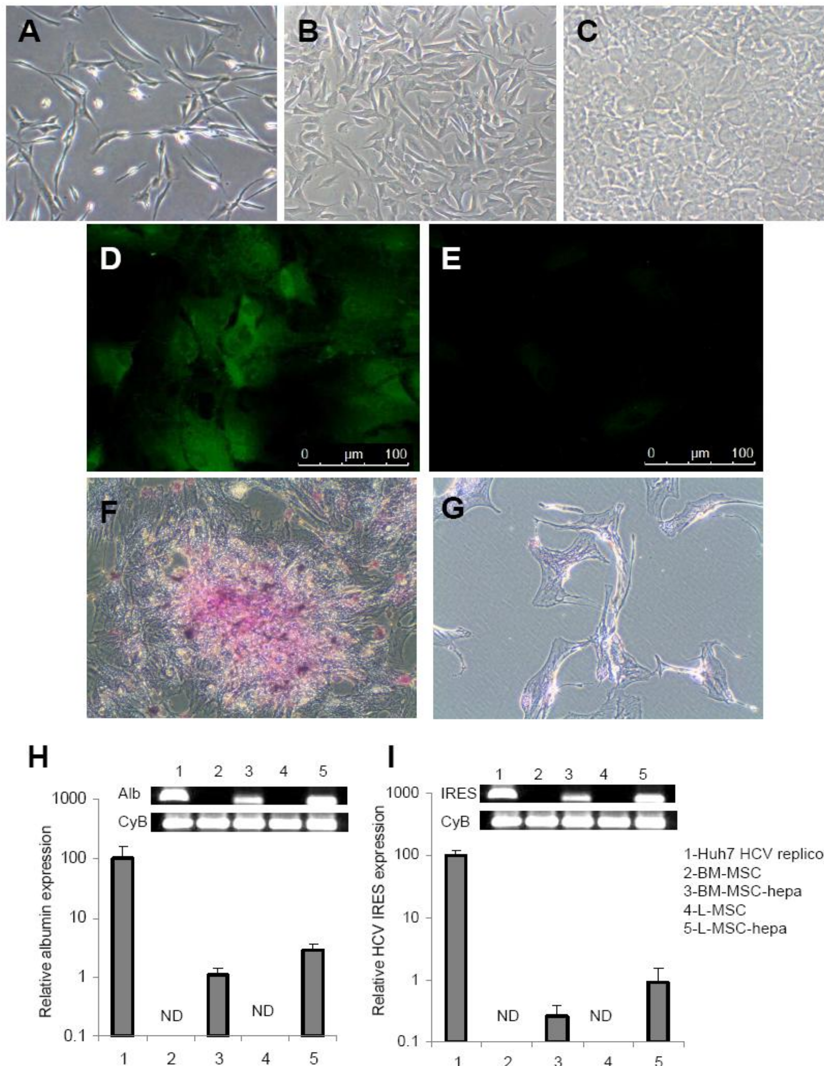


Figure 5. L- MSCs differentiate into hepatocyte-like cells that permit HCV infection

A. In contrast to the fibroblast-like spreading of undifferentiated L-MSC, **B-C.** hepatogenic differentiation of L-MSC induced a polygonal morphology with granular cytoplasm at 15 and 25 days of differentiation, respectively (magnification x400). Fluorescent immunocytochemistry showed human albumin staining (green) in **D.** hepatic differentiated MSCs on day 30, but not **E.** in undifferentiated MSCs. Glycogen storage (pink) was seen **F.** in hepatically differentiated MSCs, but not **G.** in undifferentiated MSCs (magnification x100); **H.** Gene expression analysis of cultured MSCs showed clear expression of hepatocyte-specific albumin gene after hepatogenic differentiation (MSC-hepa) by quantitative real-time RT-PCR. Albumin mRNA was not detectable (ND) in undifferentiated BM-MSCs or L-MSCs, and the Huh7 hepatoma cell line served as a positive control; **I.** For further characterization of the MSC-derived hepatocyte-like cells, differentiated and undifferentiated cells were incubated with a conditioned medium containing infectious HCV particles (cell culture-produced HCV). Real-time RT-PCR analysis for the HCV IRES sequence showed that differentiated MSCs could be infected by HCV, whereas undifferentiated BM-MSCs and L-MSCs did not permit infection. Huh7 replicon cells (Huh7-ET) with high-level HCV replication served as positive controls.

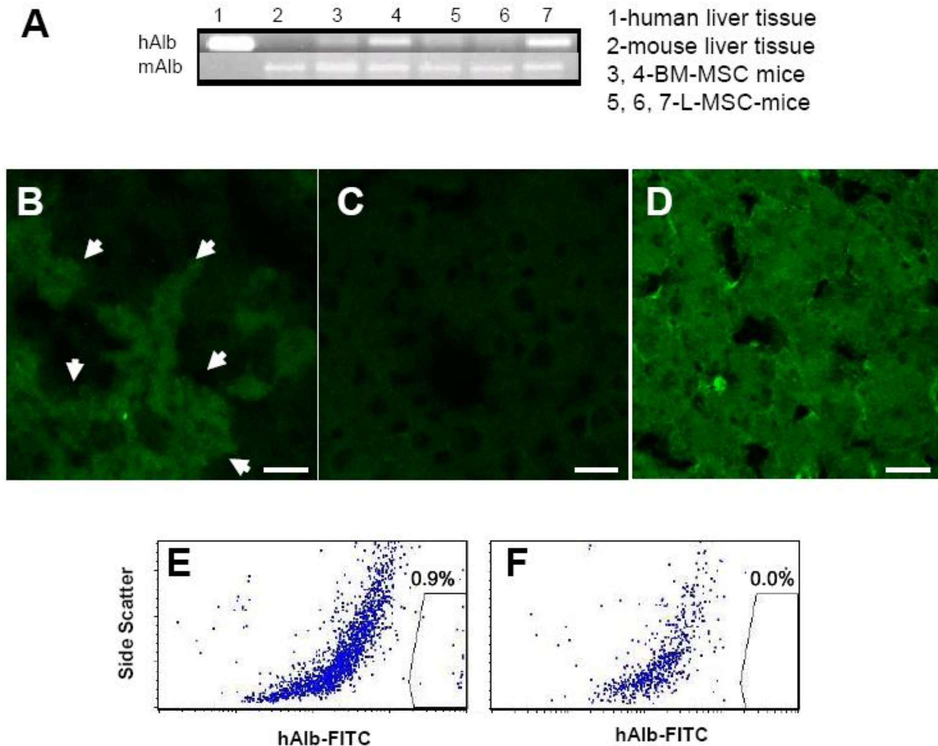


Figure 6. MSC differentiation into hepatocyte-like cells in vivo

L-MSCs or BM-MSCs were engrafted into the livers of NOD/SCID mice subjected to CCL_4-induced liver toxicity. Four weeks after the MSC administration, the mouse livers were harvested and analyzed for evidence of hepatic differentiation. **A.** RT-PCR analysis for human- and mouse-specific albumin RNA. Human albumin expression was observed in all MSC-engrafted livers (lanes 3-5) and was not observed in the control mouse livers (lane 2). Human liver RNA served as a positive control (lane 1), and mouse albumin was detected in all mouse livers (lanes 2-7) but not in the human liver; **B.** Immunohistochemical staining of the mouse livers confirmed the presence of human albumin–positive cell clusters (indicated by arrows) in mice engrafted with BM-MSCs or L-MSCs (n=7); **C.** Control mouse and **D.** human liver tissue served as negative and positive controls, respectively, for the albumin staining (bar=20 µm). The concentration of human-specific albumin-positive cells, as quantified by flow cytometry, was **E.** 1.09% ± 0.39% in the dissociated mouse livers (n=5) and **F.** not detectable (ND; <0.01%) in the sham-treated control mice (n=3).

(76%, P<0.001) and 1:16 (53%, P<0.05; n=6). Comparable inhibition of proliferation was observed with PHA-stimulated PBMCs (Figure 7B). Similar results were obtained when BM-MSCs were used and when purified $CD4^{+}CD25^{-}$ T cells were used as responder cells (data not shown). These findings indicate that L-MSCs, like other MSC populations, are potent inhibitors of T cell proliferation and may contribute to allo-immune regulation after liver transplantation.

DISCUSSION

Currently, the therapeutic potential of stem cells such as MSCs are being explored at an incredible pace for the treatment of liver disease as well as many other diseases.[4, 14] In vitro hepatic differentiation has been described for MSCs derived from different sources, including the BM, fat, lungs, cord blood, and amniotic fluid.[25, 36-38] In the current study, we found evidence of the presence of MSCs in the adult human liver itself. Gene expression profiling showed a high degree of similarity between L-MSCs and BM-MSCs. Overall, only 82 genes (0.25% of the transcriptome) were more than 2-fold different in expression between L-MSCs and BM-MSCs. Their high similarity, which included the expression profiling of most of these MSC markers, supports the idea that these isolated cells constitute a bona fide MSC population. In addition, the distinct genomic signature provides an indication of their distinct origin, which in turn provides potential markers for distinguishing these 2 MSC populations.

In order to determine hepatic differentiation of L-MSCs in vitro, we combined the conventional methods with a novel approach of infecting the differentiated cells with HCV. We found that hepatic differentiation occurred in a subpopulation of L-MSCs that not only changed morphology and expressed albumin, but also supported HCV infection. Because HCV infection is largely restricted to mature hepatocytes, the results strongly indicate that at least some of the MSC-derived hepatocytes were fully differentiated and functionally supported viral entry and replication.

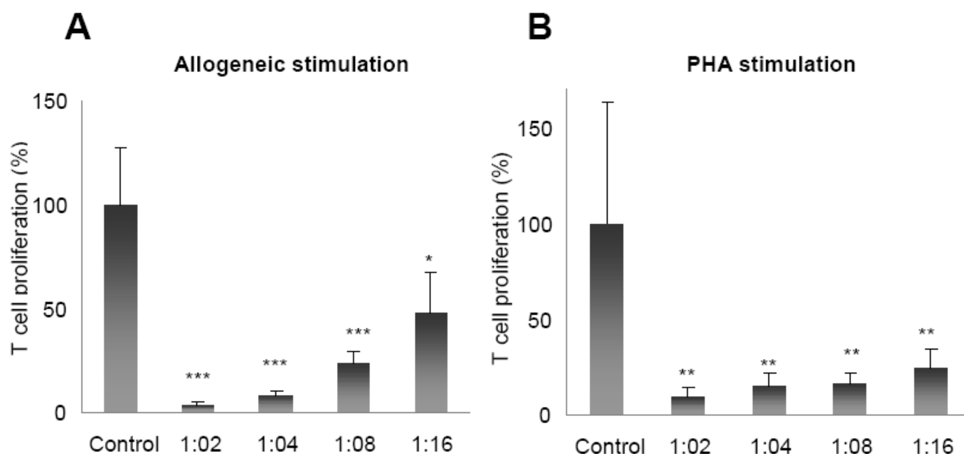


Figure 7. Suppression of T cell proliferation by L-MSCs

A. T cells were stimulated with irradiated allogenic PBMCs and co-cultured with allogeneic MSCs in 1:2, 1:4, 1:8 and 1:16 MSC/PBMC ratios for 5 days; **B.** T cells were stimulated with PHA and co-cultured with MSCs for 5 days at different ratios. Significant inhibition of cell proliferation was observed with different ratios. Similar results were observed when BM-MSCs were used and when purified CD4+CD25- T cells were used as responders (data not shown). The means and standard deviations of 6 experiments are shown. *P<0.05, **P<0.01, ***P<0.001.

Consistently, hepatic differentiation of MSCs has been reported in vivo as well. Several studies mentioned the occurrence of MSC-oriented hepatic differentiation in models of liver injury and regeneration.[14, 25, 37, 39] Although hepatic differentiation generally occurs at a relatively low frequency and concerns have been raised about the fate of stem cells after transplantation in immune-competent hosts[40], these findings still bring new hope for MSC-based cell therapies for regenerative liver diseases. Notably, the hepatic differentiation of MSCs appears to be less robust that seen with a different, more committed population of hepatic stem cells identified from human fetal and postnatal livers.[8] But the in vivo hepatic differentiation capacity of MSCs could be further improved by pre-differentiation in vitro before engraftment.[41] If MSCs are used to treat liver diseases, both hepatic differentiation and in vivo survival of the stem cells are crucial. In this study, we found that L-MSCs not only can differentiate towards hepatocytes but can also be retrieved 4 weeks after transplantation into NOD/SCID mice; this indicates their long-term survival properties in vivo.

Notably, MSCs can migrate to injured tissue and contribute to tissue repair and wound healing. This mobilization is likely regulated by specific danger signals and chemotactic factors.[42, 43] MSCs have a profoundly greater capacity to survive under conditions of ischemia, because in the absence of oxygen MSCs can survive using anaerobic ATP production.[44] Our previous studies have shown that several types of liver-derived hematopoietic cells are mobilized during perfusion of the graft and are continuously released into the recipient after liver transplantation.[23, 27] The continuous migration of donor leukocytes into recipient's circulation, which leads to chimerism, occurs more often in liver transplantation than other organ transplantation procedures and has been associated with graft acceptance.[45] We hypothesize that ex vivo vascular ischemic perfusion of liver grafts may stimulate MSC mobilization at the time of transplantation. Indeed, substantial numbers of L-MSCs were isolated from liver perfusates by culturing the cell fraction, and this demonstrated the migration of graft MSCs during cold storage and perfusion. Thus, liver preservation fluid can be considered a novel source of MSCs that is particularly important because normal healthy human liver tissue is usually not available. Because L-MSCs possess potent immunomodulatory properties, we speculate that like many liver leukocytes, graft-derived MSCs may also migrate to the recipient after liver transplantation and subsequently play a role in immunoregulation, tissue repair, and regeneration.

In conclusion, this study provides evidence for the presence of MSCs in human adult liver. These cells have characteristics similar to those of BM-MSCs with respect to phenotype and function. The migration of graft MSCs at the time of liver transplantation provides an alternative source of L-MSCs, and this also suggest that a continuous release of graft MSCs and a systemic contribution may occur after transplantation in a recipient. We believe that our observations have paved the way for further study on the role of these cells in physiological and particular pathological conditions.

Acknowledgements

The authors thank Professor Takaji Wakita (National Institute of Infectious Diseases, Tokyo, Japan) for generously providing the full-length JFH1-derived infectious HCV replicon; Professor Ralf Bartenschlager and Dr. Volker Lohmann (University of Heidelberg, Heidelberg, Germany) for providing the Huh7 subgenomic HCV replicon cells; Dr. Thanyalak Tha-In, Dr. Scot Henry and Dr. Wendy van Veelen for providing technical support; Dr. Yuedan Li and Dr. Martijn Brugman for performing the gene array data analysis; and Dr. Martin Hoogduijn for critically reading the manuscript.

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Chapter 6

Detection of spontaneous tumorigenic transformation during culture expansion of human mesenchymal stromal cells

Qiuwei Pan[1], Suomi M.G. Fouraschen[2], Petra E. de Ruiter[2], Winand N.M. Dinjens[3], Jaap Kwekkeboom[1], Hugo W. Tilanus[2] and Luc J.W. van der Laan[2]

[1]Department of Gastroenterology & Hepatology, [2]Department of Surgery, Laboratory of Experimental Transplantation and Intestinal Surgery and [3]Department of Pathology, Erasmus MC-University Medical Center, Rotterdam, The Netherlands

(Experimental Biology and Medicine, in press)

ABSTRACT

Human mesenchymal stem/stromal cells (MSCs) have been explored in a number of clinical trials as a possible method of treating various diseases. However, the effects of long-term cell expansion *in vitro* on physiological function and genetic stability are still poorly understood. In this study, MSC cultures derived from bone marrow and liver were evaluated for the presence of aberrant cells following long-term expansion. In 46 independent cultures, four batches of transformed MSCs (TMCs) were found, which were all beyond the culture period of five weeks. These aberrant cells were first identified based on the appearance of abnormal cytology and the acquirement of growth advantage. Despite common MSC markers being diminished or absent, TMCs remain highly susceptible to lysis by allogeneic NK cells. When transplanted into immunodeficient mice, TMCs formed sarcoma-like tumors, whereas parental MSCs did not form tumors in mice. Using a combination of high-resolution genome-wide DNA array and short-tandem repeat profiling, we confirmed the origin of TMCs and excluded the possibility of human cell line contamination. Additional genomic duplication and deletions were observed in TMCs, which may be associated with the transformation event. Using gene and microRNA expression arrays, a number of genes were identified that were differentially expressed between TMCs and their normal parental counterparts, which may potentially serve as biomarkers to screen cultures for evidence of early transformation events. In conclusion, the spontaneous transformation of MSCs resulting in tumorigenesis is rare and occurs after relatively long-term (beyond five weeks) culture. However, as an added safety measure, cultures of MSCs can potentially be screened based on a novel gene expression signature.

INTRODUCTION

Mesenchymal stromal cells (MSCs), originally identified as a heterogeneous population of non-hematopoietic cells in the bone marrow (BM)[1], have the capacity for extensive expansion *in vitro* and differentiate into multiple mesenchymal lineages, including adipocytes, osteoblasts, and chondrocytes.[2, 3] In addition to BM, MSCs have been identified in a number of postnatal organs where they occupy a perivascular niche.[4] Our group recently demonstrated that the adult human liver harbors a resident cell that is phenotypically and functionally similar to BM-MSCs.[5] Owing to their multi-lineage potency together with immunomodulatory properties[6, 7], MSCs have attracted widespread interest as a method of treating a number of diseases.

Despite compelling data showing efficacy in preclinical studies using MSCs for the treatment of various diseases in small animals, there is a lack of positive data reported in clinical trials to date.[8] One conceivable explanation could be that insufficient amounts of MSCs are applied in the clinical setting, which may not be a major issue in small animal models. Therefore, optimizing expansion of MSCs in culture may be critical for achieving significant clinical benefit by applying sufficient numbers of MSCs in patients. Extensive expansion of any cell *in vitro* however raises important safety issues, as increasing cell doublings and culture times not only increase the risk of altering the phenotype of MSCs but also increase the risk of the acquisition of genetic abnormalities.[9, 10] Thus, there is an ongoing debate regarding the tumorigenic risk associated with long-term expansion of MSCs *in vitro*.

Murine MSCs have been reported to be susceptible to spontaneous transformation in culture.[11-13] In addition, spontaneous transformation of MSCs derived from cynomolgus macaques[14] has been recently reported. Using short tandem repeat (STR) analysis, a type of DNA profiling methodology, the authors verified the origin of the transformed cells excluding human cell line contamination.[15] Human MSCs were thought to be genetically stable in culture even following long-term expansion.[16] The first evidence that human adipose-derived MSCs could undergo spontaneous malignant transformation sparked the flame of a scientific controversy.[17] Another independent study subsequently reported that human BM-MSCs frequently undergo spontaneous transformation after long-term culture[18], whereas an additional study reported that MSCs remain stable after long-term culture.[16] However, these two studies have caused turmoil in the MSCs field, because they later reported that the appearance of transformed malignant cells was due to cross-contamination of human MSCs cultures with a tumor cell line.[19, 20] A recent large-scale analysis of various adult stem cells, including MSCs, has recently demonstrated that they can acquire chromosomal aberrations, which may provide a growth advantage.[9] Because human MSCs can acquire chromosomal abnormalities and MSCs have been reported to give rise to sarcomas[21], there is an urgent need to assess the safety of MSCs following

long-term expansion in culture and to better investigate the possibility of spontaneous malignant transformation during cell culture.

The aim of this study was to investigate the occurrence of spontaneous cellular transformation in an independent series of MSC cultures obtained either from primary human BM or liver. Following long-term expansion of BM- and liver-derived MSCs, four independent cultures out of 46 tested produced transformed cells with tumorigenic potential. High-resolution genome-wide DNA array and STR profiling were used to confirm a shared origin of the transformed cells and parental MSCs. Furthermore, using gene expression analysis and microRNA (miRNA) arrays, we identified a gene expression signature that can be potentially useful for screening of transformation in MSC cultures.

MATERIALS AND METHODS

Isolation and culture of MSCs

The source of the MSCs are described in Supplementary Table 1. Liver MSCs were obtained from explanted livers from patients with end-stage liver disease or from liver graft preservation fluid (perfusates) collected at the time of transplantation. Liver grafts were routinely screened for malignancy. BM-MSCs were either obtained from healthy patients undergoing total hip replacement (provided by the Department of Orthopaedics, Erasmus MC, Rotterdam, The Netherlands)[22] or commercially purchased from Lonza (Breda, The Netherlands). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Lonza, Verviers, Belgium) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 100 IU/ml penicillin and 100 µg/ml streptomycin. The use of liver tissue, perfusates and BM was approved by the medical ethical committee of Erasmus MC. We obtained written informed consent for this original human work.

Flow cytometry

Cells were stained for 30 minutes at 4°C with directly-labeled mouse monoclonal antibodies directed against CD13-PECy7, CD34-APC, CD45-PERCP, HLA-I-APC (BD Biosciences), CD73-PE, CD166-PE (BD Pharma, San Jose, CA) and CD105-FITC (R&D Systems, Abingdon, United Kingdom). Flow cytometry analysis was performed using the FACSCanto II (BD Biosciences) and 10.000 events were collected for analysis performed using FlowJo software.

Gene and miRNA expression profiling by microarray

The total RNA (including small RNA) from parental and transformed MSC cultures was isolated using a Qiagen miRNeasy Mini Kit (Qiagen, Venlo, The Netherlands), and used for genome-wide gene expression analysis with Affymetrix HuGene 1.0 ST Genechips or miRNA Arrays (Affymetrix, Santa Clara, CA) according to the manufacturer's instructions. Transcript levels were generated using RMA as implemented in the Affymetrix Gene Expression Console and probeset annotations were retrieved from NetAffx using the same

software. Probesets differentially expressed among conditions were analyzed using Partek Genomics Suite (Partek Inc., Saint Louis, MO). Pathway analysis was performed using Ingenuity Pathway Analysis (Ingenuity Systems, Redwood City, CA).

High-resolution genome-wide DNA analysis

Genomic DNA of parental and transformed MSCs was isolated using a QIAamp DNA Blood Mini Kit (Qiagen) according to the manufacturer's instructions. High-resolution genomic DNA profiling was performed using the Affymetrix Cytogenetics Whole-Genome 2.7M Array. This array provides unbiased, whole-genome coverage with the highest density of 2.7 million markers to enable superior resolution for the detection of chromosome aberrations. Data analysis was performed with Affymatrix Chromosome Analysis Suite that contains a build-in human genome as a reference. To generate a karyoview of TMCs and MSCs, a 750 KB cutoff was used to detect genome loss or gain variations.

Short Tandem Repeat profiling

The parental and transformed MSCs were genotyped by short tandem repeat (STR) profiling using the PowerPlex 16 System (Promega), as previously described.[23] The PowerPlex 16 system is composed of 15 STR loci. Amplification was done using 1 ng of template DNA, which was applied to the PowerPlex 16 system following the manufacturer's recommendations. Multiplex PCR reactions were carried out using fluorescent dye–linked primers. Labeled products were detected by electrophoretic size fractionation on an ABI 3100 genetic analyzer. The data were analyzed using Genescan and Genotyper software (Perkin-Elmer) to categorize peaks according to their size in relation to an internal standard run. This analysis enabled every peak to be allocated a size corresponding to the number of repeat units present.

Real time-quantitative polymerase chain reaction

Total RNA was isolated by a Qiagen miRNeasy Mini Kit and quantified using a Nanodrop ND-1000 (NanoDrop Technologies, Wilmington, DE). For gene expression, complementary DNA (cDNA) was prepared from the total RNA using an iScript cDNA Synthesis Kit from Bio-Rad (Bio-Rad Laboratories, Stanford, CA). Gene expression was quantified using real-time PCR (MJ Research Opticon, Hercules, CA) using SYBR Green (Sigma-Aldrich, Zwijndrecht, The Netherlands) according to manufacturer's instructions. GAPDH was used as a reference gene to normalize gene expression. For miRNA expression, cDNA was prepared by a TaqMan microRNA reverse transcription kit (Applied Biosystems, Carlsbad, CA). RT-qPCR reactions were performed using a TaqMan Universal PCR Master Mix and microRNA-specific PCR primers (Applied Biosystems) according to the manufacturer's instructions. A small nuclear RNA, RNU43, served as a reference gene.

MTT assay

Parental MSCs or TMCs (5 x 10^3 cells) were plated onto 96-well plates. At the indicated times, the number of metabolically active cells was quantified by the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay (0.5 mg/ml).

Cytotoxicity-mediated lysis by europium release assay

Cytotoxicity-mediated lysis of target cells was determined by europium release assays as previously described.[24] For effector cells, PBMCs derived from healthy volunteers were stimulated with allogeneic γ-irradiated PBMCs and 200 IU/ml IL-2 (proleukin; Chiron BV, Amsterdam, The Netherlands) in round-bottom 96-well plates for 7 days at 37°C. MSCs were used as target cells.

Maximum release of europium by target cells was measured by incubation of 5 × 10^3 labeled target cells with 1% triton (Sigma-Aldrich) for 4 hours. Spontaneous release of europium by target cells was measured by incubation of labeled target cells without effector cells. The percentage of leakage was then calculated as follows: (spontaneous release/maximum release) × 100%. Finally, the percentage of cytotoxicity-mediated lysis was calculated as follows: % lysis = [(measured lysis − spontaneous release)/(maximum release − spontaneous release)] × 100%.

Engraftment of cells into immunodeficient mice

Immunodeficient NOD/SCID mice (Erasmus MC institutional breeding) were maintained in the Erasmus MC animal facility on a 12/12 hour light/dark schedule. The animals had free access to food and drinking water. When reaching the age of 6-8 weeks, the mice were subcutaneously injected with 1 × 10^6 parental MSCs or TMCs (two independent batches into four mice) labeled with or without luciferase reporter gene, as previously described.[5] Luciferase activity was measured at the indicated time points by an IVIS camera in living animals. One pair of luciferase labeled TMCs and MSCs were injected at different sites in the same recipient NOD/SCID mouse.

To avoid discomfort caused by the overgrowing tumor, mice were sacrificed after 4 weeks by CO_2 inhalation. The tumors were resected for histologic evaluation and partly dissociated for tumor cell cultures. The use of animals was approved by the institutional animal ethics committee.

Statistical analysis

Statistical analysis was performed with either the paired nonparametric (Wilcoxon signed-rank) test, or the unpaired nonparametric (Mann–Whitney) test, using GraphPad InStat software (GraphPad Software Inc., San Diego, CA). P-values <0.05 were considered statistically significant.

RESULTS

Spontaneous transformation of human BM- and liver-derived MSCs after long-term culture expansion

MSC cultures were obtained from human bone marrow (n=5), liver biopsies (n=12) or liver graft preservation fluid (n=29). Donor and culture characteristics are shown in Supplementary Table 1. In four independent MSC cultures beyond five weeks of *in vitro* expansion, we observed aberrant looking cells (transformation) with an epithelial-like cytology. Initially, these aberrant MSCs appeared as a few scattered colonies (Figure 1A), but gradually these cells acquired a growth advantage and overgrew the normal appearing MSCs in the culture (Figure 1B). Quantification of the viable cells confirmed the accelerated proliferation of the aberrant MSCs compared to parental MSC cultures (Figure 1C). These aberrant MSCs could be expanded in serum-containing medium for at least 30 passages over a three month period without any evidence of senescence (data not shown). For liver-derived MSCs, obtained from either preservation fluid or tissue biopsies, we observed two cultures with aberrant cells in forty-one independent cultures (Supplementary Table 1). For BM-derived MSCs, we observed two out of five cultures positive for aberrant cells. Importantly, all samples were obtained from donors with no evidence of pre-existing malignancy.

Aberrant MSCs have tumorigenic potential

We next investigated the tumorigenic potential of the aberrant MSCs. For this, 1.0 x 10^6 unlabeled aberrant MSCs or MSCs labeled with a luciferase reporter gene were subcutaneously injected in NOD/SCID mice. Within a period of four weeks, both unlabeled and luciferase-labeled aberrant MSCs formed solid tumors following subcutaneous injection (n=4; Figure 2A). The formation of solid tumors under the skin was visible as early as two weeks after injection. Due to symptoms related to tumor burden, mice were sacrificed 4-5 weeks post-injection and the tumors were harvested. Histological examination of the tumor tissue revealed a sarcoma-like pathology (Figure 2B).

To confirm that the tumorigenicity was due to spontaneous transformation, we simultaneously injected luciferase labeled TMCs and normal MSCs derived from the same origin at different sites in the recipient NOD/SCID mouse. As shown in Figure 2C, the luciferase signal from TMCs dramatically increased with time, resulting in the formation of palpable tumors. In contrast, the signal from the normal parental MSCs diminished over time and by 4 weeks was no longer visible. This is in agreement with our previous data[5] showing no tumor formation after engraftment of both BM- or liver-derived MSCs, either subcutaneously or intrahepatic (n=14). Following dissociation of the explanted tumor tissue, viable cells were cultured, which were termed T-TMCs. These cells demonstrated a similar cytology and proliferation capacity as primary TMCs prior to injection, and could be expanded for at least five passages tested so far (Figure 3). These data confirmed the tumorigenicity and malignancy status of these transformed cells.

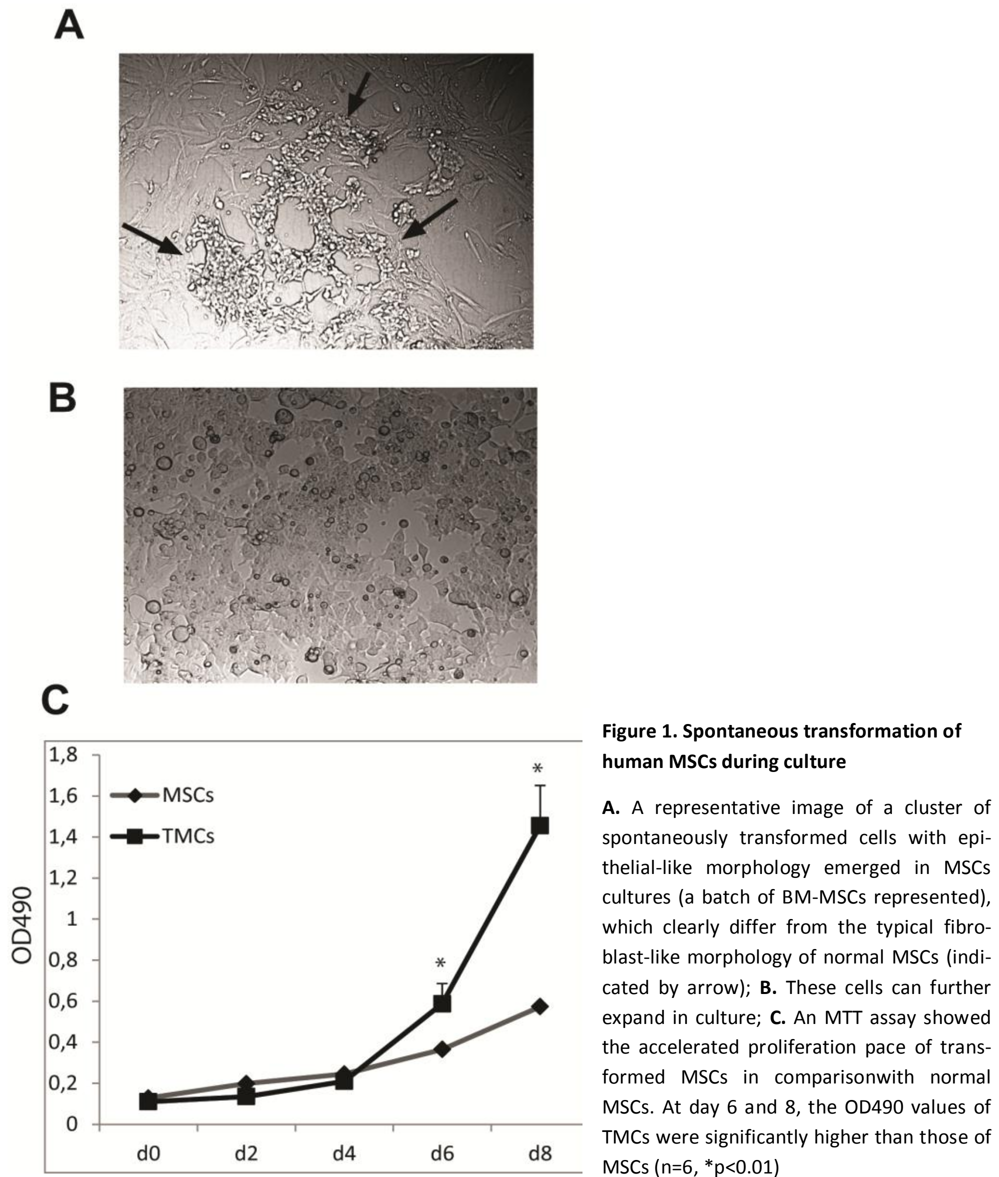


Figure 1. Spontaneous transformation of human MSCs during culture

A. A representative image of a cluster of spontaneously transformed cells with epithelial-like morphology emerged in MSCs cultures (a batch of BM-MSCs represented), which clearly differ from the typical fibroblast-like morphology of normal MSCs (indicated by arrow); **B.** These cells can further expand in culture; **C.** An MTT assay showed the accelerated proliferation pace of transformed MSCs in comparisonwith normal MSCs. At day 6 and 8, the OD490 values of TMCs were significantly higher than those of MSCs (n=6, *p<0.01)

Common MSC markers are diminished in TMCs, and TMCs remain susceptible to cell lysis by natural killer cells

We next compared the antigenic profile of TMCs to parental MSCs using flow cytometry. The common MSC markers CD13, CD73, CD105, and CD166 were downregulated in TMCs compared to their normal parental counterparts (Figure 4). However, the common hematopoietic markers CD34 and CD45 remained negative. In addition, we also showed that T-TMCs harvested from tumors grown in NOD/SCID mice and expanded *in vitro* possess a similar antigenic profile as the TMCs, which was unexpected.

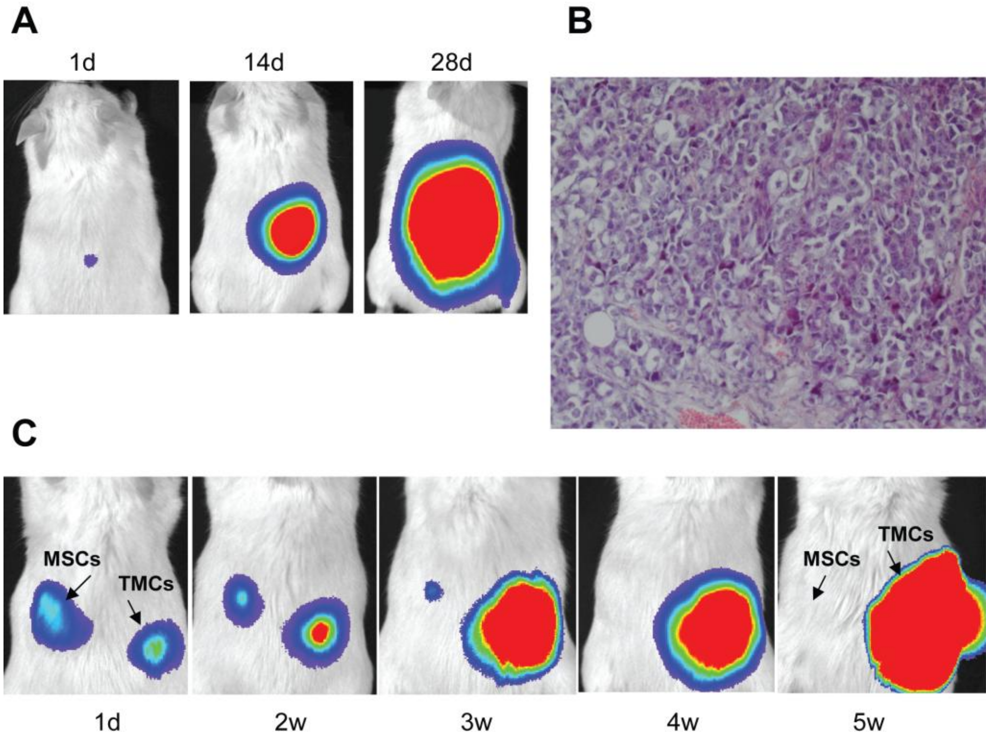


Figure 2. Transformed, but not parental MSCs, are tumorigenic in immunodeficient mice

A. BM- or liver-derived TMCs were labeled with a lentiviral expressed luciferase gene and subcutaneously injected into immunodeficient NOD/SCID mice (n=4). Luciferase activity was longitudinally monitored by an IVIS camera in living mice at indicated time points; **B.** H&E stained tumor sections showing a liposarcoma-like histology; **C.** Simultaneous engraftment of one pair of luciferase labeled TMCs and MSCs into the right and left lower flanks of NOD/SCID mice, respectively. The luciferase signal from TMCs dramatically increased, whereas the signal from MSCs decreased over time.

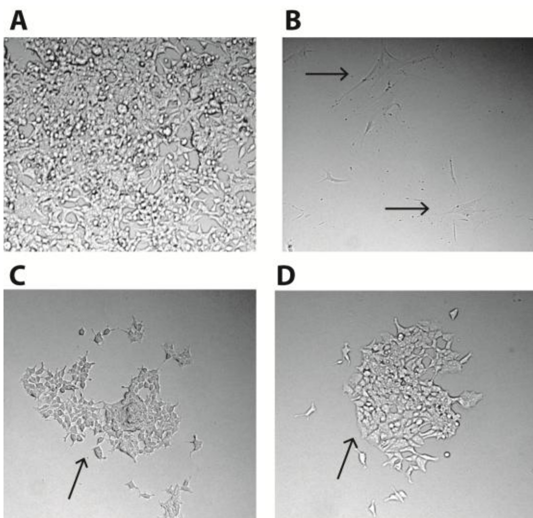


Figure 3. Cell culture expansion of T-TMCs

A. T-TMCs harvested from tumors formed by TMCs in NOD/SCID mice could be expanded in cell culture. A cell proliferation assay performed by plating 1000 cells in a 12-well plate and culturing for 8 days showed the growth superiority of **C.** TMCs and **D.** T-TMCs, compared with **B.** MSCs.

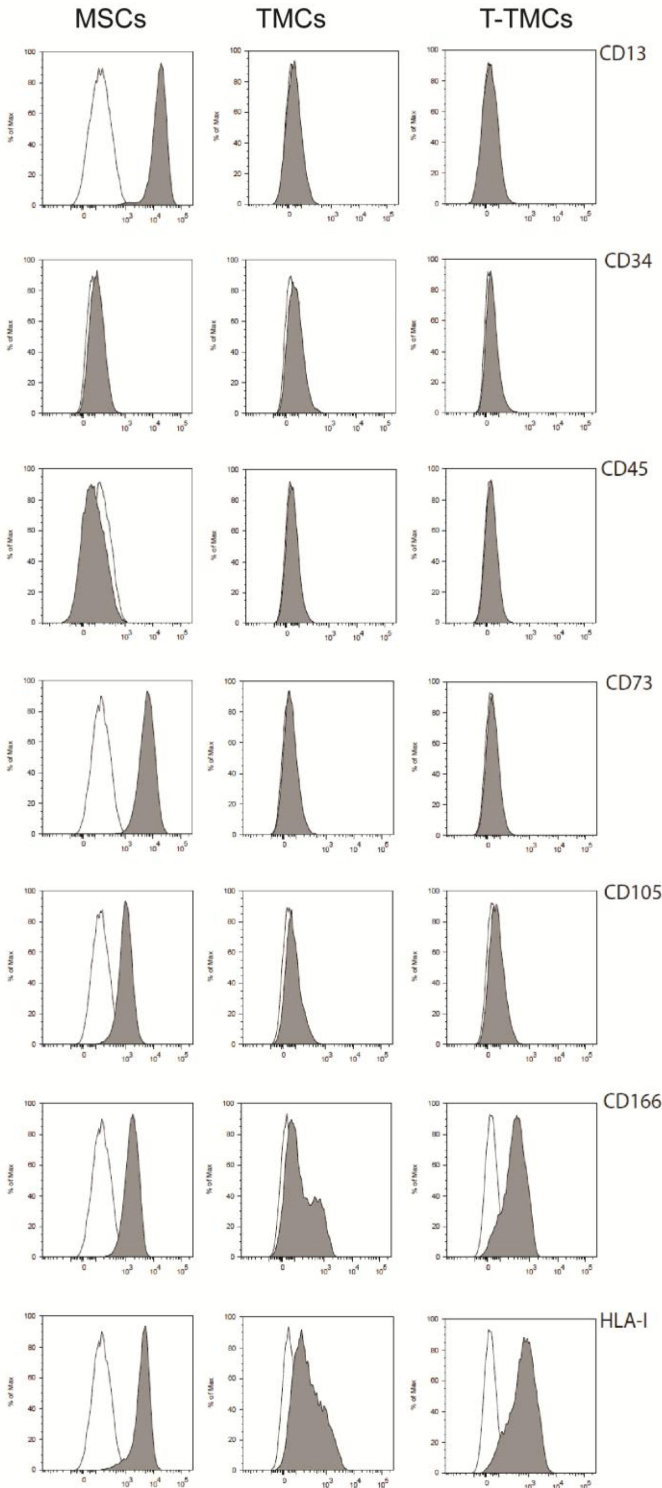


Figure 4. Transformed MSCs lose mesenchymal markers

Flow cytometric analysis revealed downregulation of the common MSC markers CD13, CD73, CD105 and CD166, whereas the hematopoietic markers CD34 and CD45 remained negative. MSCs, normal MSCs; TMCs, transformed MSCs and T-TMCs, tumor cells isolated from TMCs-formed solid tumors in mice. This figure shows one representative transformed BM-MSCs batch.

It is known that MSCs are susceptible to rapid killing by natural killer (NK) cells, which is presumed to contribute to the destruction of allogenic MSCs after transplantation into an immune competent host.[25, 26] We next investigated whether transformed MSCs remain susceptible for lysis by NK cells. As shown in Figure 5, TMCs were highly susceptible to lysis by activated NK cells. In addition, T-TMCs cultured from explanted tumor tissue from NOD/SCID mice were lysed by NK cells, comparable to MSCs and TMCs.

Short tandem repeat analysis authenticates the origin of the transformed MSCs excluding the possibility of human cell line contamination.

To address the identity of the TMCs, we used DNA profiling and short tandem repeat (STR) analysis. Genomic DNA from both TMCs and parental MSCs was subjected to high-resolution genome DNA profiling using the Affymetrix Cytogenetics Whole-Genome 2.7M Array. As shown in Figure 6A, an identical signature in copy number variation (CNV) was observed between one pair of TMCs and MSCs, providing evidence that both cell types were of the same origin. Of note, these common CNVs were either inherited or gained *de novo* during the culture of parental MSCs before transformation. In contrast, the karyoview of two individuals, generated from genomic DNA of peripheral blood mononuclear cells (PBMCs) and analyzed by 750 KB cutoff as well, displayed distinct CNV patterns (data not shown).

To authenticate the origin of the TMCs, we profiled 15 STR loci and one sex chromosome marker Amelogenin from two independent pairs of TMCs (Figure 6B). Based on the STR analysis, parental and transformed MSCs have an identical STR signature. We also compared the data obtained from the STR profiling to the ATCC STR database and no hits were obtained, which further excluded the possibility of human cell line contamination.

Further analysis of the cytogenetics array showed additional unique CNVs present in TMCs (data not shown). In particular, chromosome 1 abnormalities were present in TMCs compared with parental MSCs. Chromosome 1 abnormalities are a frequent occurrence in cancer[27], however, the role this plays in contributing to the growth advantage and tumorigenic potential in our study requires further clarification.

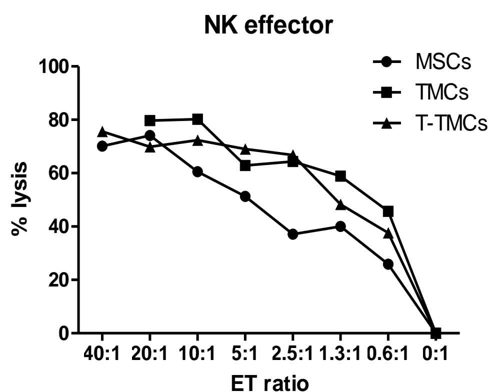


Figure 5. TMCs are susceptible for lysis by NK cells

Effector and target cells were cultured at indicated ratios. MSCs, normal MSCs; TMCs, transformed MSCs and T-MCs, tumor cells isolated from TMCs-formed solid tumors in mice.

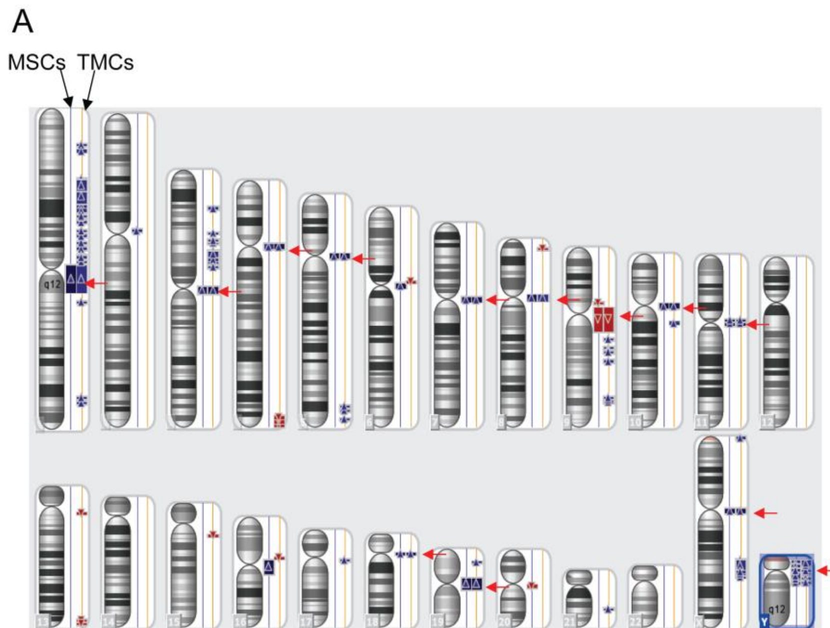


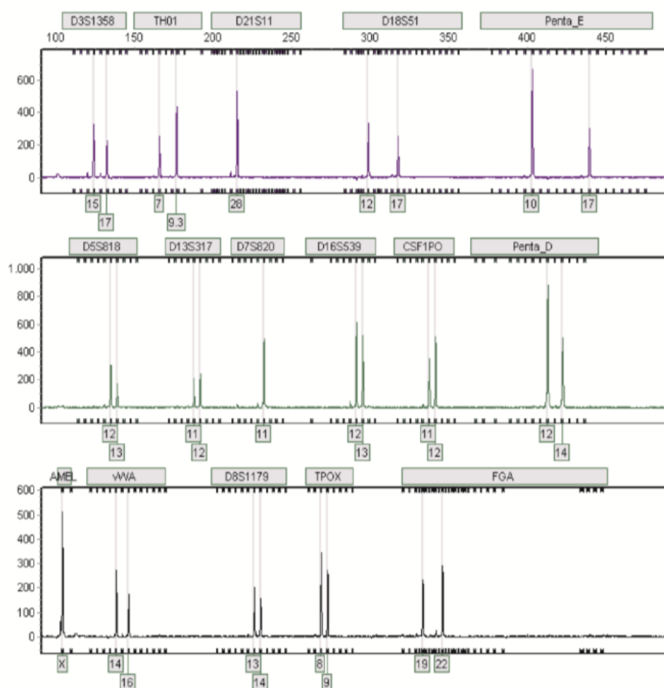
Figure 6. Genomic DNA comparison of parental and transformed MSCs

A. High-resolution Affymetrix Cytogenetics Whole-Genome 2.7M Array was used to map the genomic DNA. To generate a clear karyoview of transformed MSCs (TMCs) and parental MSCs, a 750 KB cutoff was used to detect genome loss (red) or gain variations (blue) as referred to the human genome built in the Affymatrix Chromosome Analysis Suite software. An identical signature (arrow indicated) of copy number variation (CNV) was observed between TMCs and MSCs, supporting the same origin of the cells. These common CNVs were either inherited or gained *de novo* during the culture of parental MSCs before transformation; **B.** STR profiling of transformed and parental MSCs. STR loci are indicated in boxes above the electropherogram; numbers of repeat units are indicated below the peaks. Two pairs of transformed and parental MSCs showed identical profiles, suggesting that *de novo* transformation, not cell-contamination, occurred.

TMCs demonstrate unique global gene and miRNA expression changes

We next characterized the genomic features of the TMCs by performing genome-wide gene expression and miRNA arrays using total RNA isolated from TMCs and parental MSCs. The expression of 3598 genes (28870 probesets in total) were found to be up- or downregulated more than two fold in TMCs versus MSCs. The global function of these differentially expressed genes was subjected to a functional and pathway analysis using Ingenuity Pathway Analysis software, which identified 31 canonical pathways that were significantly altered between MSCs and TMCs (B-H multiple testing correction $P<0.05$; Figure 7A). Many of these pathways were associated with cell cycling or cancer signaling.

Genome-wide profiling identified 23 miRNAs that were upregulated more than 10 fold (Figure 7B) and 41 that were downregulated over 10 fold in TMCs compared to parental MSCs (Figure 7C). Overall, a greater number of miRNAs were downregulated in TMCs, which is consistent with a previous report describing a general down regulation of miRNAs

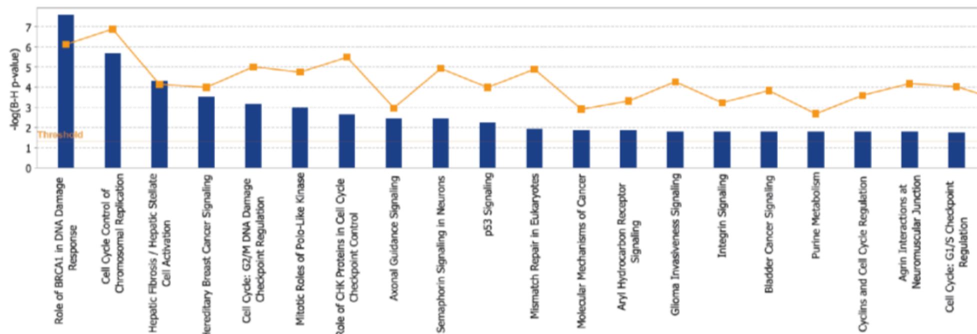


	Penta_E	D18S51	D21S11	TH01	D3S1358	FGA	TPOX	D8S1179	vWA	Penta_D	CSF1PO	D16S539	D7S820	D13S317	D5S818	Amg
MSC-1	10, 17	12, 17	28	7, 9.3	15, 17	19, 22	8, 9	13, 14	14, 16	12, 14	11, 12	12, 13	11	11, 12	12, 13	X
MSCT-1	10, 17	12, 17	28	7, 9.3	15, 17	19, 22	8, 9	13, 14	14, 16	12, 14	11, 12	12, 13	11	11, 12	12, 13	X
MSC-2	7, 15	17, 18	28, 30.2	7, 9.3	15, 16, 17	23	10, 1 1	11, 12, 14	17, 19, 20	9, 10	11, 12	9, 13	10, 11	12	8, 9	X
MSCT-2	7, 15	17, 18	28, 30.2	7, 9.3	15, 16, 17	23	10, 1 1	11, 12, 14	17, 19, 20	9, 10	11, 12	9, 13	10, 11	12	8, 9	X

in various cancers.[28] In particular, the let-7 family, comprised of 12 family members (let-7-a1, a2, a3, b, c, d, e, f1, f2, g, i and miR-98), have been described as major guardians against pluripotency and cancer progression, and are often found to be downregulated during cancer progression.[29] Four of these members were down regulated over ten fold in TMCs, namely let-7i (58-fold), let-7b (43-fold), let-7c (14-fold) and miR-98 (11-fold) (Figure 7C). Notably, among the most highly upregulated miRNAs in TMCs (Figure 7B), miR-378 (178-fold) is known to promote cell survival, tumor growth and angiogenesis[30] and miR-183 (61-fold) has oncogene function and been shown to be overexpressed in tumor tissues.[31] MiRNA-199a-3p was downregulated in TMCs by 195 fold; this is consistent with a recent study that miRNA-199a-3p is downregulated in human osteosarcoma and regulates cell proliferation and migration.[32]

For integrative analysis of gene and miRNA regulation, we combined the 2-fold differentially expressed gene list with the 10-fold differentially expressed miRNA list and then subjected them to Ingenuity Pathway Analysis. Within the 25 networks analyzed, 7 networks were composed of an interaction of miRNAs with genes, including the top second,

A



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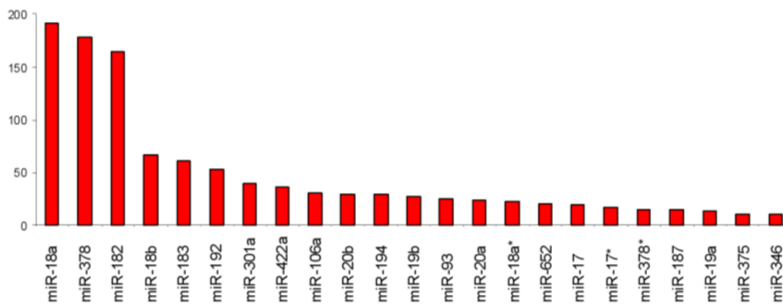

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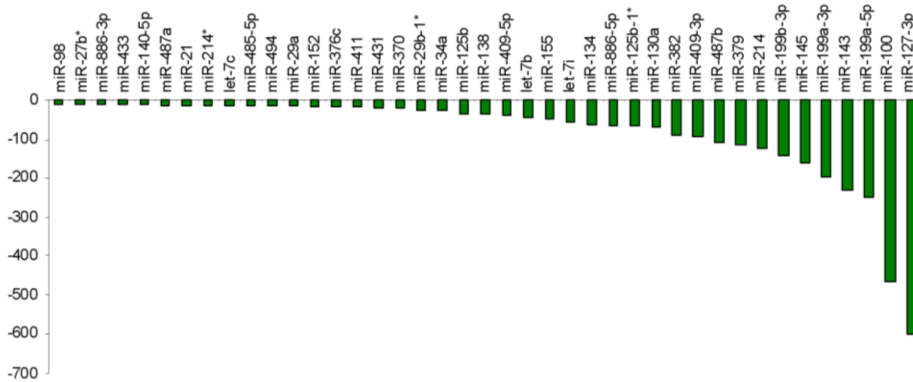


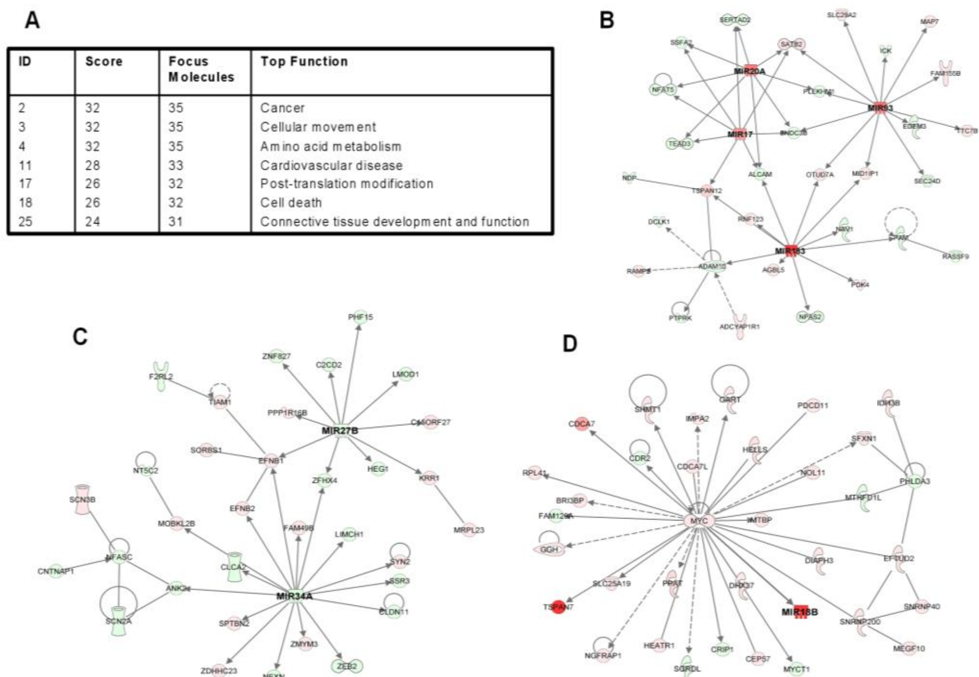
Figure 7. Gene/miRNA expression profiling and pathway analysis

A. The expression of 3598 genes (28870 probesets total) were found to be up- or downregulated more than two fold in a paired TMCs versus MSCs analysis. Pathway analysis identified 31 canonical pathways significantly enriched for these genes (B-H multiple testing correction P<0.05), that were associated with cell cycling or cancer signaling; **B.** Genome-wide profiling of 853 human miRNAs identified 23 miRNAs that were upregulated more than ten fold and **C.** 41 that were downregulated over ten fold in TMCs compared to parental MSCs.

third and fourth scored networks (Figure 8A). The functions of these top significant networks are associated with cancer (Figure 8B), migration (Figure 8C) and amino acid metabolism (Figure 8D). Taken together, the integrative molecular mapping reflects the transformed status of the MSCs.

Screening for spontaneous transformation using a gene and miRNA expression signature

Plastic adherence is a defining feature of MSCs.[33] However, we observed that aberrant cells readily detached from the plastic surface upon applying shear stress in each independent culture. As shown in Figure 9A, applying shear stress resulted in the complete detachment of TMCs cells without affecting the attachment of the normal MSCs present in the same culture flask. Since the culture of clinical grade MSCs often use adherent plastic flasks[34], potentially transformed cells can be removed by mechanically detaching low-adhesive cells and these cells can be harvested by centrifugation of the discarded culture medium.



ID	Score	Focus Molecules	Top Function
2	32	35	Cancer
3	32	35	Cellular movement
4	32	35	Amino acid metabolism
11	28	33	Cardiovascular disease
17	26	32	Post-translation modification
18	26	32	Cell death
25	24	31	Connective tissue development and function

Figure 8. Integrative analyze genome-wide gene and miRNA regulation in TMCs Vs MSCs

The combined 2-fold differentially expressed gene list with 10-fold differentially expressed miRNA list was subjected to Ingenuity Pathway Analysis; **A.** Within the 25 networks analyzed, 7 networks were composed of the interaction between miRNAs and genes, including the top second, third and fourth scored networks. The functions of these most significant networks are associated with **B.** cancer, **C.** cellular movement and **D.** amino acid metabolism. Red, upregulation; Green, downregulation.

We next selected a set of genes and miRNAs, which we had found to be highly expressed in TMCs (Figure 7) and confirmed their relative expression in three independent pairs of MSCs and TMCs using qRT-PCR. As shown in Figure 9B, CKMT1A was elevated over ten thousand-fold, and several other genes were also elevated over a thousand fold in TMCs. Similarly, miR-182 and miR-378 were elevated approximately 500- and 100-fold respectively (Figure 9C). To determine the sensitivity of detection of TMCs, we performed mixed culture experiments with different ratios of TMCs and MSCs. After mechanical pipetting to dissociate low adhering cells, the supernatant was collected and the cells were harvested. Quantitative RT-PCR was performed on the genes CKTM1A, IRS4 and HOXD10, to detect transformed MSCs (Figure 9D). Transformed cells could be identified at a resolution of 1:10.000 by screening the collected low adhesion cells using this panel of genes and miRNAs by quantitative RT-PCR.

DISCUSSION

The study of transformation of mammalian cells in culture has flourished over decades. Virus-induced, carcinogen-induced or spontaneous transformation has been investigated mainly for the purpose of serving as an assay for titrating viruses, evaluating specific carcinogens or studying the mechanisms of carcinogenesis.[35] The first report of the spontaneous malignant transformation of cultured primary cells was published in 1941, where transformed rat fibroblasts in culture were able to form sarcomas when transplanted into rats.[36] Meanwhile, another study reported that transformed mouse fibroblasts also gave rise to sarcomas after injection into the mice of the inbred strain of origin.[37]

In the 1950s, it was discovered that the bone marrow harbors mesenchymal stem/stromal cells, which have a typical fibroblast-like morphology. More recent studies report the presence of MSCs in other compartments including adult adipose tissue, dermal tissues, spleen, liver as well as umbilical cord blood and various fetal tissues. Since the therapeutic application of MSCs requires an extensive expansion of the cells in culture, the study of tumorigenic transformation of MSCs is an important clinical issue. Spontaneous transformation of murine and monkey MSCs has previously been observed[11-15], and the accumulation of chromosomal instability as a result of expansion in culture has been implicated as the potential mechanistic cause.[11] Although human MSCs are genetically more stable compared with murine MSCs, genomic instability has also been reported in cultured human MSCs.[38] However, controversy regarding the transformation of MSCs remains, as two recent reports that initially described MSC transformation following cell culture were retracted due to the discovery of contamination of the primary cell cultures with tumor cell lines.[19, 20] In our study, we observed the spontaneous transformation of human liver- and BM-derived MSCs after long-term (more than five weeks) expansion *in vitro* with tumorigenic consequences. The epithelial-like cells in MSC cultures acquired a growth advantage and eventually overgrew the normal MSCs. These transformed cells formed sarcoma-like tumors after injection into NOD/SCID mice, supporting their tumorigenicity *in*

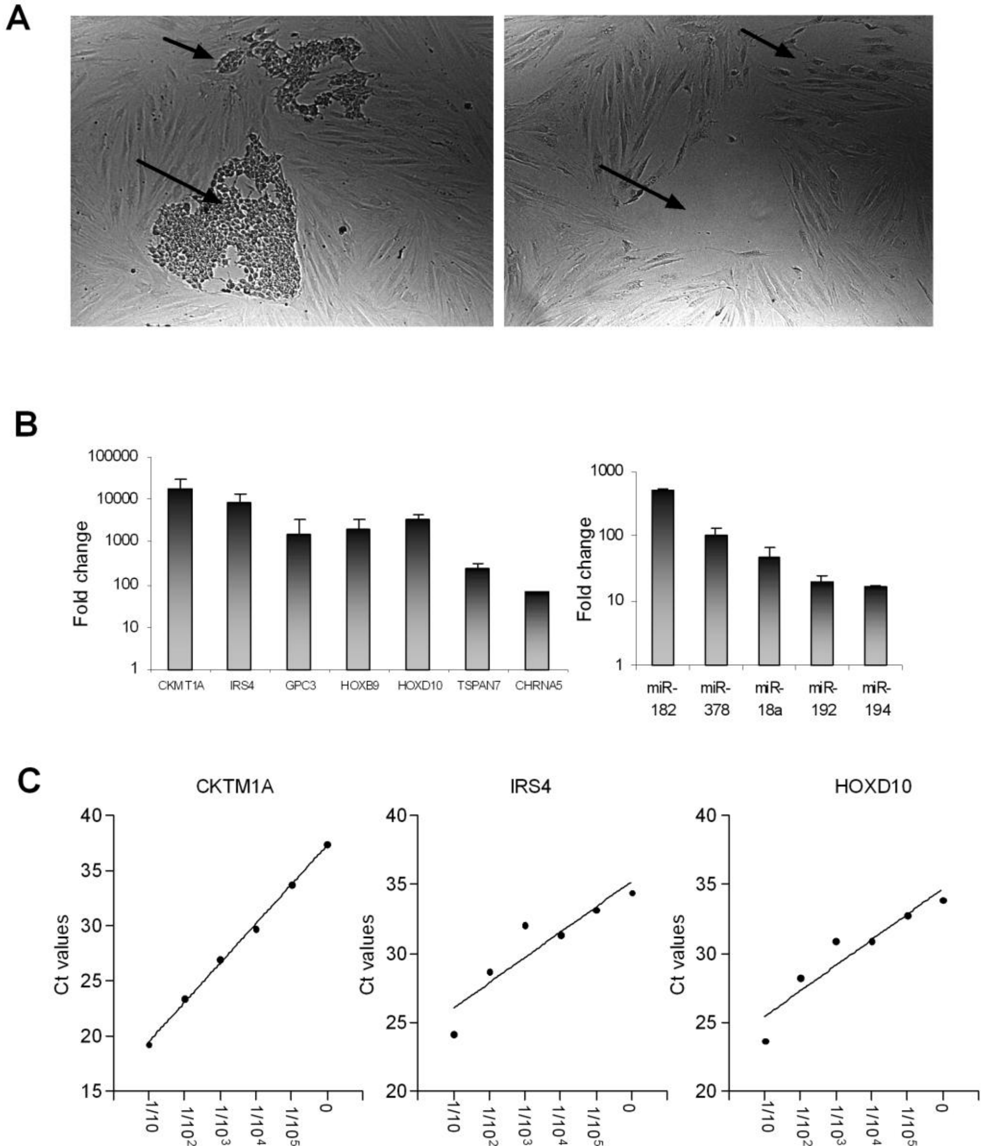


Figure 6 Screening transformed MSCs using biomarkers. (A) Transformed MSCs (arrow, a batch of BM MSC represented) were observed as low plastic adhering cells, which can be mechanically detached without affecting normal MSCs. A set of genes (B) and miRNAs (C) were found to be dramatically up-regulated in transformed MSCs. (D) TMCs and MSCs were cultured at indicated ratios. After mechanical pipetting to dissociate low adhering cells, the supernatant was collected and the cells were harvested by centrifugation. QRT-PCR was carried out using CKTM1A, IRS4 and HOXD10 to detect transformed MSCs. The X-axis indicates the Ct value of qRT-PCR and the Y-axis indicates the ratio of mixed to transformed MSCs. The data represents one of the three independent experiments.

vivo. We authenticated the origin of the transformed MSCs using both high-resolution genome-wide DNA analysis and STR profiling, a standard technique for characterizing cell identity[39], and our results demonstrate that the transformed MSCs originated from the original MSCs donors, but not due to tumor cell contamination as previously described.[19, 20] Further characterization of transformed MSCs by genome-wide gene and miRNA expression with integrated pathway analysis confirmed their tumorigenic status. Interestingly, solid evidence based on transgenic mice and the genetic investigation of MSCs has placed these cells as the most likely cell of origin for certain sarcomas.[21] In addition, a clinical study reported the development of osteosarcoma in a recipient 17 years after bone marrow transplantation. They further demonstrated the osteosarcoma cell line established from the recipient's tumor carried a marker of chimerism and expressed a panel of MSC markers, suggesting its origin as being from donor MSCs.[40]

The therapeutic potential of adult stem cell therapies, including MSCs, is promising due to the growing need for new medicins to treat a number of debilitating diseases, which currently lack adequate treatments.[41-43] However, there is a growing need to develop novel methods to assess the safety of expanded adult stem cells. One of the major concerns, yet still controversial, is the tumorigenic potential of adult stem cell cultures, including MSCs. Although limited in their differentiation potential, there is increasing evidence that MSCs do contribute, in part, to the growth and spread of solid tumors. However, one of the limiting factors in the MSC field is bioequivalence. It is still not known whether BM-MSCs, which represent the main cell type used in the human clinical trials to date, and stromal cells found in the tumor microenvironment are bioequivalent. Second, and even more controversial, is whether MSCs themselves are tumorigenic, such as has been suggested for certain soft tissue sarcomas. Clearly, the debate still continues and needs to be clarified.

One of the defining features of human MSCs is their dependence on anchorage, allowing their adherence to plastic surfaces. We noted that in the MSC cultures, aberrant cells had low adhesive properties. Transformed MSCs appeared after long-term culture, indicating that early passage cultures are likely safe to use. Furthermore, we have identified a panel of highly upregulated miRNAs and genes in transformed MSCs using genome-wide miRNA and gene expression arrays, which could be used as potential biomarkers to screen for transformed cells during culture. In fact, the establishment of safety evaluation makers is one of top priorities in the stem cell field.[44] Using the low adhesive properties of transformed MSCs, the sensitivity of screening assays could be dramatically increased by harvesting only the low adhesive cells. Obviously, heterogeneity probably exits that not all the transformed cells express the same markers. Thus, the utility of this approach still requires further investigation and validation.

In summary, we observed the tumorigenic transformation of human MSCs during long-term culture expansion, which was not due to contamination of human cell lines. Furthermore, we identified a number of genes and miRNAs using gene arrays and qRT-PCR

that may potentially be used to screen for transformation events in long-term cell culture. This approach would be a significant step towards the clinical application of stem cell therapy, and would help to alleviate the concern of transplanting malignant cells into patients.

Acknowledgments

The authors thank Dr. Martin Hoogduijn for technical support and Dr. Ram Siddappa (University of Leiden) for critical discussions. General financial support was provided by The Foundation for Liver and Gastrointestinal Research (SLO) Rotterdam. Support from the Netherlands Organization for Scientific Research (NWO), in the form of VENI-grant 916-13-032, is gratefully acknowledged by Q. P.

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Chapter 7

Studying paracrine effects of mesenchymal stromal/stem cell-derived factors *in vivo* on liver injury and regeneration

Suomi M.G. Fouraschen, Jeroen de Jonge and Luc J.W. van der Laan

Department of Surgery, Laboratory of Experimental Transplantation and Intestinal Surgery, Erasmus MC-University Medical Center, Rotterdam, The Netherlands

(Methods in Molecular Biology, book chapter, conditionally accepted)

ABSTRACT

Mesenchymal stromal/stem cells (MSCs) have multilineage differentiation potential and as such are known to promote regeneration in response to tissue injury. However, accumulating evidence indicates that the regenerative capacity of MSCs is not via transdifferentiation but mediated by their production of trophic and other factors that promote endogenous regeneration pathways of the tissue cells. In this chapter, we provide a detailed description on how to obtain trophic factors secreted by cultured MSCs and how they can be used in small animal models. More specific, in vivo models to study the paracrine effects of MSCs on regeneration of the liver after surgical resection and/or ischemia and reperfusion injury will be described.

INTRODUCTION

The potential role of stem and progenitor cells as a therapeutic strategy for tissue injury or disease is widely being investigated. In recent years, stem/progenitor cells have been successfully applied in experimental models to treat several inflammatory and autoimmune diseases, including graft-versus-host disease, systemic lupus erythematosus, multiple sclerosis, type I diabetes and inflammatory bowel disease.[1-5] Treatment with stem/progenitor cells resulted in decreased immune cell infiltration, reduced production of auto-antibodies and improvement of survival. Furthermore, multiple studies have shown that stem/progenitor cells are also effective in mediating tissue repair. Beneficial effects have been reported in case of myocardial infarction, cornea damage as well as spinal cord, lung and skin injury.[6-10]

Another research area in which stem cell-based treatment strategies have been brought forward as a promising new therapeutic intervention is the field of liver regeneration. The liver has the remarkable capacity to regenerate in order to compensate for lost or damaged liver tissue after injury, a process that enables large (oncologic) liver resections and living donor liver transplantation. However, after surgery for malignancies, regeneration is often compromised due to neo-adjuvant chemotherapy, poor nutritional status and increasing age of the patient population, thereby restricting surgical treatment options.[11-14] In the setting of living donor liver transplantation, on the other hand, both donor and recipient end up with a small-for-size liver, associated with significant morbidity and mortality.[15-17] In this situation, both loss of a substantial part of the liver mass as well as oxidative stress after ischemia and reperfusion are major mechanisms of hepatic injury.[18, 19] Potential therapeutic strategies to improve liver regeneration and stimulate recovery are therefore most welcome.

Several studies describe the ability of stem cells, especially MSCs, to promote liver regeneration after toxic injury and protect against fulminant hepatic failure.[20-23] MSCs have the ability to differentiate into hepatocytes and cholangiocytes and induce immunomodulatory and anti-inflammatory responses.[9, 24-27] Furthermore, they are described to promote angiogenesis by upregulating the expression of pro-angiogenic factors.[28, 29]

MSCs can be obtained from multiple different sources.[30, 31] The first described and most widely used source of MSCs for regenerative purposes is bone marrow. Alternative and more accessible sources include cord blood and adipose tissue. Our group has shown that the adult human liver harbors a population of MSCs, which is mobilized from liver grafts at time of transplantation.[32] These liver-derived MSCs (L-MSCs) can be retrieved from the organ preservation solution and, similar to bone-marrow MSCs, appear to have immunosuppressive capacities as well as multi-lineage differentiation potential. Furthermore, we have reported that the trophic factors secreted by these L-MSCs stimulate liver regeneration after surgical resection, mainly by promoting hepatocyte proliferation and altering expression levels of regeneration-related genes.[33]

Beneficial effects of MSC-secreted factors have also been reported in the setting of toxic liver injury and hepatic failure.[34, 35] In a clinical setting, the use of MSC-derived factors may have several advantages over the use of MSCs, since there is no risk of rejection or possible malignant transformation and the factors can be produced in large clinical grade quantities. In this chapter, we describe the technical aspects to produce MSC-conditioned culture medium (MSC-CM), including serum free culturing and the concentration of MSC-CM. Furthermore, we outline several procedures to study the effects of MSC-derived factors in vivo on liver regeneration, using partial hepatectomy and/or ischemia and reperfusion injury models in mice.

MATERIALS

The materials listed do not include standard equipment used in cell culture labs or animal facilities. Tubes, vials and reagents used for cell culture should be sterile. Reagents should be stored according to the manufacturers' description, unless otherwise described. Materials in italic are only needed if chosen to freeze cells down for storage prior to use.

Isolation of MSCs for primary cultures

1. 50 ml conical tubes
2. DMEM medium (Lonza)
3. Ficoll-Paque Plus (GE Healthcare)
4. Trypan Blue
5. *1 ml cryovials*
6. *Freezing container for cryovials*
7. *Medium A: 80% DMEM + 20% fetal bovine serum (FBS; Sigma-Aldrich); store at 4°C (see* ***Note 1****)*
8. *Medium B: 60% DMEM + 20% FBS + 20 % dimethylsulfoxide (DMSO); store at 4°C (see* ***Note 1****)*

Culturing system for MSCs

1. Serum-containing culture medium: DMEM, 10-15% FBS, 1% L-Glutamin (Lonza) and 1% penicillin/streptomycin solution (Invitrogen); store at 4°C (see **Note 2**)
2. T75 culture flasks (Corning)
3. Trypsin/EDTA (Invitrogen)
4. *15 ml tubes*
5. *FBS*
6. *Phosphate buffered saline (PBS; Sigma-Aldrich)*
7. *Trypan Blue*

Concentration of MSC-secreted factors

1. PBS
2. Serum-free culture medium: MEM-alpha (Invitrogen), 0.05% bovine serum albumin (Sigma-Aldrich), 1% L-Glutamin, 1% penicillin/streptomycin solution; store at 4°C
3. 50 ml syringes
4. 0.45 μm syringe filters (Corning)
5. 50 ml tubes
6. 3-kD molecular cut-off filters (Amicon Ultra, Millipore, Carrigtwohill, Ireland)
7. 2 ml vials

In vivo liver injury and regeneration models

1. Male C57Bl/6 mice (preferably young adults, age around 8-14 weeks) maintained in the animal facility on a 12/12 hour light/dark schedule with free access to food and drinking water.
2. Isoflurane vaporizer with induction chamber and suitable mouth piece for mice
3. Isoflurane
4. Heating plate (to keep mice at body temperature during the procedure)
5. Thin polystyrene foam or cork pad
6. Shaver
7. Tape
8. 70% ethanol
9. Microsurgery instruments: curved blunt forceps, straight dissecting forceps, curved needle holder, half-curved scissors, microvascular clamps, microvascular clamp holder
10. Other surgical instruments: operating scissors, two paper clips (partly unfolded to be used as retractors), 2 needles
11. 0.9% NaCl or phosphate buffered saline (PBS)
12. Cotton tips
13. Cotton gauzes (5x5 cm)
14. Silk sutures: 4-0 for liver lobe resections, 5-0 for abdominal wall closure
15. 1 ml syringes with injection needles
16. Heparin

METHODS

Culture procedures should be performed in a culture grade flow cabinet to keep reagents and cultures sterile. Reagents should be stored according to the manufacturer's description, unless otherwise described. Procedures in italic are only needed if chosen to freeze cells down for storage prior to use. Animal experiments should be performed according to national laws and with approval of the institutional animal welfare committee.

Primary cultures of MSCs from human tissue

MSCs can be obtained from various tissue samples, including bone marrow, dental marrow, adipose tissue and organs like lung, liver and hearth. The different methods for processing these tissues for primary cell cultures depend on the tissue type and have been described elsewhere. As an alternative for invasive techniques to obtain healthy tissue for the isolation of MSCs, organs and tissues used for transplantation are an attractive source. In this setting not only tissue biopsies but also the graft preservation solution can be used as a source of cells. This following section describes the procedure for primary MSC cultures from liver graft preservation solution.

1. Collect the organ preservation solution of human liver grafts for transplantation (UW solution; Viaspan, Bristol-Myers Squibb, Woerden, Netherlands) in sterile tubes or cups at the end of the cold storage period and store at 4°C till further processing in the lab (see **Note 3**).
2. *Put the freezing container and cryovials at 4°C to cool down before use.*
3. Distribute the organ preservation solution in 50 ml tubes, spin down (1500 rpm; 4°C; 5 min.) and discard the supernatant.
4. Pool the pellets in one 50 ml tube using ice-cold DMEM up to a final volume of 30 ml (see **Note 4**).
5. Fill another 50 ml tube with 15 ml Ficoll, pipet the cell suspension gently onto the Ficoll and spin down (1800 rpm; 20°C; 19 min.; acceleration 9; brake 1).
6. Harvest the enriched cell fraction (ring of cells on Ficoll) into a new 50 ml tube and wash the fraction: add DMEM, spin down (1500 rpm; 4°C; 10 min.) and discard the supernatant (see **Note 5**).
7. Resuspend the pellet in 10 ml DMEM and count the number of cells with Trypan Blue (vital and dead cells; standard protocol).
   If chosen to freeze the cells down for storage prior to use, continue to the next step; otherwise continue to the second step of the culture expansion protocol.
8. *Freeze the cells at a concentration of approximately 10x10^{6} cells per cryovial:*
   a. *Spin down (*1500 rpm; 4°C; 5 min.*) and discard the supernatant.*
   b. *Add the desirable amount of medium A (0.5 ml per cryovial) and put the cells on ice for 30 min.*
   c. *Slowly add the desirable amount of medium B (0.5 ml per cryovial) and divide the cells over the cryovials (1 ml per cryovial) (see* ***Note 6****).*
   d. *Put the cryovials directly in the freezing container and store at -80°C. The next day, cells can be transferred to regular -80°C storage boxes until further use (see* ***Note 7 and 8****).*

Culture expansion of MSCs

If chosen to use frozen cells, start with the first step; otherwise continue to the second step.

1. *Prepare frozen cells for culture:*
   a. *Fill 15 ml tubes with 3 ml ice-cold FBS per tube (1 tube per cryovial frozen cells).*
   b. *Thaw cryovials in a 37°C waterbath till a small piece of ice is left (see* ***Note 9****).*
   c. *Transfer the cell suspensions onto the ice-cold FBS.*
   d. *Wash the cryovials with 2 ml ice-cold PBS and transfer the PBS onto the FBS.*
   e. *Spin down the 15 ml tubes (1500 rpm; 4°C; 7 min) and discard the supernatant.*
   f. *Resuspend the pellets using ice-cold serum-containing culture medium, spin down (1500 rpm; 4°C; 7 min.) and discard the supernatant.*
   g. *Pool the pellets in one 15 ml tube using serum-containing culture medium up to a final volume of 10 ml (unless using frozen cells from different liver grafts and chosen to culture them separate) and count the number of cells with Trypan Blue (vital and dead cells; standard protocol).*
2. Spin down (1500 rpm; 4°C; 7 min.) and discard the supernatant.
3. Resuspend the pellet in serum-containing culture medium with a maximum of 12 ml per $10x10^6$ cells.
4. Transfer the cell suspension into T75 culture flasks (approximately $10x10^6$ cells per flask), add up to 12 ml serum-containing culture medium per flask and store them in a 37°C incubator (see **Note 10**).
5. Change the culture medium every 3 days:
   a. Pre-warm the serum-containing medium in a 37°C waterbath.
   b. Gently rinse the cell layer with serum-containing medium to get rid of debris (see **Note 11**).
   c. Put 12 ml of fresh serum-containing medium in the culture flasks and place them in the incubator.
6. After approximately 7-10 days, the cultures will show several fibroblast-like cells, which will multiply. Dependent on how quickly they multiply, these MSCs can be transferred to larger culture flasks using a standard Trypsin/EDTA protocol (around 70-80% confluence; Figure 1) (see **Note 12-15**).

Collection and concentration of MSC-secreted factors

1. Expand the cultures until the desired amount of concentrated MSC-conditioned culture medium (MSC-CM) for in vivo use of MSC-secreted factors can be prepared (preferably passage 5-10). One T75 culture flask with at least 70-80% MSC

confluence will result in approximately 400 μl MSC-CM (in our experiments 100 μl per mouse per treatment was used) (see **Note 16 and 17**).

2. Three days before collection, remove the serum-containing culture medium, gently rinse the culture flasks with pre-warmed PBS and change to 10 ml pre-warmed serum-free (or serum-containing if preferred) culture medium per T75 culture flask (see **Note 18-20**).
3. After 3 days of culture, collect the conditioned culture medium in a 50 ml syringe, connected to a 0.45 μm syringe filter and placed on a 50 ml tube (see **Note 21**).
4. Filter the conditioned culture medium to remove any cells and cell debris possibly present.
5. Transfer the filtered conditioned culture medium to the 3-kD molecular cut-off filters and spin down (4000 rpm; 4°C; 60 min; brake 1; this will concentrate the conditioned culture medium approximately 25-fold) (see **Note 22**).
6. Transfer the concentrated MSC-CM above the filter to 2 ml vials and put on ice until further use.

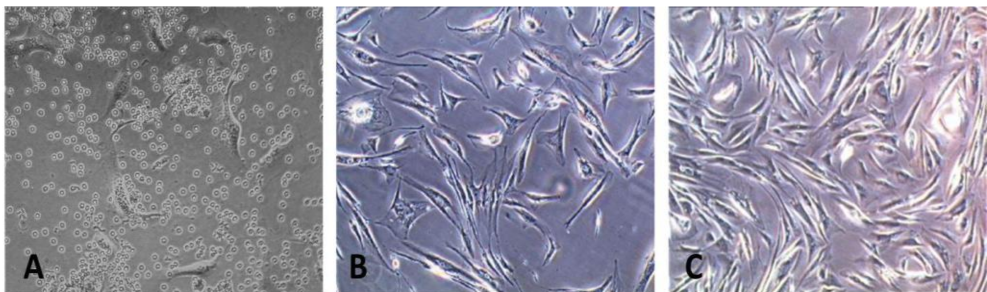

Figure 1. MSC cultures

A. In the majority of cultures, cells with a fibroblast-like morphology appear within ten days; **B.** Fibroblast-like cells e.g. MSCs rapidly proliferate and can be sub-cultured and expanded for up to 10-20 passages. **C.** MSC cultures with 70-80% confluence are optimal for collecting MSC-CM.

In vivo models to study paracrine effects of MSC-CM

Partial hepatectomy model

1. Place the mouse into the induction chamber of the Isoflurane vaporizer and use 2-3 liters/min oxygen flow until anesthesia is induced.
2. Shave the abdominal skin, transfer the mouse onto the heating plate covered with the foam/cork pad (pre-heated at 37°C) and continue the anesthesia by using the mouth piece connected to the vaporizer.
3. Fixate the mouse with the abdominal wall upward by taping the stretched legs to the plate and desinfect the abdominal skin with 70% ethanol (see **Note 23**).
4. Make a midline incision (2.5-3 cm) using the curved blunt forceps and operating scissors: gently lift the skin cq. peritoneum when cutting to avoid damaging the

intestines; start in the lower abdominal area and work your way up until the xyphoid is exposed.

5. Place the two unfolded paperclips in the midline incision in such a position that they retract the abdominal wall lateral and upward, thereby exposing the liver, and fixate them to the pad with the needles.
6. Gently lift up the left lateral lobe using saline-moistened cotton tips, cut the membrane connecting this lobe to the caudate lobe with the half-curved scissors and hold the left lobe in this upward position (if needed by retracting it with the tip of a saline-moistened gauze) (see **Note 24**).
7. Use the blunt forceps to place a 4-0 silk thread close to the hilum under the left lateral lobe and flip the lobe back to its original position, thereby wrapping the silk thread around the base of the lobe.
8. Use the curved needle holder and forceps to tie the ends of the silk thread close to the hilum (the color of the lobe will darken due to interruption of the blood flow) and cut the lobe close to the knot. Make sure there's no bleeding from the remnant (see **Note 25**).
9. Place a second silk thread underneath the median lobe, gently pull down this lobe and cut the falciform ligament.
10. Pull one end of the thread in the groove on the right side of the gallbladder and the other end around the back of the right part of the median lobe, tie the ends together (which will cut across the liver tissue) and remove the right part of the median lobe by cutting close to the knot (see **Note 26**).
11. Perform the same procedure for the left part of the median lobe, which is smaller than the right part, and shorten the ends of all remnant silk threads.
12. Close the peritoneum and skin separately with 5-0 silk sutures, clean the abdominal skin from blood remnants and place the animals in a warm environment to recover (see **Note 27 and 28**).
13. If chosen to treat the animals at time of surgery, inject the MSC-CM/treatment solution when the animals are still anesthetized (see **Note 29 and 30**).
14. Sacrifice the animals at the preferred time point and collect blood and/or tissue for further analysis (see **Note 31**).

Ischemia/reperfusion model with or without partial hepatectomy

1. Inject the mouse approximately 15 minutes prior to surgery intraperitoneally with heparin (100 U/kg; solution of 10 U/mL in PBS) to prevent intravascular thrombus formation.
2. Follow steps 1-5 as described in the partial hepatectomy protocol. For the ischemia/reperfusion model, it is best to perform the surgical procedures using a microscope (especially the vascular clamping).

3. Lift the intestines from the abdominal cavity and wrap them in a saline-moistened gauze to protect them from dehydrating.
4. Gently lift the median and left lateral lobes using saline-moistened cotton tips, cut the membrane connecting the left lateral lobe to the caudate lobe with the half-curved scissors, and hold the lobes in this upward position by retracting it with a moistened gauze (see **Note 24**).
5. Use another moistened gauze to gently retract the right lateral and caudate lobes in the caudal direction to expose the portal triad.
6. Using the straight dissecting forceps, carefully dissect the space behind the portal triad without puncturing the aorta.
7. After creating a sufficiently large opening behind the portal triad, place a microvascular clamp just above the branch of the right lateral lobe using the clamp holder (see **Note 32**).
8. Reposition the liver lobes and the intestines in their anatomic position, inject 0.5 mL of saline in the abdominal cavity and cover the animal with a moistened gauze.
9. Keep the animals anaesthetized and on the heating plate during the ischemic time and make sure the gauze stays moist (see **Note 33**).
10. At the end of the ischemic period, gently remove the microvascular clamp to reperfuse the median and left lateral lobes.
11. Follow steps 12-14 of the partial hepatectomy protocol to finish the procedure, unless chosen to combine ischemia and reperfusion injury with a (approximately) 50% partial hepatectomy, leaving only ischemic liver tissue.
12. Gently remove the intestines and lift the median and left lateral liver lobes as described in step 3 and 4 of this protocol.
13. Using moistened cotton tips, the blunt forceps and the half-curved scissors, carefully dissect the right lateral and caudate lobes from their surroundings, so they can be lifted.
14. Place a 4-0 silk thread underneath the right lateral lobe, close to the base and flip the lobe back to its original position, thereby wrapping the silk thread around the base of the lobe.
15. Use the curved needle holder and forceps to tie the ends of the silk thread close to the base and cut the lobe close to the knot. Make sure there's no bleeding from the remnant (see **Note 25**).
16. Perform the same procedure for the caudate lobes as well as the right part of the median lobe (step 9 and 10 of the partial hepatectomy protocol) and shorten the ends of all remnant silk threads (see **Note 34**).
17. Close the peritoneum and skin separately with 5-0 silk sutures, clean the abdominal skin from blood remnants and place the animals in a warm environment to recover (see **Note 27 and 28**).

18. If chosen to treat the animals at time of surgery, inject the MSC-CM/treatment solution when the animals are still anesthetized (see **Note 29 and 30**).
19. Sacrifice the animals at the preferred time point and collect blood and/or tissue for further analysis (see **Note 31**).

NOTES

1. Do not use medium A and B older than 2 weeks.
2. Try what percentage of FBS works best in your cultures.
3. In our institution liver grafts are flushed twice before implantation into the recipient: prior to preparation at the backbench and between backbench preparation and implantation. Both batches of preservation solution can be used, however, the first batch contains most cells.
4. If preferred, RPMI or MEM-alpha can also be used.
5. Plastic pipettes are convenient for controlled collection of the cell fraction.
6. Slowly drip the medium along the side of the tube while rotating the tube, to evenly distribute medium B over the cell suspension. If added to quickly the DMSO will damage the cells.
7. Transfer the cryovials to a -80°C environment as soon as possible, as DMSO (especially if not kept cold) damages cells.
8. If planning to store cells for a longer period of time, transfer them to a -135°C freezer to keep maximum cell condition.
9. Leaving a small piece of ice will ensure a relatively low temperature. If the temperature will get too high, the DMSO in the cell freezing suspension will damage too many cells. Perform the thawing steps b-d therefore as quickly as possible.
10. Serum-free culture medium often doesn't stimulate the cells enough in the initial phase of the culture.
11. One needs to find a balance between rinsing too gentle (thereby not removing the debris) and rinsing too rough (thereby removing adherent living cells from the culture flask).
12. The MSC light up under the microscope (phase contrast setting) and are larger than other cells in the culture flask; they develop from an oval shape into long, sprouted cells with a fibroblast-like morphology.
13. Split cell cultures 1:2 or 1:3, otherwise the cell concentration can become too low to multiply and cells may go into a resting phase.
14. Every graft preservation yields a different amount of cells and a different percentage of L-MSC. If the cultures do not show the fibroblast-like cells after 2 weeks, try starting new cultures with different cell concentrations. For example: divide the cells from a cryovial over the wells a 6- or 12-well culture plate: well 1 with 50% of the cells, well 2 with 25% of the cells, well 3 (and 4) with 12.5% of

the cells. This way the differences in cell concentration as well as culture surface will reveal the best concentration for the specific graft preservation cell yield.

15. If the cultures show fibroblast-like cells but these cells do not multiply, the cell concentration might be too low. A possible solution is to transfer the cells to a smaller culture surface (6-well plate, T25 flask) or to pool multiple flasks, thereby increasing the cell concentration.
16. Cells can be cultured up to 10-20 passages. Below passage 5 MSC numbers are usually too low to perform in vivo experiments. Above passage 10 cell cultures may show many resting cells or even (malignant) transformation of cells.
17. Factors like MSC batch, passage number, differences in cell density and amount of proliferating and resting cells in a culture can influence the quality of the MSC-CM. It is therefore very important to standardize the culture conditions as much as possible. Other options to mimimize these effects are by combining MSC-CM from multiple culture flasks and different batches. Furthermore, quality control by ELISA, mass spectrometry or gel electrophoresis is recommendable.
18. Rinsing the culture flasks with PBS will remove remnants of the serum-containing culture medium, thereby preventing these factors to influence the experiment results.
19. The fetal bovine serum in serum-containing culture medium contains several growth factors that improve survival, growth and proliferation of cultured cells. These proteins, especially if concentrated, may affect experimental data and make a well thought-out experimental setup with the proper vehicle control group essential.
20. If feasible, plan the collection and concentration of MSC-CM on the same day as the experiments to prevent possible brake down of MSC-secreted factors.
21. Filter units can be used for large volumes.
22. If a lower concentration factor is preferred, shorten the spinning time accordingly.
23. The forelegs can also be fixated ‘embracing’ the mouth piece. The advantage is that the rib cage and diaphragm are positioned a bit more cranial, thereby exposing the liver slightly better.
24. The liver tissue is very delicate and easily damaged. Using moistened cotton tips and gauzes will reduce the chance of damaging the tissue.
25. If the remnant is bleeding, tighten the knot to stop the bleeding.
26. The right and left part of the median lobe can also be removed together, using one silk thread and thereby also removing the gall bladder. However, if the knot is placed too close to the hilum/supra-hepatic caval vein, the resection will cause venous obstruction resulting in congestion, necrosis and failure of regeneration. On the other hand, the further away the knot is placed, the more functional liver tissue is left in situ. By removing the right and left part of the median lobe sepa-

rately, leaving the gall bladder in situ, the risk of causing venous obstruction or leaving too much liver tissue is reduced.

27. Mice tend to bite the sutures. If the abdomen is closed in one layer, knots are too easily accessible (the lower, the easier) or not properly tied, this will result in evisceration.
28. A warm recovery environment can be created using an incubator designed for animals (keep at 37oC) or heating lamps. Be careful if using heating lamps, they tend to get very hot and dehydrate/overheat the animals.
29. The site and time of MSC-CM administration possibly affect its therapeutic effects. Stem cell derived factors have been injected intravenously as well as intraperitoneally and at time points prior to, during or after the induction of injury. The best time and route of administration still need to be elucidated. However in our study on liver regeneration after partial hepatectomy, pre-treatment 4 hours prior to surgery seemed beneficial over treatment at time of resection.[33] A possible explanation could be that the liver is already primed in those 4 hours and can therefore immediately respond to the loss of liver mass (Figure 2).

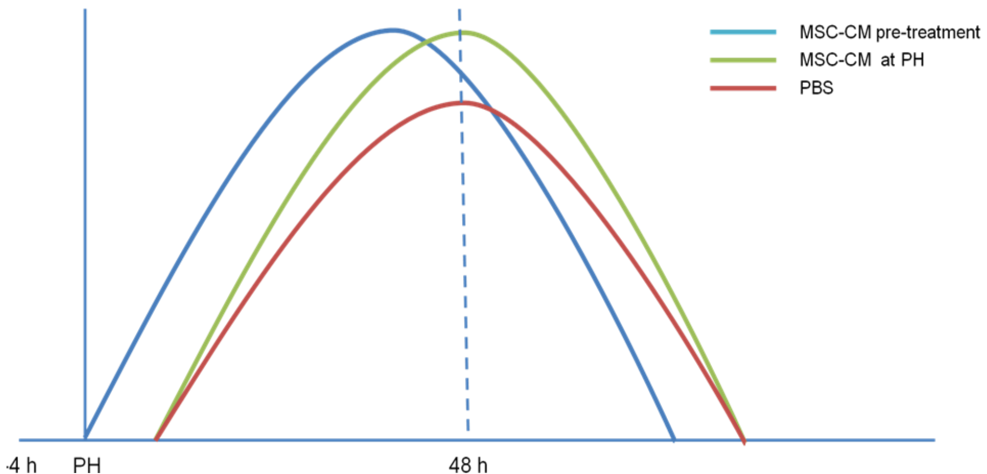

Figure 2. Postulated kinetics of hepatocyte proliferation after MSC-CM treatment

In our study on the paracrine effects of MSC-derived factors on liver regeneration after partial hepatectomy, treatment 4 hours prior to surgery was compared with treatment at time of resection. Based on the hepatocyte proliferation and gene expression data, treatment at time of resection seemed most effective in stimulating liver regeneration. Interestingly however, a significant increase in liver to body weight ratio was found after pre-treatment, whereas no significant difference compared to control treated animals was found if animals were treated at time of resection. We hypothesize that MSC-CM pre-treatment shifts the regenerative response of the liver after surgical injury forward and thus accelerates liver regeneration.

30. Always look for strategies/alternatives that least harm the animals.
31. Dependent on the field of interest, multiple read-outs for regeneration can be used, for instance:
    a. Immunohistochemical staining for BrdU (5-bromo-2'-deoxyuridine), PCNA (proliferating cell nuclear antigen) or Ki67 to determine the percentage of proliferating cells. In case of using BrdU, the animals have to be injected at least 30 minutes prior to sacrifice with 50 mg/kg dissolved BrdU, as this compound needs to be incorporated into the DNA of proliferating cells.
    b. qRT-PCR techniques to investigate cell cycle related, inflammatory and pro-angiogenic gene expression levels.
    c. Western blotting or ELISA techniques to detect levels of relevant proteins in tissue or blood (for example serum transaminases and bilirubin).
32. Only the median and left lateral lobes should show a change in color. If the right lateral lobe shows any signs of ischemia, the clamp is placed too close to its branch.
33. The duration of the ischemic time period depends on the preferred amount of injury. Most studies describe an ischemic time in rodents between 60 and 90 minutes. Below 60 minutes, the injury inflicted is often not severe enough to show clear beneficial effects of a treatment. Above 90 minutes, the survival rate of the animals drops, which is often only preferred in survival studies. While developing our model, we noticed that ischemia and reperfusion injury combined with a 50% partial hepatectomy allowed ischemic times up to 60 minutes before the survival rates went down.
34. Part of the caudate lobes can best be approached by flipping the intestines to the right.

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Chapter 8

Secreted factors of human liver-derived mesenchymal stem cells promote liver regeneration early after partial hepatectomy

Suomi M.G. Fouraschen[1], Qiuwei Pan[2], Petra E. de Ruiter[1], Waqar R.R. Farid[1], Geert Kazemier[1], Jaap Kwekkeboom[2], Jan N.M. Ijzermans[1], Herold J. Metselaar[2], Hugo W. Tilanus[1], Jeroen de Jonge[1, #] and Luc J.W. van der Laan[1, #]

[1]Department of Surgery, Laboratory of Experimental Transplantation and Intestinal Surgery and [2]Department of Gastroenterology & Hepatology, Erasmus MC-University Medical Center, Rotterdam, The Netherlands; [#]Both authors contributed equally to this study

(Stem cells and Development, 2012 Sep 1;21(13):2410-9)

ABSTRACT

Rapid liver regeneration is required after living donor liver transplantation and oncologic liver resections to warrant sufficient liver function and prevent small-for-size syndrome. Recent evidence highlights the therapeutic potential of mesenchymal stem cells (MSCs) for treatment of toxic liver injury, but whether MSCs and their secreted factors stimulate liver regeneration after surgical injury remains unknown. Therefore, the aim of this study is to investigate the effect of human liver-derived MSC-secreted factors in an experimental liver resection model.

C57BL/6 mice were subjected to a 70% partial hepatectomy and treated with either concentrated MSC-conditioned culture medium (MSC-CM) or vehicle control. Animals were analyzed for liver and body weight, hepatocyte proliferation and hepatic gene expression. Effects of MSC-CM on gene expression in a human hepatocyte-like cell line (Huh7 cells) were analyzed using genome-wide gene expression arrays.

Liver regeneration was significantly stimulated by MSC-CM as shown by an increase in liver to body weight ratio and hepatocyte proliferation. MSC-CM upregulated hepatic gene expression of cytokines and growth factors relevant for cell proliferation, angiogenesis and anti-inflammatory responses. *In vitro*, treatment of Huh7 cells with MSC-CM significantly altered expression levels of approximately 3000 genes. Functional analysis revealed strong effects on networks associated with protein synthesis, cell survival and cell proliferation.

This study shows that treatment with MSC-derived factors can promote hepatocyte proliferation and regenerative responses in the early phase after surgical resection. MSC-CM may represent a feasible new strategy to promote liver regeneration in patients undergoing extensive liver resection or after transplantation of small liver grafts.

INTRODUCTION

One of the most remarkable features of the liver is the regenerative capacity in response to liver injury. In healthy individuals the liver can compensate an acute loss of up to 70-75% of its total mass.[1-3] This regenerative capacity enables living donor liver transplantation and (oncologic) liver resections. Either situation requires rapid liver regeneration to warrant sufficient liver function and homeostasis. In case of liver surgery, advantages in surgical techniques have enabled progressively larger resections. However, after surgery for malignancies, regeneration is often compromised due to neo-adjuvant chemotherapy, poor nutritional status and increasing age of the patient population, thereby restricting surgical treatment options.[4-7] Living donor liver transplantation, on the other hand, was introduced to help overcome donor organ scarcity and reduce waitlist mortality. In this situation, both donor and recipient end up with a small-for-size liver, associated with significant morbidity and mortality.[2, 8, 9] The considerable risks hamper large scale use of living donors in the western world. The use of smaller liver grafts would decrease the donor risk and thereby increase the donor pool, but would simultaneously increase the risk of recipients to develop small-for-size syndrome. Potential therapeutic strategies to improve liver regeneration after surgical injury therefore need to be investigated.

Recent studies describe the potential role of stem cells, especially mesenchymal stem cells (MSCs), to promote liver regeneration after toxic injury and protect against fulminant hepatic failure.[10-13] MSCs were initially thought only to reside in the bone marrow, providing a supportive niche for hematopoietic stem cells. More recently, MSCs have been identified in multiple tissue compartments, including lung, liver and adipose tissue, and described to provide pleiotropic effects in response to tissue injury.[14-16] MSCs have the ability to differentiate into hepatocytes and cholangiocytes and have immunomodulatory and anti-inflammatory properties.[17-21] Furthermore, they are described to promote angiogenesis by upregulating the expression of pro-angiogenic factors.[22, 23] There is an ongoing discussion on whether MSCs contribute to liver regeneration by (trans)differentiation into liver cells or by paracrine effects of their trophic factors. Research by Van Poll and colleagues shows a stimulating effect of bone marrow-derived MSC (BM-MSC)-secreted factors on liver regeneration in the setting of toxic liver injury and hepatic failure.[24, 25] In a clinical setting, application of MSC-derived factors to improve regeneration might be more beneficial than the use of MSCs, since there is no risk of rejection or possible malignant transformation. In addition, the factors can be produced in large clinical grade quantities. However, whether MSC-secreted factors have beneficial effects on liver regeneration after surgical injury is unknown.

Recently we showed that the adult human liver harbors a population of MSCs.[15] These liver-derived MSCs (L-MSCs) were found to mobilize from liver grafts at time of transplantation and could be retrieved from the organ preservation solution. L-MSCs were found to be highly similar to BM-MSCs with regard to adipogenic and osteogenic differentiation potential and Wnt-stimulated proliferative responses. Moreover, their genome-wide gene

expression profile was highly similar to that of BM-MSCs, with less than 0.3% of genes showing a two- or more-fold difference in expression. Like BM-MSCs, these L-MSCs have immunosuppressive capacities and the potential to differentiate into hepatocyte-lineages in a mouse model of liver injury. Therefore, L-MSCs from adult human liver have regenerative and immune regulatory potential. The aim of the current study is to investigate whether trophic factors secreted by L-MSCs stimulate liver regeneration after surgical liver injury.

MATERIALS AND METHODS

Animals

Male C57Bl/6 mice (age 13-18 weeks) were obtained from Charles River (Maastricht, Netherlands) and maintained in the animal facility on a 12/12 hour light/dark schedule. The animals had free access to food and drinking water. All animal experiments were performed with approval of the institutional animal welfare committee.

Human L-MSC cultures and conditioned medium

Liver-derived MSCs were obtained from the organ preservation solution (Viaspan, Bristol-Myers Squibb, Woerden, Netherlands), collected after cold storage of human liver grafts for transplantations performed at the Erasmus Medical Center, Rotterdam, The Netherlands. The Medical Ethical Council of the Erasmus Medical Center approved the use of human donor material for medical research. Mononuclear cells were isolated from the collected preservation fluids by density gradient centrifugation using Ficoll-Paque Plus (GE Healthcare, Uppsala, Sweden) and put into culture as previously described.[15] Culture medium consisted of DMEM (Lonza, Verviers, Belgium) supplemented with 10% fetal bovine serum (Sigma-Aldrich, Taufkirchen, Germany), L-Glutamin (Lonza), penicillin and streptomycin (Gibco, Invitrogen, Paisley, United Kingdom). When L-MSCs were cultured under serum free conditions, culture medium consisted of MEM-alpha (Gibco) supplemented with 0.05% bovine serum albumin (Sigma-Aldrich), L-Glutamin, penicillin and streptomycin. The conditioned culture medium was collected three days after medium change of L-MSC cultures from passage 6-10. MSC-CM was concentrated approximately 25-fold by filtration with 3-kD molecular cutoff filters (Amicon Ultra, Millipore, Carrigtwohill, Ireland).

Partial hepatectomy and MSC-CM treatment

Liver regeneration was induced by subjecting C57BL/6 mice to a 70% PH as first described by Higgins and Anderson in 1931. Animals were anaesthetized with isoflurane and, after a midline laparotomy, the left lateral and median lobes of the liver were ligated and resected. The peritoneum and skin were sutured separately. All procedures were performed under clean conditions. Animals were treated intraperitoneally with 100 μl concentrated MSC-CM in PBS (Lonza; total volume 0.5 ml) or with PBS alone as vehicle con-

trol, starting four hours prior to or at time of PH, and in both cases 24 hours after resection.

In addition, a serum free experiment was performed in which animals were injected at time of and 24 hours after PH with the concentrated MSC-CM collected from serum free cultured L-MSCs, to exclude possible effects on liver regeneration caused by fetal bovine serum. The control group in this serum free experiment was treated with concentrated culture medium treated exactly the same as the serum free MSC-CM (SF-MSC-CM), but without the presence of L-MSCs (serum free unconditioned medium; SF-UM). All animals were sacrificed two days after PH and their livers and blood were collected to further investigate the effects of MSC-CM on hepatocyte proliferation, hepatic gene expression and serum markers of liver function and injury in the early phase of liver regeneration.

Weight calculations

The animals were weighed daily prior to treatment and the resected liver mass was weighed after PH. The initial total liver weight was calculated as follows:

resected liver weight/70*100 (g)

At time of sacrifice, animals and their regenerated liver mass were weighed and the percentage of reconstitution of the liver was calculated by:

regenerated liver weight/initial total liver weight*100 (%)

The liver to body weight ratio was calculated by:

regenerated liver weight/body weight at time of harvest*100 (%)

Immunohistochemistry

One hour prior to sacrifice, animals were injected intraperitoneally with 50 mg/kg BrdU (5-Bromo-2'-deoxyuridine; B5002, Sigma-Aldrich). Livers were harvested and processed to 4 μm thick formalin fixed, paraffin embedded slides. Antigen retrieval was achieved by boiling the slides in 0.01 M sodium citrate; pH 6.0 (microwave 1000 Watt; 1x7 and 2x3 minutes). Endogenous peroxidase was blocked by 0.6% H_2O_2 in PBS for 30 minutes at room temperature, after which DNA was denaturated by incubation for 1 hour at 37°C in 0.1 M HCl in aqua dest. Aspecific binding was prevented by 0.5% milk powder supplemented with 0.15% glycin in PBS (blocking buffer). Slides were incubated overnight at 4°C with monoclonal mouse anti-BrdU (Bu20a; DakoCytomation, Glostrup, Denmark; 1:80 in blocking buffer). The next day slides were incubated for 30 minutes at room temperature with polyclonal rabbit anti-mouse IgG/HRP (P0161; DakoCytomation; 1:1000 in blocking buffer). After antibody incubation slides were incubated with DAB-solution and counterstained with hematoxylin. Per animal 4 high power fields (HPF; 400x) were analyzed for BrdU positive hepatocytes.

Serum analysis of enzyme levels

Blood samples were collected at time of sacrifice in heparin coated micro tubes. After collection, samples were centrifuged (19 minutes, 1800 rpm) to separate the serum, which was then further analyzed at the clinical chemical core facility of the Erasmus MC-University Medical Center to determine albumin, bilirubin, ALT and AST levels.

Real-time quantitative RT-PCR

At time of sacrifice, liver tissue was stored overnight at 4°C and thereafter at -80°C in Allprotect Tissue Reagent (Qiagen, Hilden, Germany) for RNA preservation. Total RNA was extracted using Trizol (Qiagen) and chloroform after mechanical disruption of the tissue. RNA was precipitated in 75% ethanol and dissolved in RNase-free water. RNA quantity and quality was analyzed using a NanoDrop ND-1000 (Thermo Fisher Scientific, Wilmington, DE, USA). One microgram of RNA was reverse-transcribed to cDNA using an iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA). PCR primers (presented in Table 1) were synthesized by Isogen Life Science (Maarssen, Netherlands) and Biolegio (Nijmegen, Netherlands). Real-time quantitative RT-PCR was performed with a SensiMix SYBR & Fluorescein Kit (Bioline, London, United Kingdom) and MyIQ real time PCR detection system (Bio-Rad Laboratories) according to the manufacturer's instruction.

Table 1. Primer sequences

Gene	Name	Accession number	Primer (forward/reverse)
TNFA	Tumor necrosis factor alpha	NM_013693	CCCTCACACTCAGATCATCTTCT GCTACGACGTGGGCTACAG
IL6	Interleukin 6	NM_031168	TAGTCCTTCCTACCCCAATTTCC TTGGTCCTTAGCCACTCCTTC
HGF	Hepatocyte growth factor	NM_010427	ATGTGGGGGACCAAACTTCTG GGATGGCGACATGAAGCAG
CCND1	Cyclin D1	NM_007631	GCGTACCCTGACACCAATCTC CTCCTCTTCGCACTTCTGCTC
PCNA	Proliferating cell nuclear antigen	NM_011045	CTTGGTACAGCTTACTCTGCG AGTTGCTCCACATCTAAGTCCAT
TGFB	Transforming growth factor beta	NM_011577	CTCCCGTGGCTTCTAGTGC GCCTTAGTTTGGACAGGATCTG
IL10	Interleukin 10	NM_010548	GCTCTTACTGACTGGCATGAG CGCAGCTCTAGGAGCATGTG
IL1RN	Interleukin 1 receptor antagonist	NM_031167	GCTCATTGCTGGGTACTTACAA CCAGACTTGGCACAAGACAGG
VEGFA	Vascular endothelial growth factor A	NM_009505	GCACATAGAGAGAATGAGCTTCC CTCCGCTCTGAACAAGGCT
FLT1	Vascular endothelial growth factor receptor 1	NM_010228	TGGCTCTACGACCTTAGACTG CAGGTTTGACTTGTCTGAGGTT
KDR	Vascular endothelial growth factor receptor 2	NM_010612	TTTGGCAAATACAACCCTTCAGA GCAGAAGATACTGTCACCACC
ANGPT1	Angiopoietin 1	NM_009640	CACATAGGGTGCAGCAACCA CGTCGTGTTCTGGAAGAATGA
ACTB	Beta-actin	NM_007393	GGCTGTATTCCCCTCCATCG CCAGTTGGTAACAATGCCATGT

Genome-wide gene expression profiling and proliferation of Huh7 cells cultured with MSC-CM

For genome-wide gene expression profiling, Huh7 cells were cultured for 48 hours with MSC-CM or unconditioned medium (final concentration 50%; 6-well plate; 3 wells/condition; 2.5 x 10^5 cells/well). Total RNA from these cultures was isolated using the RNeasy Micro Kit (Qiagen) and used for gene expression analysis using Human Gene 1.0 ST Genechips (Affymetrix, Santa Clara, USA) according to the manufacturer's instructions.

The effect on proliferation of Huh7 cells was determined using an MTT assay, for which Huh7 cells were cultured up to 4 days with MSC-CM or UM (final concentration 50%; 96-well plate; 3 wells/time point/condition; 2 x 10^4 cells/well). At day 1, 2, 3 and 4 after starting treatment, the number of metabolically active cells was quantified by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 0.5 mg/ml) colorimetry.

Statistical analysis

For genome-wide gene expression analysis, normalized signals were generated using RMAExpress. Class predictions were performed with Partek Genomics Suite software and functional analyses were performed using Ingenuity Pathway Analysis (Ingenuity Systems, Redwood City, CA, USA). With these array analyses, p-values <0.005 using a two-way ANOVA test were considered significant. All other data are presented as mean ± SEM and statistical analyses were performed using the Mann–Whitney test with GraphPad Prism software and p≤0.05 was considered statistically significant.

RESULTS

MSC-CM treatment enhances hepatocyte proliferation and liver weight gain after partial hepatectomy

All treatment groups showed 100% survival. Hepatocyte proliferation, quantified by the percentage of BrdU positive hepatocyte nuclei, was significantly increased at day 2 after PH in animals treated at time of PH with MSC-CM compared to animals treated at time of PH with PBS (20.0% vs. 12.1%, p=0.02; Figure 1A, B). Animals pre-treated four hours prior to PH with MSC-CM showed a similar trend compared to animals pre-treated with PBS (19.3% vs. 14.7%; data not shown), however this difference did not reach statistical significance. Interestingly, the liver to body weight ratio was significantly increased in animals pre-treated with MSC-CM compared to animals pre-treated with PBS (3.1% vs. 2.8% of total body weight, p=0.05; data not shown). No significant differences were seen between animals treated at time of PH with MSC-CM or PBS.

Even though proliferation was stimulated, known to cause a shift away from metabolic functions, MSC-CM treatment did not cause changes in liver function compared to PBS treatment, as shown by serum albumin and bilirubin levels (Figure 1C, D). Neither did MSC-CM treatment induce or reduce hepatocyte cell injury after PH compared to PBS treatment, as shown by ALT and AST levels (Figure 1E, F).

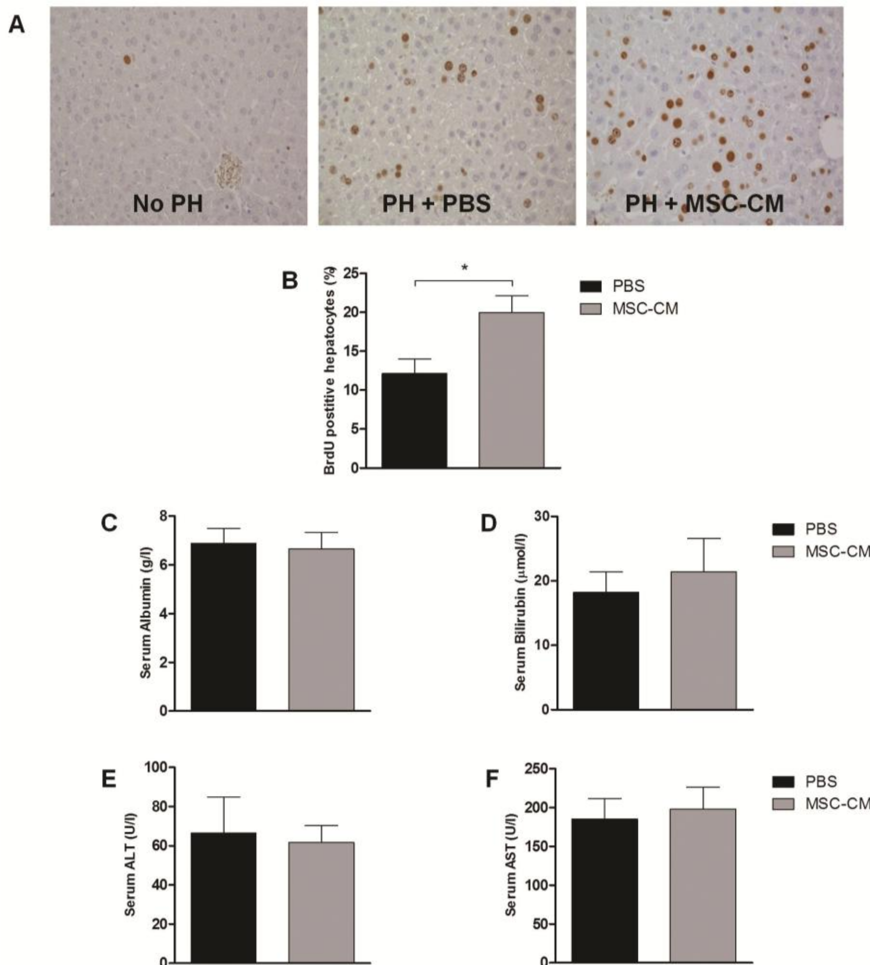

Figure 1. Effects of MSC-CM on hepatocyte proliferation, function and injury

A-B. Livers were processed for immunohistochemistry on BrdU to quantify hepatocyte proliferation. **A.** Representative pictures of hepatocyte proliferation in liver tissue before and two days after PH; **B.** Quantification of hepatocyte proliferation at day 2 after PH and MSC-CM treatment at time of PH; **C-F.** Serum levels at day 2 after PH and MSC-CM treatment at time of PH for **C.** albumin, **D.** bilirubin, **E.** ALT and **F.** AST. n=9 per group; *P≤0.05 versus PBS.

MSC-CM induces expression of genes relevant for hepatocyte proliferation

Previous studies have shown that tumor necrosis factor alpha (TNF-α) and interleukin 6 (IL-6) are crucial priming factors for hepatocytes to enter the cell cycle, whereas hepatocyte growth factor (HGF) is important in the proliferative phase.[26-28] Downstream in their signal transduction cascades, the passage of hepatocytes from the G1 into the S phase is associated with upregulation of several cyclins including Cyclin D.[29] Transforming growth factor beta (TGF-β) is known to be involved in the negative feedback on hepatocyte prolif-

eration.[30] Figure 2A shows the hepatic gene expression levels of these factors as well as of proliferating cell nuclear antigen (PCNA), known to be upregulated during DNA synthesis. MSC-CM treatment at time of PH significantly upregulated hepatic gene expression levels of TNF-α (2.6-fold, p=0.02), HGF (1.9-fold, p=0.03), PCNA (4.8-fold, p<0.01) and TGF-β (2.1-fold, p=0.02) compared to PBS treatment. IL-6 and Cyclin D1 gene expression did not show a statistically significant upregulation.

Pre-treatment with MSC-CM, compared to pre-treatment with PBS, only resulted in significant upregulation of TGF-β gene expression (2.6-fold, p<0.01; data not shown). A possible explanation for these findings could be that pre-treatment with MSC-CM might accelerate the regenerative response of the liver after surgical injury, with a shift forward in induction of abovementioned genes. Therefore, effects of pre-treatment with MSC-CM on the hepatic gene expression at time of resection were evaluated. As shown in Figure 2B, pre-treatment with MSC-CM had no effect on HGF, Cyclin D1, PCNA or TGF-β gene expression, but caused a significant upregulation of TNF-α (6.6-fold, p=0.02) and IL-6 gene expression (2.0-fold, p=0.05) at time of resection.

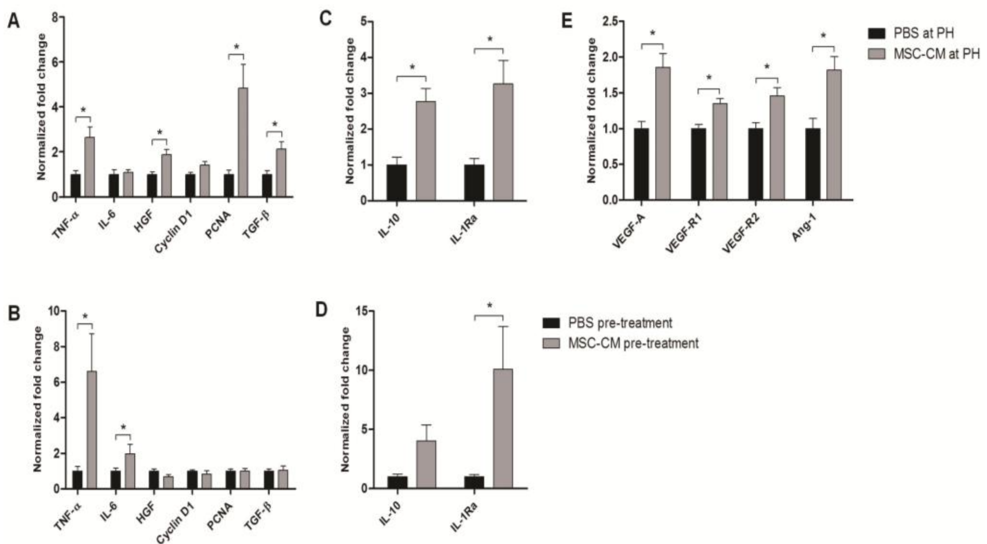

Figure 2. Effects of MSC-CM on hepatic gene expression

Hepatic gene expression levels were determined by quantitative RT-PCR and normalized against β-actin. **A-B.** Expression levels of hepatocyte proliferation related genes at **A.** day 2 after PH and MSC-CM treatment at time of PH and **B.** time of resection after pre-treatment. **C-D.** Expression levels of anti-inflammatory genes at **C.** day 2 after PH and MSC-CM treatment at time of PH and **D.** time of resection after pre-treatment. **E.** Expression levels of angiogenesis related genes at day 2 after PH and MSC-CM treatment at time of PH. *P≤0.05 versus PBS.

MSC-CM treatment induces an increased anti-inflammatory response

MSC are described to have anti-inflammatory capacities with an important role for interleukin 10 (IL-10) and interleukin 1 receptor antagonist (IL-1Ra).[21, 31] Therefore, effects on the anti-inflammatory response after PH were evaluated by analyzing gene expression levels of these cytokines. MSC-CM treatment at time of PH induced a significant upregulation of both IL-10 (2.8- fold, p=0.05) and IL-1Ra gene expression (3.3-fold, p=0.02) compared to PBS treatment (Figure 2C). Pre-treatment with MSC-CM, compared to PBS pre-treatment, also showed a significant upregulation of IL-1Ra gene expression at day 2 after PH (3.5-fold, p=0.05), but not of IL-10 (data not shown). Upregulation of IL-1Ra gene expression was already observed at time of resection, four hours after pre-treatment with MSC-CM (10.1-fold, p=0.02; Figure 2D).

Treatment with MSC-CM upregulates gene expression of pro-angiogenic factors

Besides hepatocyte proliferation, regeneration of damaged or lost vasculature plays an important role in liver regeneration. We therefore investigated effects on hepatic gene expression of the pro-angiogenic factors vascular endothelial growth factor A (VEGF-A), vascular endothelial growth factor receptor 1 and 2 (VEGF-R1 and -R2) as well as angiopoietin 1 (Ang-1). Figure 2E shows that in animals treated with MSC-CM at time of PH, a significant upregulation in hepatic gene expression of the pro-angiogenic factors VEGF-A (1.9-fold, p<0.01), VEGF-R1 (1.4-fold, p<0.01), VEGF-R2 (1.5-fold, p<0.01) and Ang-1 (1.8-fold, p=0.02) was seen at day 2 after PH compared to PBS treated animals. Pre-treatment with MSC-CM, compared to PBS pre-treatment, showed a similar induction in gene expression of these factors (VEGF-A 1.4-fold, VEGF-R2 1.9-fold and Ang-1 1.5-fold), but differences were only statistically significant for VEGF-R1 (1.4-fold, p=0.03; data not shown). Pre-treatment with MSC-CM did not cause significant changes in angiogenic gene expression levels at time of resection (data not shown).

Treatment of Huh7 cells with MSC-CM induces gene pathways involved in liver regeneration

To validate our findings in an *in vitro* model, a hepatocyte-like cell line (Huh7 cells) was stimulated with MSC-CM. MSC-CM treatment up to four days had no significant effect on cell viability, though there was a slight trend towards increased cell numbers, as determined by an MTT-assay (data not shown). Genome-wide gene expression analysis showed that in Huh7 cells exposed to MSC-CM approximately 3000 genes (<10% of the genome) were significantly differentially expressed compared to Huh7 cells exposed to UM. Functional analysis revealed that these genes are associated with pathways and networks relevant for protein synthesis, cell survival and cell proliferation (Figure 3).

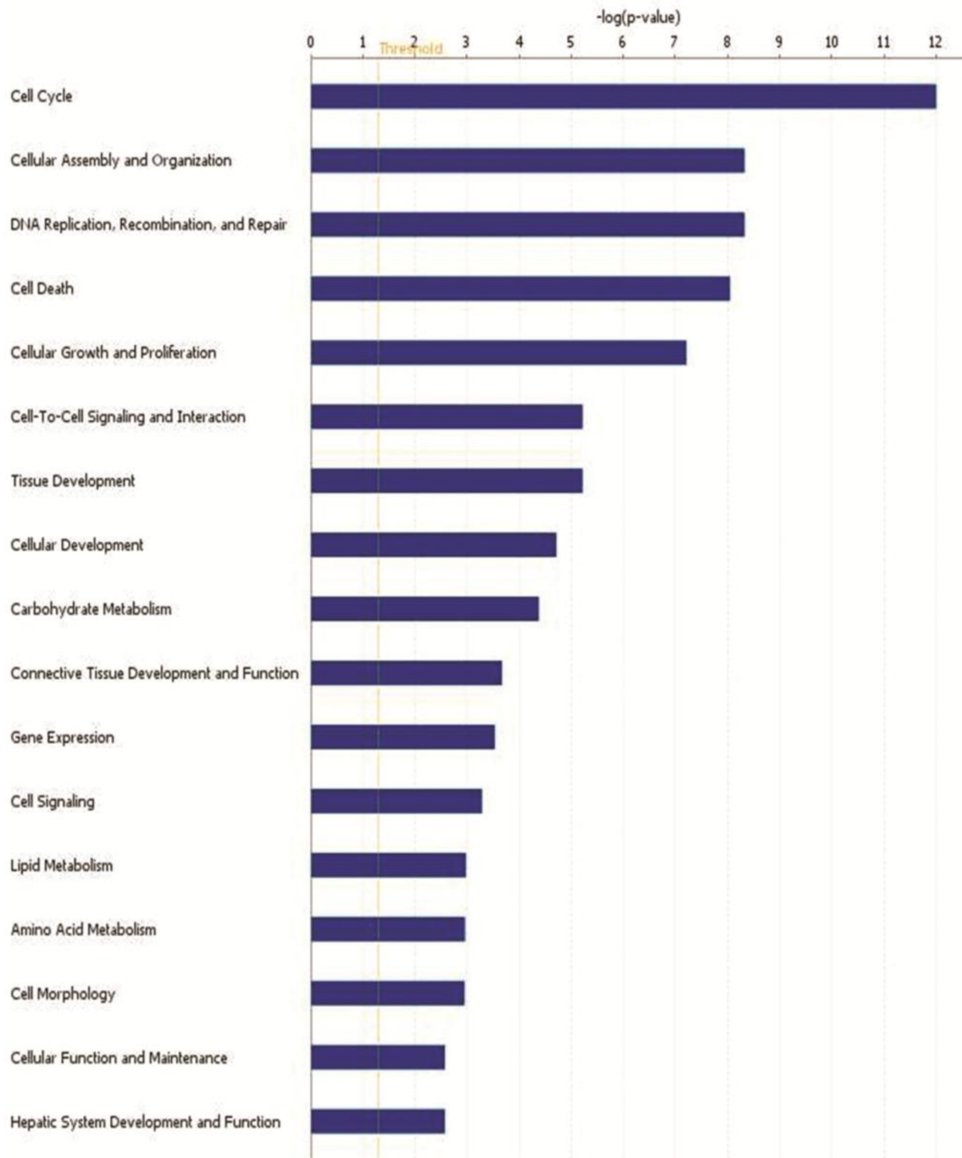

Figure 3. Molecular and cellular functions affected in Huh-7 cells cultured with MSC-CM

Genome-wide gene expression profiles in Huh-7 hepatoma cells after 48 hours culturing with MSC-CM were analyzed using Affymetrix genechips. Functional analysis using Ingenuity Pathway Analysis of the approximately 3000 significantly differentially expressed genes ($P<0.005$) after MSC-CM treatment revealed their involvement in pathways and networks relevant for cell growth, proliferation and survival.

Serum free MSC-CM causes similar effects on liver regeneration as serum containing MSC-CM

The use of bovine serum in the culture medium of MSCs will not be suitable for clinical applications of MSC-CM in humans, because of concerns regarding prions, zoonosis and xenogenic immune responses. Therefore, the effects of concentrated serum free MSC-CM were investigated in liver regeneration after PH and compared to the effects of concentrated serum free unconditioned medium. Similar to treatment at time of PH with serum containing MSC-CM, no significant changes in body weight, liver to body weight ratio or serum levels of albumin, bilirubin, ALT and AST were seen in animals treated with SF-MSC-CM compared to animals treated with SF-UM (data not shown). SF-MSC-CM treatment caused a significant stimulation of hepatocyte proliferation at day 2 after PH compared to SF-UM treatment (17.3% vs. 9.1%, $p<0.01$; Figure 4), which was similar to the effect of serum containing MSC-CM. These results show that MSC-CM can be produced under conditions suitable for clinical application in humans and furthermore that positive effects of MSC-CM on liver regeneration are not caused by factors present in serum.

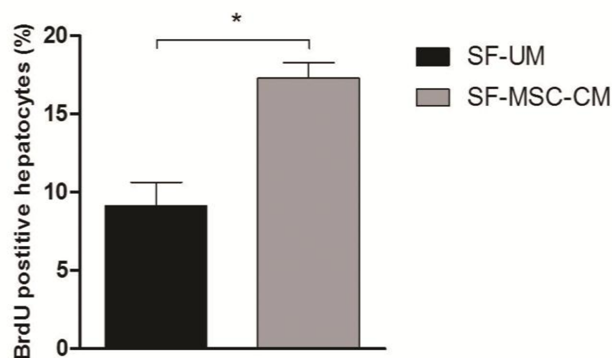

Figure 4. Effects of serum free MSC-CM on hepatocyte proliferation

Two days after PH, livers were processed for immunohistochemistry on BrdU to quantify hepatocyte proliferation after SF-MSC-CM and SF-UM treatment. n=7 in the SF-MSC-CM group and n=5 in the SF-UM group; $*P\leq0.05$ versus SF-UM.

DISCUSSION

In this study we found that treatment with the concentrated conditioned culture medium of human liver-derived MSCs stimulates the already robust liver regeneration in mice after 70% PH. Hepatocyte proliferation was increased by 65% and the tightly regulated liver to body weight ratio by 11%. These effects are in line with findings of Van Poll et al., who observed a three-fold increase in the number of proliferating hepatocytes in animals treated with MSC-CM from BM-MSC after toxic liver injury.[24]

Possible underlying molecular mechanisms responsible for the stimulating effects of MSC-CM could be altered expression levels of cytokines and growth factors involved in liver regeneration. Indeed, MSC-CM upregulated gene expression levels of the cytokine TNF-α and growth factor HGF, known to be required for initiation of and progression through the hepatocyte cell cycle.[1, 26, 28] MSC-CM furthermore upregulated gene expression of the pro-angiogenic factors VEGF-A, VEGF-R1 and -R2 and Ang-1, suggesting that MSC-CM promotes neo-angiogenesis after liver resection. This is in line with findings of Lai and co-workers, who found increased capillary density and better heart function after MSC-CM administration to pigs with a myocardial infarction.[32]

Another interesting effect is the induction of an anti-inflammatory response, as shown by upregulated gene expression of the cytokines IL-10 and IL-1Ra. These findings are in line with previous reports suggesting the immunomodulatory effect of MSCs can be attributed to their stimulating effects on IL-10 expression and their production of IL-1Ra.[21, 31, 33, 34] The anti-inflammatory response may prevent adverse effects of the pro-inflammatory cytokine TNF-α, which is needed for the initiation of liver regeneration, but also mediates cell death.[35] This is supported by the findings of Cosgrove et al., showing that IL-1Ra enhances TNF-α-stimulated hepatocyte proliferation.[36]

Overall, our results show that MSC-CM stimulates liver regeneration by influencing signalling pathways relevant for hepatocyte proliferation, angiogenesis and reduction of inflammation. This is supported by mass spectrometry analysis of the MSC-CM, which revealed the presence of IL-6 and TGF-β, among other extracellular and cytoplasmic components known to be related to cellular growth, proliferation and cellular homeostasis (see Supplementary Methods and Supplemental Table 1). The hypothesis that factors present in MSC-CM influence signalling pathways in hepatic cells is further supported by *in vitro* experiments showing that MSC-CM strongly affects genomic pathways associated with protein synthesis, cell survival and cell proliferation in human hepatocyte-like Huh7 cells. In these short-term culture experiments a trend towards increased cell numbers was found, though this did not reach statistical significance despite upregulation of cell proliferation-related pathways. It is relevant to note that not all genes upregulated by MSC-CM treatment in mouse livers, were found to be upregulated in Huh7 cells. A possible explanation for this discrepancy could be the fact that mouse liver tissue contains more cell types than just hepatocytes (i.e. Kupffer cells, stellate cells, cholangiocytes and others), which could each trigger different gene expression profiles in response to MSC-CM.

The most critical phase in patients undergoing partial liver resection or transplantation of a partial graft is during the early post-operative phase. Potential new treatment strategies to enhance or accelerate liver regeneration should therefore act in this early post-operative period. For that reason, we chose to investigate the effects of MSC-CM at day 2 after partial hepatectomy, known to show the early peak in hepatocyte proliferation in mice. Furthermore, both treatment with MSC-CM at time of PH as well as pre-treatment four hours prior to resection were investigated. Based on hepatocyte prolifera-

tion and gene expression data, treatment at time of PH seemed most effective in stimulating liver regeneration. Interestingly however, we found a significant increase in liver to body weight ratio after pre-treatment, whereas no significant difference was found if animals were treated at time of resection. We hypothesize that MSC-CM pre-treatment shifts the regenerative response of the liver after surgical injury forward and thus accelerates liver regeneration. Gene expression data collected at day 2 after PH in both treatment groups, combined with data collected at time of PH from pre-treated animals, gave insight in the (timely) underlying mechanism by which MSC-CM seems to stimulate liver regeneration. The effects of MSC-CM pre-treatment on gene expression in the resected liver tissue indeed showed a significant increase in TNF-α, IL-6 and IL-1Ra expression at time of PH. Although further evaluation is required, these data support the possibility that pre-treatment with MSC-CM is able to prime the liver prior to surgery and accelerate regeneration after liver resection. This is also supported by the finding that two days after PH only gene expression of TGF-β, known to be important in the termination phase of liver regeneration, was significantly upregulated in animals pre-treated with MSC-CM. This in contrast to significantly upregulated gene expression levels of TNF-α, HGF and PCNA, relevant in earlier stages of hepatocyte proliferation, found in animals first treated at time of PH.

The abovementioned hypothesis that pre-treatment with MSC-CM primes the liver prior to surgery and thereby accelerates the regenerative response, seems the most likely explanation for the different results in liver weight gain and hepatocyte proliferation between animals pre-treated and treated at time of PH with MSC-CM. However, a possible other underlying mechanism could be that pre-treatment stimulates the early recruitment or activation of stem/progenitor cells, which in turn aid to the regeneration process.

Aside from hepatocyte proliferation and liver weight gain, the functional and metabolic recovery of the liver is an essential component of liver regeneration after injury. At any given time, liver function reflects a complex balance between cellular proliferation and metabolic homeostasis.[37-39] During the early phase of liver regeneration, a large part of the liver’s energy is needed for rapid proliferation of parenchymal and non-parenchymal cells, thereby decreasing the amount of energy available for metabolic functions. Because of the increase in the percentage of proliferating cells, one could therefore expect a decrease in metabolic activity in MSC-CM treated animals compared to control treated animals. However, in our experiments, no decrease in serum albumin or increase in bilirubin was found. We can therefore conclude that MSC-CM increases liver regeneration without negatively affecting metabolic homeostasis.

In this study we deliberately chose to use stem cell-conditioned medium, rather than a cell transplantation strategy. Compared with MSC-CM, cell transplantation has the potential advantage of regeneration via (trans)differentiation or cell fusion as well as possible prolonged effects on the micro-environment by long-term engrafted MSCs. However, although differentiation of MSCs into hepatocyte-like cells has been described, differentia-

tion into fully functional hepatocytes is rare. Moreover, engraftment levels of MSCs are often insignificant and cell transplantation may lead to sensitization.[40] Although our findings show the effectiveness of MSC-CM in stimulating liver regeneration, concerns regarding the safe application in humans remain. Especially the use of (bovine) serum in the culture medium of MSCs raises concerns about transmission of prions, zoonosis and xenogenic immune responses. However, our experiments with MSC-CM from serum free cultured L-MSCs showed similar effects on liver regeneration as MSC-CM from serum containing cultures. This illustrates that MSC-CM can be produced under conditions suitable for safe application in humans and furthermore that stimulation of liver regeneration by MSC-CM is not caused by factors present in serum.

In summary, MSC-secreted factors are effective in stimulating liver regeneration after surgical resection by influencing expression levels of cytokines and growth factors relevant for cell proliferation, angiogenesis and anti-inflammatory responses. Therefore, MSC-secreted factors represent a promising therapeutic strategy to improve liver regeneration in patients with a small-for-size liver graft or after extensive liver resections.

Acknowledgments

The authors would like to thank dr. Wendy van Veelen, Jasper Deuring and Werner Helvensteijn for technical support, dr. Yuedan Li and dr. Martijn Brugman for help with gene array data analyses and dr. Jeroen Demmers for helping with mass spectrometry (Erasmus MC Proteomics Center). This study has been financially supported by the Astellas Trans(p)la(n)t(at)ional Research Prize and the Liver Research Foundation (SLO) Rotterdam.

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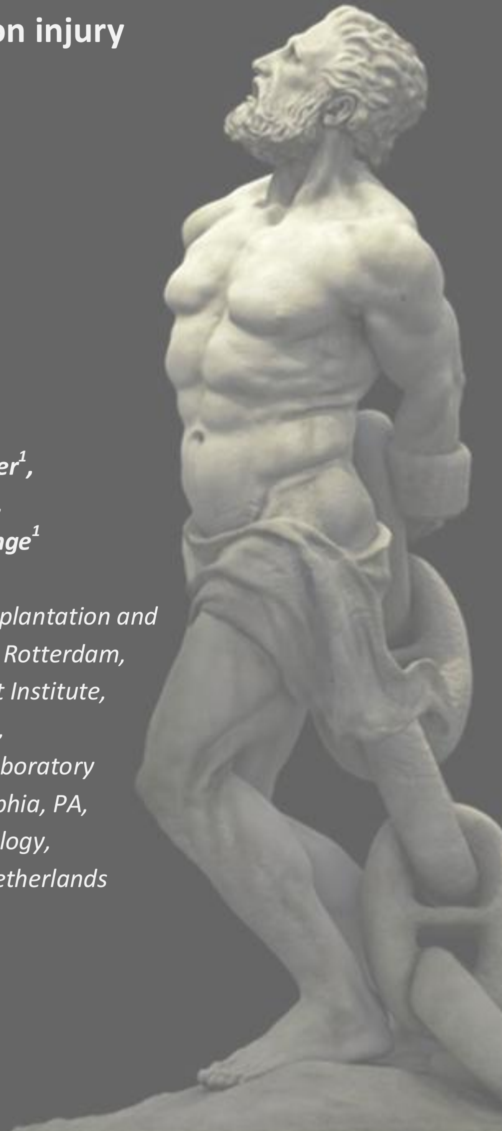
Chapter 9

Mesenchymal stromal cell-derived factors promote tissue repair in a small-for-size ischemic liver model, but do not protect against early effects of ischemia and reperfusion injury



Suomi M.G. Fouraschen[1, 2], Joshua Wolf[2], Petra E. de Ruiter[1], Wayne Hancock[3], Herold J. Metselaar[4], Hugo W. Tilanus[1], Luc J.W. van der Laan[1], Kim M. Olthoff[2] and Jeroen de Jonge[1]

[1]Department of Surgery, Laboratory of Experimental Transplantation and Intestinal Surgery, Erasmus MC-University Medical Center, Rotterdam, The Netherlands; [2]Department of Surgery, Penn Transplant Institute, University of Pennsylvania, Philadelphia, PA, United States, [3]Department of Transplant Immunology, Pathology and Laboratory Medicine, The Children's Hospital of Philadelphia, Philadelphia, PA, United States; [4]Department of Gastroenterology & Hepatology, Erasmus MC-University Medical Center, Rotterdam, The Netherlands

(Manuscript in preparation)

ABSTRACT

Post-resectional liver failure and small-for-size syndrome can occur after extensive liver resections and partial liver transplantation. In these settings, combined loss of a substantial part of the initial liver mass and ischemia/reperfusion injury (IRI) are major contributing mechanisms. Recent studies describe the potential of trophic factors produced by mesenchymal stromal cells (MSCs) to stimulate regeneration after surgical or toxic liver injury. The aim of this study is to investigate if human liver-derived MSC-secreted factors also protect against hepatic IRI as well as promote liver regeneration in a combined injury model.

C57BL/6 mice underwent IRI of 70% of their liver mass, alone or in combination with 50% partial hepatectomy (PH), leaving only ischemic tissue. Mice were treated with MSC-conditioned culture medium (MSC-CM) or unconditioned medium (UM) and sacrificed after 6 or 24 hours in the IRI group and after 48 hours in the IRI+PH group. Their blood and liver tissue were analyzed for hepatocyte proliferation, hepatic injury and gene expression.

In the IRI model, serum ALT and AST levels as well as hepatic tissue damage scores and inflammatory cytokine gene expression levels showed no significant differences after 6 or 24 hours between the MSC-CM and UM treatment group. In the IRI+PH model, a significant reduction in hepatic tissue damage scores after 48 hours was observed in the MSC-CM treatment group. In addition, MSC-CM treatment caused a significant increase in hepatocyte proliferation (2.7 fold increase in BrdU-positive nuclei, p=0.002) in this group.

Conclusion: MSC-derived factors do not show significant benefit on the early effects of ischemia and reperfusion injury. In a small-for-size ischemic liver model however, tissue damage was significantly decreased and regeneration of the liver stimulated by trophic factors secreted by liver-derived MSCs. MSC-derived factors represent a promising ready-to-use strategy to decrease injury and improve regeneration in the setting of small-for-size syndrome and post-resectional liver failure.

INTRODUCTION

Advances in surgical techniques have enabled large liver resections as well as split and living donor liver transplantation (LDLT). Transplantation of partial (living donor) liver grafts was introduced to help overcome donor organ scarcity and reduce waitlist mortality. Living donors undergo resection of approximately 40-60% of their liver volume, which is transplanted into the recipient. Without the exceptional capacity of the liver to regenerate and thereby compensate for tissue loss and restore homeostasis, these extensive resections and partial graft transplantations would not be possible.[1-3] Nevertheless, in case of adult to adult living donor liver transplantation both donors and partial graft recipients end up with a small-for-size liver, which is still associated with significant morbidity and mortality.[2, 4, 5] In an attempt to decrease donor risk, smaller grafts (such as the left lobe of the liver) can be used, but this is limited by the increased risk of the recipient to develop small-for-size syndrome.[6]

In these settings, both loss of a substantial part of the liver mass as well as the inevitable ischemia and reperfusion injury (IRI) are major mechanisms of hepatic injury.[7, 8] Effective therapeutic strategies to protect against IRI, enhance regeneration and stimulate recovery could minimize donor and recipient risk. This benefits liver transplantation using size-mismatch grafts and could increase the donor pool.

A promising new therapeutic intervention can be found in mesenchymal stromal/stem cell (MSC) based strategies.[9-12] Initially, MSCs were thought only to provide a supportive niche for hematopoietic stem cells in the bone marrow. Over the last years, they have been reported to reside in multiple tissue compartments, including lung, liver and adipose tissue.[13-15] Our group showed that the adult human liver harbors a population of MSCs, highly similar to bone-marrow MSCs, which is mobilized from liver grafts at time of transplantation.[12] These liver-derived MSCs (L-MSCs) can be retrieved from the organ preservation solution and appear to have immunosuppressive capacities as well as multilineage differentiation potential. Furthermore, we have reported that the trophic factors secreted by these L-MSCs stimulate liver regeneration after surgical resection, mainly by promoting hepatocyte proliferation and altering expression levels of regeneration-related genes.[16]

The use of MSC-derived factors in a clinical setting may have important advantages over the use of MSCs: there is no risk of rejection or possible malignant transformation and the factors can be produced ready-to-use in large clinical grade quantities. Beneficial effects of MSC-secreted factors have also been reported in the setting of toxic liver injury and hepatic failure.[17, 18] The aim of this study is to investigate whether L-MSC-derived factors are as effective to ameliorate hepatic ischemia and reperfusion injury as well as to promote regeneration in a clinically relevant model of combined IRI and partial liver resection.

MATERIALS AND METHODS

Animals

Male C57Bl/6 mice (age 7-10 weeks) were obtained from Jackson Laboratories (Bar Harbor, ME, USA) and maintained in the animal facility on a 12/12 hour light/dark schedule. The animals had free access to food and drinking water. All animal experiments were performed with approval of the institutional animal welfare committee.

Human L-MSC cultures and conditioned medium

Liver-derived MSCs were obtained from the UW organ preservation solution (Viaspan, Bristol-Myers Squibb, Woerden, Netherlands), collected after cold storage of human liver grafts for transplantations performed at the Erasmus Medical Center, Rotterdam, The Netherlands. The Medical Ethical Council of the Erasmus Medical Center and the Institutional Biological Safety Committee of the Children's Hospital of Philadelphia approved the use of human donor material for medical research.

Mononuclear cells were isolated from the collected preservation fluids by density gradient centrifugation using Ficoll-Paque Plus (GE Healthcare, Uppsala, Sweden) and put into culture as previously described.[14] Culture medium consisted of MEM alpha (Invitrogen, Grand Island, NY, USA) supplemented with 15% fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA), L-Glutamin (Invitrogen), penicillin and streptomycin (Invitrogen). The last three days before collecting the supernatant, L-MSCs were cultured under serum free conditions. Culture medium was therefore changed to MEM-alpha supplemented with 0.05% bovine serum albumin (Sigma-Aldrich), L-Glutamin, penicillin and streptomycin. The MSC-conditioned culture medium (MSC-CM) was collected three days after medium change of L-MSC cultures from passage 6-10. MSC-CM was concentrated approximately 25-fold by filtration with 3-kD molecular cut-off filters (Amicon Ultra, Millipore, Carrigtwohill, Ireland).

Surgical procedures and MSC-CM treatment

C57Bl/6 mice were anesthetized and injected intraperitoneally with 100 U/kg heparin. After a midline laparotomy, ischemia and reperfusion injury (IRI) with or without partial hepatectomy (PH) was induced. All procedures were performed under clean conditions.

In the IRI alone group, 90 minutes of ischemic injury of 70% of the liver was induced by clamping the blood supply to the left lateral and median lobes with microvascular clamps. This way the right lateral and caudate lobes served as a porto-caval shunt, allowing survival of the animals during the ischemic period.

In the combined (IRI+PH) group, 60 minutes of ischemic injury was induced as described above, after which the right part of the median lobe, the right lateral lobe and the caudate lobes were ligated and resected, leaving approximately 50% ischemic liver tissue. The combination of ischemic injury with 50% hepatectomy did not allow an ischemic period of more than 60 minutes, without affecting survival. During the ischemic period in both

groups, the abdominal cavity was covered with saline-moistened gauzes and the animals were kept under anesthesia on a warming plate to conserve body temperature. At the end of the surgical procedures the peritoneum and skin were sutured separately.

In both groups, part of the animals was treated with 200 μl of the concentrated serum-free L- MSC-CM, injected intraperitoneally at the end of the surgical procedure. The other animals were treated similarly with concentrated serum-free unconditioned medium (UM). This unconditioned medium consisted of culture medium treated exactly the same as the serum free MSC-CM, but without the presence of L-MSCs. The animals in the IRI group were sacrificed either 6 or 24 hours after surgery. The animals in the IRI+PH group were treated a second time with MSC-CM or UM after 24 hours and were sacrificed 48 hours after surgery. From all animals (n=8 per group) blood and liver tissue were collected to further investigate the effects of MSC-CM on serum markers of liver function, tissue injury, hepatocyte proliferation and hepatic gene expression in the early phase after liver injury.

Weight calculations

Animals were weighed daily prior to treatment. In the IRI+PH group the resected liver mass was weighed after PH. The initial total liver weight was calculated as follows:

Resected liver weight/50*100 (g)

At time of sacrifice the animals and, in the IRI+PH group, their regenerated liver mass were weighed. The percentage of reconstitution of the liver was calculated by:

Regenerated liver weight/initial total liver weight*100 (%)

The liver to body weight ratio was calculated by:

Regenerated liver weight/body weight at time of harvest*100 (%)

Immunohistochemistry

One hour prior to sacrifice, animals were injected intraperitoneally with 50 mg/kg BrdU (5-Bromo-2'-deoxyuridine; B5002, Sigma-Aldrich). Livers were harvested and processed to 4 μm thick formalin fixed, paraffin embedded sections and stained with Hematoxylin and Eosin (H&E) using a standard staining protocol, and for BrdU using the following protocol: Antigen retrieval was achieved by boiling the sections in 0.01 M sodium citrate; pH 6.0 (microwave 1000 Watt; 1x7 and 2x3 minutes). Endogenous peroxidase was blocked by 0.6% H_2O_2 in PBS for 30 minutes at room temperature, after which DNA was denaturated by incubation for 1 hour at 37°C in 0.1 M HCl in aqua dest. Aspecific binding was prevented by 0.5% milk powder supplemented with 0.15% glycin in PBS (blocking buffer). Sections were incubated overnight at 4°C with monoclonal mouse anti-BrdU (Bu20a; DakoCytomation, Glostrup, Denmark; 1:80 in blocking buffer). The next day sections were incubated for 30 minutes at room temperature with polyclonal rabbit anti-mouse IgG/HRP (P0161; DakoCytomation; 1:1000 in blocking buffer). After antibody incu-

bation sections were incubated with DAB-solution and counterstained with hematoxylin. Per animal 4 high power fields (HPF; 400x) were analyzed for BrdU positive hepatocytes.

Serum analysis of transaminase levels

Blood samples were collected at time of sacrifice in heparin coated micro tubes. After collection, samples were centrifuged (19 minutes, 1800 rpm) to separate the serum, which was then further analyzed at the clinical chemical core facility of The Children's Hospital of Philadelphia to determine alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels.

Real-time quantitative RT-PCR

At time of sacrifice, liver tissue was stored overnight at 4°C and thereafter at -80°C in Allprotect Tissue Reagent (Qiagen, Valencia, CA, USA) for RNA preservation. Total RNA was extracted using Trizol (Qiagen) and chloroform after mechanical disruption of the tissue. RNA was precipitated in 75% ethanol and dissolved in RNase-free water. RNA quantity and quality was analyzed using a NanoDrop ND-1000 (Thermo Fisher Scientific, Wilmington, DE, USA). One microgram of RNA was reverse-transcribed to cDNA using an iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA). PCR primers (presented in Table 1) were synthesized by Isogen Life Science (Maarssen, Netherlands) and Biolegio (Nijmegen, Netherlands). Real-time quantitative RT-PCR was performed with a SensiMix SYBR & Fluorescein Kit (Bioline, London, United Kingdom) and MyIQ real time PCR detection system (Bio-Rad Laboratories) according to the manufacturer's instruction.

Table 1 RT-PCR primer sequences

Gene	Name	Accession number	Primer (forward/reverse)
TNFA	Tumor necrosis factor alpha	NM_013693	CCCTCACACTCAGATCATCTTCT GCTACGACGTGGGCTACAG
IL6	Interleukin 6	NM_031168	TAGTCCTTCCTACCCCAATTTCC TTGGTCCTTAGCCACTCCTTC
IL1RN	Interleukin 1 receptor antagonist	NM_031167	GCTCATTGCTGGGTACTTACAA CCAGACTTGGCACAAGACAGG
IL10	Interleukin 10	NM_010548	GCTCTTACTGACTGGCATGAG CGCAGCTCTAGGAGCATGTG
CCND1	Cyclin D1	NM_007631	GCGTACCCTGACACCAATCTC CTCCTCTTCGCACTTCTGCTC
TGFB	Transforming growth factor beta	NM_011577	CTCCCGTGGCTTCTAGTGC GCCTTAGTTTGGACAGGATCTG
KDR	Vascular endothelial growth factor receptor 2	NM_010612	TTTGGCAAATACAACCCTTCAGA GCAGAAGATACTGTCACCACC
ANGPT1	Angiopoietin 1	NM_009640	CACATAGGGTGCAGCAACCA CGTCGTGTTCTGGAAGAATGA
VEGFA	Vascular endothelial growth factor A	NM_009505	GCACATAGAGAGAATGAGCTTCC CTCCGCTCTGAACAAGGCT
FLT1	Vascular endothelial growth factor receptor 1	NM_010228	TGGCTCTACGACCTTAGACTG CAGGTTTGACTTGTCTGAGGTT
TBP	TATA binding protein	NM_013684	AGAACAATCCAGACTAGCAGCA GGGAACTTCACATCACAGCTC

Statistical analysis

All data are presented as mean ± SEM and statistical analyses were performed using the Mann-Whitney test with GraphPad Prism software and $p \leq 0.05$ was considered statistically significant.

RESULTS

Body and liver weight after IRI with or without PH are not affected by MSC-CM

In the IRI alone group, no significant differences in body weight change were observed (data not shown). In the IRI+PH group, all animals showed a decrease in body weight on post-operative day 1 and 2, but without statistically significant differences between the MSC-CM and UM treated groups (9.2% vs. 10.3% decrease of initial body weight, p=0.96; Figure 1A). Liver weight after PH increased with 29.0% in the MSC-CM treated group (from 50% to 64.5% of the initial liver weight) and with 21.6% in the UM group (from 50% to 60.8%, p=0.40; Figure 1B). A similar effect was seen with regard to the liver to body weight ratio at time of sacrifice (3.0% in the MSC-CM group vs. 2.9% in the UM group, p=0.31; Figure 1C).

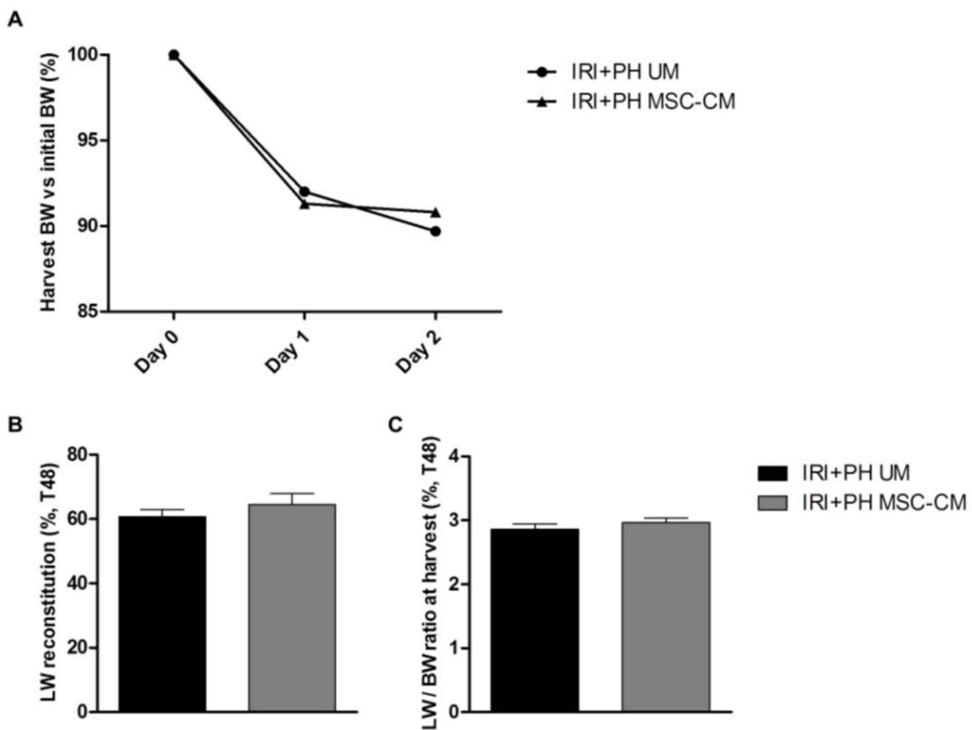


Figure 1. Effects of MSC-CM on body and liver weight after IRI+PH

A. Body weight change from surgery to harvest; **B.** Harvest liver weight versus initial liver weight; **C.** Harvest liver weight to body weight ratio.

MSC-CM treatment provides cyto-protective effects

We investigated hepatic tissue injury 6 and 24 hours after IRI as well as 48 hours after IRI+PH by analyzing H&E stained liver tissue sections for signs of injury. Sections were classified based on the percentage of affected tissue (no injury, 0-25%, 25-50%, 50-75% or >75% of liver tissue affected).

At 6 hours after IRI, no statistically significant differences in tissue injury score were found in animals treated with MSC-CM compared to UM treatment, though a trend toward reduced hepatic injury was seen (MSC-CM vs. UM treatment: no injury 43% vs. 38%, 0-25% injury 57% vs. 25% and >25% 0% vs. 38%; p=0.18; Figure 2A). Similar results were found 24 hours after IRI (MSC-CM vs. UM treatment: no injury 63% vs. 50%, 0-25% injury 25% vs. 50% and >25% injury 13% vs. 0%; p=1.00; Figure 2B). After IRI+PH however, MSC-CM treatment significantly decreased hepatic tissue injury compared to UM treatment, with 38% versus 10% of animals showing no microscopic signs of injury, 63% versus 50% showing 0-25% injury and 0% versus 40% with >25% injury at 48 hours after surgery (p=0.04; Figure 2C).

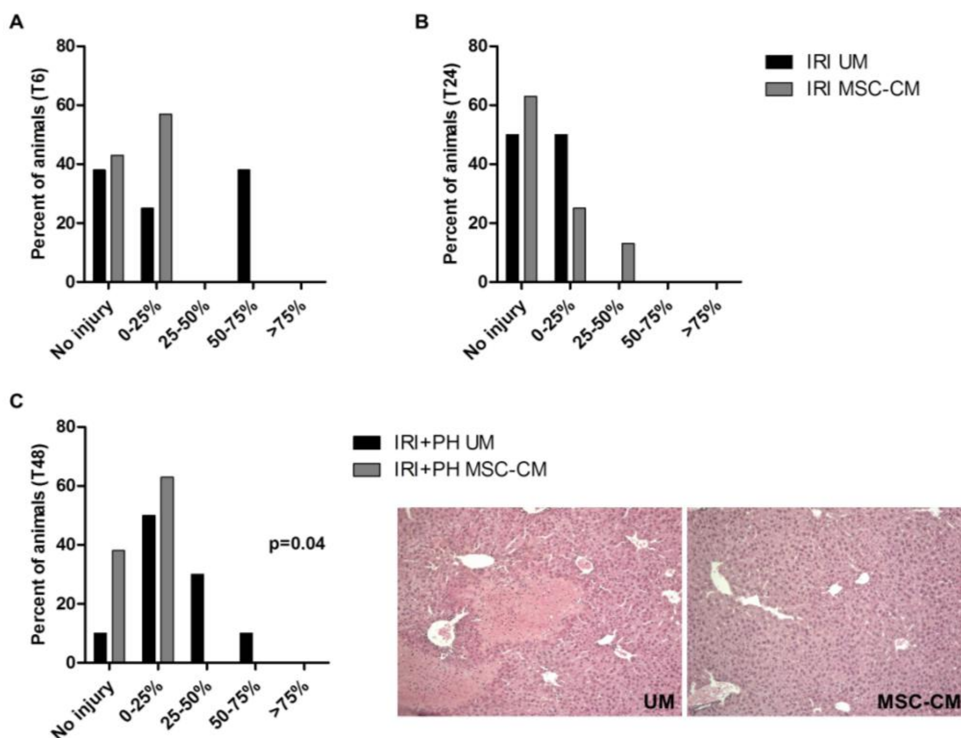


Figure 2. Effects of MSC-CM on hepatic tissue injury

H&E stained liver tissue sections were classified based on the percentage of damaged tissue: no injury, 0-25%, 25-50%, 50-75% or >75% of liver tissue affected. This figure shows the percentage of animals with a certain injury score **A.** 6 hours after IRI, **B.** 24 hours after IRI and **C.** 48 hours after IRI+PH with representative pictures.

Additionally, serum transaminase levels were investigated as markers for hepatic injury. In the IRI group, serum ALT levels after 6 hours were 5301±1426 IU/l in the MSC-CM treated group versus 5225±1654 IU/l in the UM treated group (p=1.00; Figure 3A). After 24 hours, ALT levels were reduced to 229±147 IU/l in the MSC-CM group versus 229±77 IU/l in the UM group (p=0.23; Figure 3B). Similar results were found for AST levels (p=1.00 at 6 hours and p=0.33 at 24 hours respectively; Figure 3D, E). In contrast, 48 hours after IRI+PH serum ALT and AST levels were markedly lower in the MSC-CM treated animals compared to the UM treated animals, though differences did not reach statistical significance (MSC-CM vs. UM treatment: ALT 138±35 IU/l vs. 764±399 IU/l, p=0.18 and AST 248±41 IU/l vs. 1008±484 IU/l, p=0.14; Figure 3 C, F).

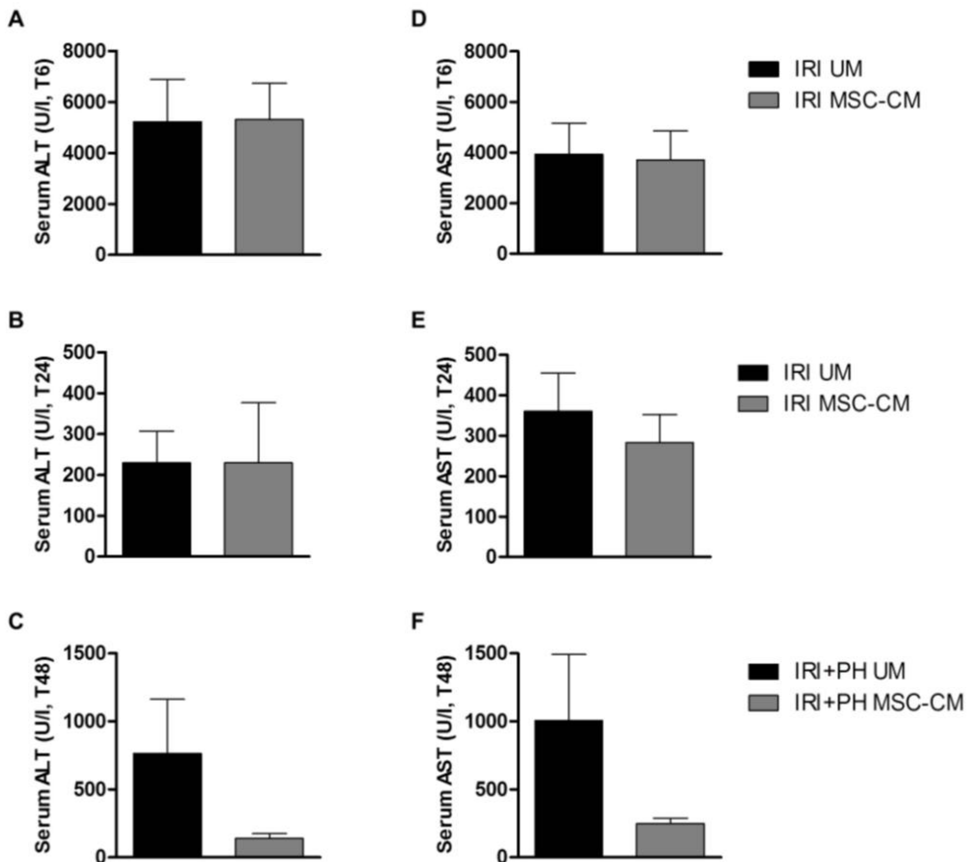


Figure 3. Effects of MSC-CM on serum injury markers

Serum ALT levels at **A.** 6 hours after IRI, **B.** 24 hours after IRI and **C.** 48 hours after IRI+PH. Serum AST levels at **D.** 6 hours after IRI, **E.** 24 hours after IRI and **F.** 48 hours after IRI+PH.

MSC-CM treatment stimulates hepatocyte proliferation after IRI+PH

In healthy individuals, nearly all hepatocytes reside in a quiescent state and only incidental proliferation is found upon investigation. After loss of liver mass, hepatocytes are triggered to enter the cell cycle and proliferate until tissue loss is compensated and homeostasis is restored, showing a peak in proliferation in rodents around day 2 after liver tissue injury.

In this study, hepatocyte proliferation in the IRI groups was not increased after 6 hours, independent of the treatment strategy (0.1% vs. 0.1%, p=0.85; Figure 4A). After 24 hours, MSC-CM treatment appeared to slightly induce hepatocyte proliferation, though proliferation levels were still low and showed no significant difference between treatment groups (0.19% after MSC-CM treatment vs. 0.06% after UM treatment, p=0.22; Figure 4B).

In contrast, IRI+PH resulted in a clear increase in hepatocyte proliferation after 48 hours, with an almost 3-fold higher proliferation index in the MSC-CM treated animals compared to the UM treated animals (13.5% vs. 5.0%, p=0.002; Figure 4C).

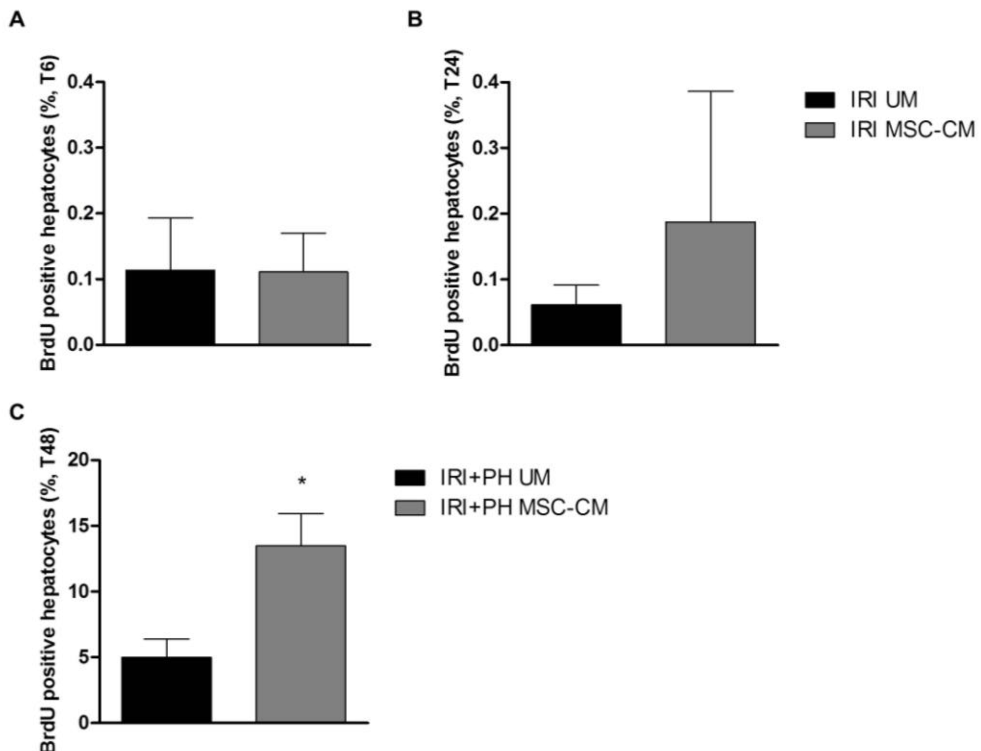


Figure 4. Effects of MSC-CM on hepatocyte proliferation

Livers were processed for immunohistochemistry on BrdU to quantify hepatocyte proliferation. This figure shows the percentage of BrdU-positive hepatocytes **A.** 6 hours after IRI, **B.** 24 hours after IRI and **C.** 48 hours after IRI+PH; *P≤0.05.

Treatment with MSC-CM does not significantly affect intrinsic gene expression levels

We investigated if treatment with MSC-derived factors affected hepatic expression levels of inflammation, proliferation and angiogenesis related genes. At 6 hours after IRI, MSC-CM treatment downregulated expression levels of the inflammatory genes TNF-α and IL-1Ra compared to expression levels in the UM treated group (TNF-α 40% reduction, p=0.33; IL-1Ra 34% reduction, p=0.51), though results were not statistically significant (Figure 5A). Similar, in the IRI+PH model, downregulation of TNF-α (50% reduction, p=0.37) and IL-1Ra (33% reduction, p=0.41) gene expression in the MSC-CM group was not statistically significant (Figure 5C). Furthermore, 48 hours after IRI+PH and MSC-CM treatment a trend toward upregulation of the cell proliferation stimulating gene Cyclin D1 (1.7-fold increase, p=0.36) was seen versus a downward trend of the cell cycle inhibiting gene TGF-β (21% reduction, p=0.10; Figure 5D). None of the pro-angiogenic genes VEGF-A, VEGF-R1, VEGF-R2 and Ang-1 showed clear differences at any time point.

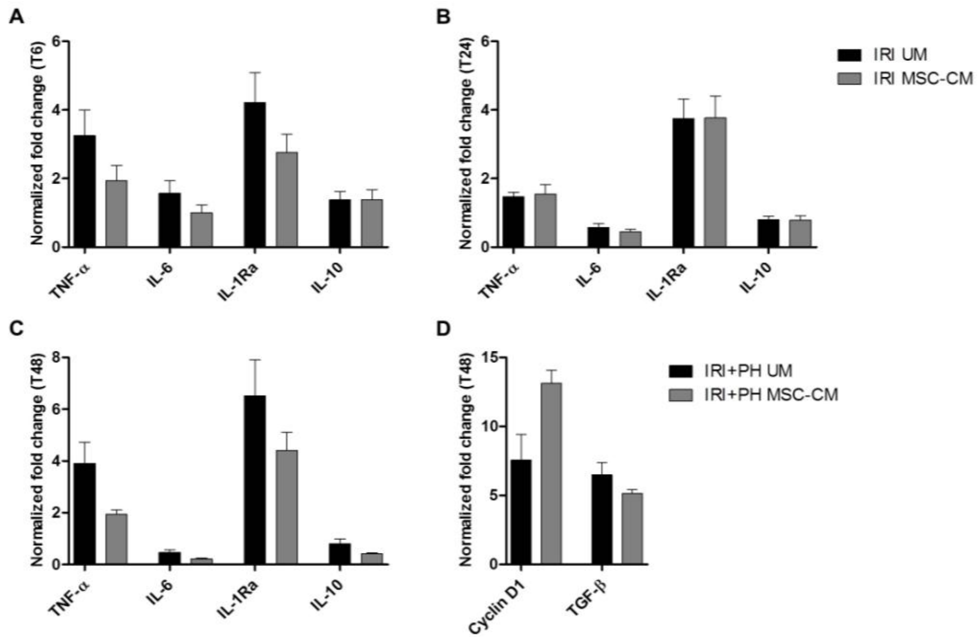


Figure 5. Effects of MSC-CM on hepatic gene expression

Hepatic gene expression levels were determined by quantitative RT-PCR and normalized against TBP. Expression levels of inflammation related genes at **A.** 6 hours after IRI, **B.** 24 hours after IRI and **C.** 48 hours after IRI+PH; **D.** Expression levels of cell cycle related genes at 48 hours after IRI+PH.

DISCUSSION

Hepatic ischemia and reperfusion injury is a common pathologic phenomenon that may occur in the situation of shock, severe liver trauma, liver resection under vascular occlusion and liver transplantation. The deprivation of blood and oxygen supply during the

ischemic period leads to deficient adenosine triphosphate (ATP) production in both parenchymal and non-parenchymal cells, resulting in intracellular ionic disturbance and cellular swelling.[19, 20] Subsequent reperfusion with oxygenated blood causes additional damage due to a fast change in the redox state of the liver tissue by increasing levels of reactive oxygen species (ROS).[7, 8, 21] These elevated ROS levels progress into oxidative stress, resulting in inflammation, damaged cellular components and induction of apoptosis and necrosis of liver cells.[22, 23] Concomitant release of cytokines and chemokines by activated Kupffer cells stimulates the infiltration of neutrophils and CD4+ T-cells into the liver tissue, resulting in a vicious circle of activation of these inflammatory cells and destruction of hepatocytes and endothelial cells.[24-27]

In case of adult-to-adult partial (living donor) liver transplantation, this hepatic IRI occurs in a graft that is per definition small-for-size for the recipient, requiring robust regeneration to provide sufficient metabolic support. Effective therapeutic strategies to protect against IRI, while enhancing regeneration, could enable donation of a smaller liver grafts and minimize donor risks. This would increase the donor pool and benefit transplantation of size-mismatch liver grafts.

In recent years the protective and regenerative effects of MSC therapy have been broadly investigated in animal models of cerebral or myocardial infarction as well as after renal IRI [28-31]. Other reports have published the effectiveness of MSCs against toxic liver injury and hepatic failure.[9-12, 17, 18] MSCs provide pleiotropic effects in response to tissue injury and appear to stimulate organ repair by affecting inflammation and inducing anti-apoptotic effects.[32-36] Furthermore, they exert immunomodulatory effects on the immune response processes triggered during reperfusion.[37] The application of MSCs in animal models of hepatic IRI resulted in reduced tissue injury by repression of oxidative damage as well as increased hepatocyte proliferation in small-for-size models.[38-43] The exact mechanism of MSC-mediated effects remains unclear, but increasing evidence suggests the involvement of paracrine effects by MSC-secreted trophic factors, as the cells are short-lived and trapped in the lung after intravenous infusion.[44]

So far, very few studies investigated the effects of MSCs on liver IRI in a combined liver resection model. Kanazawa and co-workers described their experience with bone marrow (BM-)MSCs in a model of 70% hepatectomy after 40 minutes of warm ischemia. BM-MSCs were infused in the portal vein directly after resection of the liver, resulting in less signs of liver tissue injury, including vacuolar changes and apoptosis, and accelerated regeneration. However, as the authors discuss, the optimal route of MSC administration remains unclear. Systemically transplanted MSCs are mostly trapped in the microvasculature of the lung because of their size and adhesion potential. Direct injection into the portal vein, on the other hand, seems effective but might be unsafe. The number of MSCs needed for therapeutic effects is not known and ranges from 2 to 10 million MSCs per kilogram in small animal experiments, whereas fatal embolism has been described for injections exceeding 10 million cells overall.[45] Furthermore, concern has been raised on the

possibility of malignant transformation of MSCs.[46, 47] The use of MSC-derived factors may therefore have important advantages over the use of MSCs. Other potential advantages are the elimination of the risk of rejection by the recipient's immune system, as well as the feasibility to produce the factors ready-to-use in large clinical grade quantities.

Our group previously reported that trophic factors secreted by L-MSCs increase hepatocyte proliferation and alter expression levels of regeneration-related genes after partial hepatectomy, thereby stimulating liver regeneration.[16] In the current study, similar effects were found with an almost 3-fold increase in hepatocyte proliferation, despite combined injury induced by 60 minutes of warm IRI and 50% hepatectomy. Furthermore, a significant reduction in hepatic tissue injury, as well as a decrease in serum transaminase levels and expression levels of inflammatory genes were found at 48 hours in the combined injury model after treatment with MSC-derived factors.

In a recent study, Du et al. describe the use of BM-MSC conditioned medium for the first time in a 50% rat liver transplantation model.[48] In this elegant combined IRI and partial liver resection model, the cold and warm ischemic times were kept to a minimum (approximately 60 and 16 minutes respectively), limiting the IRI component. Nevertheless, their results show similar effects: decreased inflammation at 24 hours, upregulation of angiogenesis at 72 hours, fewer apoptotic cells and overall promoted liver regeneration. Interestingly, in our model liver cell proliferation was more profound whereas in the study of Du et al. inhibition of inflammation was more obvious. This might possibly be attributed to the different source of MSCs: our liver-derived MSCs were found to be highly similar to BM-MSCs, however a small percentage (approximately 0.3%) of genes showed a two- or more-fold difference in expression.[14]

Significant differences in liver weight reconstitution were not detected in our study, which is in line with the findings of Kanazawa et al., showing a significant increase in liver weight at seven days after 50% liver transplantation in rats, but not at earlier time points.[41] Seki and co-workers, on the other hand, describe an increase in regenerated liver weight as early as day 2 in their model of MSC treatment after combined IRI and 70% hepatectomy.[39] However, they induced 15 minutes of IRI compared to 60 minutes in our model, resulting in significantly lower oxidative stress related injury and thereby preserving more functional hepatocytes that can contribute to regeneration by proliferation.

Interesting furthermore are the effects of MSC-CM after IRI without liver resection. Our results show no effects on serum ALT and AST levels and only a trend toward reduced tissue injury at 6 hours post-reperfusion. In contrast, studies using MSCs have shown that treatment with these cells significantly decreases serum ALT and AST levels after IRI.[38, 40, 49] This suggests that MSC-derived factors are not as successful in preventing tissue injury. In our combined IRI and resection model, however, a beneficial effect on both markers of tissue injury was found. Another possible explanation for these seemingly conflicting data could be the duration of ischemia and the percentage of ischemic liver mass: 90 minutes of ischemia in 70% of the liver in our model versus 60 minutes in 30-70% of the liver in the

other studies. The oxidative damage induced by 90 minutes could be too severe to detect differences at early time points, i.e. 6 and 24 hours after reperfusion.

In summary, our study confirms that MSC-CM decreases hepatic tissue injury and promotes liver regeneration after large liver resections in an ischemia/reperfusion injury environment. MSC-derived factors represent a promising strategy for safe and ready-to-use therapeutic intervention to stimulate organ repair and regeneration in the setting of small-for-size syndrome and post-resectional liver failure. However, the optimal source of MSCs for this conditioned medium as well as the dosage still need to be elucidated.

Acknowledgments

The authors would like to thank Liqing Wang for technical support. This study has been financially supported by the ASTS-ESOT exchange grant and the Foundation for Liver and Gastrointestinal Research (SLO).

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Chapter 10

Summary, General discussion & Future perspectives

Prometheus unbound?

Since the Greek myth about Prometheus' torture and the first scientific documentation of the phenomenon of liver regeneration in the 19th century, scientists have extensively investigated this intriguing process in an attempt to unravel its mystery. Numerous molecules and pathways involved in regeneration of the liver have been revealed, however the exact underlying mechanisms are still not fully elucidated. Meanwhile, the extensive regenerative capacity of the liver has been used to benefit patients with (end-stage) liver disease, as it enables oncologic liver resections and living donor cq. split liver transplantation. However, several factors like a patient's age, life style, nutritional status, disease condition, degree of injury and medication, but probably also genetic predisposition, can interfere with and limit the process of regeneration, resulting in impaired liver function and compromised homeostasis. Better understanding of the factors influencing and regulating regeneration of the liver after injury, contributes to the investigation and development of potential therapeutic strategies to prevent liver dysfunction and promote regeneration, thereby decreasing subsequent patient morbidity and mortality.

In the present thesis the mechanisms involved in the process of liver regeneration, specifically after liver resection and transplantation, were further investigated. Several aspects that influence regeneration in the setting of liver resection and transplantation are described, including ischemia and reperfusion (IR) injury and immunosuppressive medication. In addition, promising stem/progenitor cell-based treatment strategies to modulate and accelerate regeneration of the liver after surgical injury were explored.

Gene expression profiles of liver regeneration in health and dysfunction

Liver regeneration has been identified as a multi-step process, starting with the priming of hepatocytes by the release of cytokines from injured liver cells and activated Kupffer cells.[1-4] Progression through the cell cycle is then stimulated by the activation of transcription factors and subsequent release of growth factors that activate cell cycle related signaling pathways. While this process has been extensively studied in experimental animal models, it has been difficult to do so in humans. In **chapter 2**, using the Adult-to-Adult Living Donor Liver Transplant (A2ALL) database, we investigated the immediate early effects of liver resection on hepatic gene expression profiles in healthy living liver donors. These donors underwent resection of approximately 50% of their total liver mass, which overall activated pathways involved in acute phase and oxidative stress responses, triggered cell proliferation related signaling and silenced metabolic functions. These early effects on gene expression profiles support the general hypothesis that the liver balances between its two major functional mechanisms: regenerative processes to restore liver tissue after injury and metabolic processes to ensure homeostasis.[5-7]

Though none of the living donors showed clinical signs of impaired regeneration, significant differences were found in their regenerated liver mass at three months after surgery. Comparison between donors with successful and limited regeneration of their rem-

nant liver mass revealed distinct differences in their immediate early gene expression profiles. Findings suggested several pathways and mechanisms that might contribute to more robust regeneration, including better activation of the oxidative stress response and higher initiation of protein synthesis at time of liver resection. Successful regenerating donors showed mostly upregulated expression levels post-resection, resulting in activation of pathways involved in stress response, cell cycle regulation and proliferation. In contrast, donors with limited regeneration mainly showed downregulated expression levels, resulting in inhibition of pathways involved in lipid and carbohydrate metabolism. In line with the regeneration-metabolism balance hypothesis, a possible conclusion from these contrariwise gene expression profiles could be that livers with deficient liver regeneration display inhibited or delayed initiation of recovery and regeneration-related molecular pathways, as their livers mainly focus on suppression of metabolism.

This delicate balance between liver tissue repair and the ability to maintain homeostasis is even more important in liver graft recipients after transplantation. Severe impact of factors like age, nutritional status, pre-operative clinical condition, degree of tissue injury and certain medication can result in impaired liver function or even hepatic failure, and is clinically manifested by high transaminases, persistent cholestasis and prolonged coagulopathy.[8-12] The analyses described in **chapter 3** correlated the clinical phenotype of early allograft dysfunction (EAD) with the molecular signature found in the liver graft immediately following reperfusion. Comparison between EAD and non-EAD recipients suggested specific metabolism, inflammation and cellular proliferation related genes and pathways, characteristic and possibly causative of EAD. Moreover, the combination of downregulated metabolic capabilities and upregulation of pro-inflammatory molecules supported our hypothesis that at any given time the liver displays a delicate energy balance between recovery and metabolic need, which may be quickly restored to equilibrium in well-functioning livers. In the setting of EAD, however, over-activation of inflammatory processes might deviate the liver's energy away from metabolic processes. The liver may therefore not be able to fully accommodate the metabolic demands of the body, thereby leading to the symptoms of dysfunction characterized by high serum transaminases, persistent cholestasis and prolonged coagulopathy.

It would therefore be of significant clinical value to be able to detect or even predict the development of EAD at an early time point. To this end, we identified a gene expression signature, consisting of 152 genes, that could distinguish between EAD and non-EAD liver biopsies taken at time of transplantation and therefore was diagnostic of EAD. We were able to validate this diagnostic expression signature, both with an independent cohort of recipients as well as with a different gene expression detection technique, showing potential for future use of the signature in a clinical setting.

The role of mTOR and autophagy in liver regeneration

After transplantation, graft recipients are treated with immunosuppressant medication to prevent graft rejection. However, several immunosuppressants are described to affect regeneration. Especially the use of the mTOR inhibitor rapamycin has raised concerns, as mTOR (mammalian target of rapamycin) is involved in the control of protein synthesis, cell size and proliferation.[13-16] On the other hand, mTOR inhibition is an attractive alternative for current calcineurin inhibitor based immunosuppressive strategies, as the mTOR inhibitor rapamycin does not cause nephrotoxicity and has recently gained wide interest in the treatment of cancer.[17, 18]

Additionally, mTOR has been implicated to be of paramount importance in the control of autophagy, a general term for pathways in which cytoplasmic material, including soluble macromolecules and organelles, are delivered to lysosomes for degradation.[19-22] Autophagy is thought to have evolved as a stress response mechanism that allows organisms to survive during harsh conditions, probably by regulating energy homeostasis.[23] As described in **chapter 4**, mTOR inhibition resulted in severely inhibited liver regeneration after surgical resection. For the first time, we describe that mTOR inhibition also significantly increased hepatic autophagy during liver regeneration after partial hepatectomy. This effect may compensate for the decreased hepatocyte proliferation, as increased autophagy ameliorates oxidative stress and saves cellular energy. The most novel finding of this study is that this impaired regeneration can be partly reversed by treatment with exogenous IL-6 and HGF.

Mesenchymal stromal/stem cell-based treatment strategies to improve liver regeneration

Mesenchymal stromal/stem cells (MSCs) were initially thought only to provide a supportive niche for hematopoietic stem cells in the bone marrow. Over the last years, they have been reported to reside in multiple tissue compartments, including lung, liver and adipose tissue.[24-26] MSCs have the ability to differentiate into hepatocytes and cholangiocytes and have immunomodulatory and anti-inflammatory properties.[27-31] Furthermore, they are described to promote angiogenesis by upregulating the expression of pro-angiogenic factors.[32, 33] In **chapter 5** we showed that the adult human liver harbors a population of MSCs, highly similar to bone-marrow MSCs, which is mobilized from liver grafts at time of transplantation. These liver-derived MSCs (L-MSCs) can be retrieved from the organ preservation solution and appear to have immunosuppressive capacities as well as multi-lineage differentiation potential. In addition, MSCs produce a broad spectrum of soluble factors, including growth factors, extracellular matrix proteins and enzymes.

MSCs have been described to contribute to tissue repair and regeneration, however there is an ongoing discussion on whether MSCs contribute to liver regeneration by (trans)differentiation into liver cells or by paracrine effects of their trophic factors. In

chapter 6 a critical note to the use of MSCs in a clinical setting is given, as we observed tumorigenic transformation of human MSCs during long-term culture expansion, which was not due to contamination of human cell lines. Furthermore, we identified a number of genes and miRNAs using gene array and qRT-PCR that may potentially be used to screen for transformation events in long-term cell culture. This approach would bear significant implication in the clinical application of stem cell therapy and alleviate the concern of transplanting malignant cells into patients.

The use of MSC-derived factors in a clinical setting may thus have important advantages over the use of MSCs: there is no risk of rejection or possible malignant transformation and the factors can be produced 'ready-to-use' in large clinical grade quantities. We set up several experimental models to investigate the effects of MSC-derived trophic factors on regeneration of the liver after injury. **Chapter 7** provides a detailed description on how to obtain trophic factors secreted by cultured MSCs and how they can be used in small animal models. More specific, in vivo mouse models to study the paracrine effects of MSCs on regeneration of the liver after surgical resection and/or ischemia and reperfusion injury are described.

Using these MSC-derived factors in the models described, we found in **chapter 8** that MSC-secreted factors are effective in stimulating liver regeneration after surgical resection, mostly by influencing expression levels of cytokines and growth factors relevant for cell proliferation, angiogenesis and anti-inflammatory responses. **Chapter 9** revealed similar results in a model of combined surgical and IR injury, showing decreased hepatic tissue injury and stimulation of liver regeneration. MSC-derived factors therefore represent a promising strategy for safe and ready-to-use therapeutic intervention to stimulate organ repair and regeneration in the setting of small-for-size syndrome and post-resectional liver failure.

Balancing between metabolism and regeneration

Both our data on gene expression changes in living donor livers at time of resection, as well as on the differences between EAD and non-EAD patients after transplantation support our hypothesis that at any given time the liver balances between its metabolic functions to ensure homeostasis and its regenerative processes to restore liver tissue after injury. Possibly, the liver has to function with a certain amount of energy and may therefore have to prioritize its activities.

Loss of liver tissue by surgical or toxic damage compromises homeostasis and, due to its reduced functional mass, the liver may not be able to compensate for the disruption. Though not yet fully established, this disturbance in the internal milieu of the organism may be the regulatory factor that determines the necessary (re)growth cq. hypertrophy of the liver and triggers the cessation of regenerative processes when homeostasis is restored. In line with this, the concomitant activation of autophagy when hepatocyte proliferation is reduced in the setting of mTOR inhibition, may suggest an intracellular balance

between the production of new building blocks and the recycling of defect or redundant components. Possibly, this mechanism and the energy preservation thereby induced, contribute to the restoration of the homeostatic balance when the liver is unable to quickly shift between its metabolic activities and regenerative processes.

When the equilibrium is not achieved, the liver can display signs of hepatic failure and/or may not be able to fully restore its original functional liver mass. Though this thesis contributes to the current understanding of liver regeneration in the setting of liver resection and transplantation, it remains unknown which underlying factors, whether related to genetic predisposition, life style or a donors unknown disease condition, cause the differences in gene expression profiles between living donors displaying successful or limited regeneration of their remnant liver. Similar, the molecules and pathways revealed in our EAD versus non-EAD analysis may either be causative of EAD or a consequence of yet to be determined causal factors. Therefore, further investation to determine the mechanisms regulating the level of metabolic and/or regenerative activity of the liver in different circumstances is needed.

Stem/progenitor cells as promising intervention

Since not all patients recover well after surgery or transplantation, there is a clear need for preventive or therapeutic interventions to reduce morbidity and mortality. In light of the search for strategies to promote recovery of diseased tissues and organs as well as the demand for transplantable organs which far exceeds supply, stem/progenitor cells are promising because of their differentiation potential and immunomodulatory and anti-inflammatory properties. Modern stem-cell research therefore focuses on different strategies to improve disease conditions, stimulate tissue regeneration and even on the recreation of a complete functioning organ.

A recurrent topic in stem cell research is whether these cells exert their beneficial properties through their fusion with resident cells, (trans)differentiation into mature functional cells or by paracrine effects of their trophic factors. As malignant transformation of stem/progenitor cells has been reported, therapeutic strategies involving transplantation of stem/progenitor cells raise severe concerns regarding patient safety. The use of stem/progenitor cell-derived factors may therefore be beneficial. Furthermore, these trophic factors can be easily obtained from the stem/progenitor cell-conditioned culture medium, enabling production of large clinical-grade 'of-the-shelve' quantities. However, the transplantation of stem/progenitor cells instead of the use of a trophic factor solution may be advantageous in that cells may have a more long-term effect and be able to optimally adjust to specific environmental needs. On the other hand, in vitro pre-treatment of stem/progenitor cells could possibly address this issue and enable production of trophic factor solutions with specific properties.

Another discussion topic is the optimal source of the stem/progenitor cells. In this thesis, liver-derived mesenchymal stromal/stem cells (MSCs) were compared with their

bone marrow-derived counterparts. Our data showed that these cells are highly similar and have common properties. Traditionally MSCs are isolated from bone marrow, which has long been suggested to be the gold standard. However, one can speculate whether liver-derived MSCs might be better equipped to address liver-specific injury. In addition, liver-derived MSCs can be be retrieved from the organ preservation solution of liver grafts: a source of MSCs that doesn't require painful and/or invasive procedures in donors or patients.

Aside from these topics, several other topics, including the choice between autologous and allogenic cells as well as the dosing, timing, route of administration and safety of stem/progenitor cell-based treatment strategies, need to be addressed before these therapeutic interventions can be adequately applied in a clinical setting. Though promising results have been reported and the first clinical studies are currently being conducted, this will probably still be a long-term process of trial and error.

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Chapter 11

Nederlandse samenvatting / Dutch summary

Prometheus unbound?

Sinds de Griekse mythe over de marteling van Prometheus en de eerste wetenschappelijke documentatie over het phenomeen leverregeneratie (aangroei van leverweefsel na schade of verlies) in de 19e eeuw, hebben wetenschappers uitgebreid onderzoek gedaan naar dit intrigerende proces in een poging haar mysterie te ontrafelen. Talrijke moleculen en cascades betrokken bij leverregeneratie zijn aan het licht gebracht, echter de exacte onderliggende mechanismen zijn nog steeds niet volledig bekend. Ondertussen is de sterke regeneratieve capaciteit van de lever aangewend ten bate van patiënten met (eindstadium) leverziekte, daar het oncologische leverchirurgie (chirugie vanwege leverkanker) en levende donor danwel split-lever transplantatie (in beide gevallen transplantatie van een gedeeltelijke lever) mogelijk maakt. Echter, multiple factoren waaronder leeftijd, voedingsstatus, ziekte, medicatie, mate van (lever)schade, maar waarschijnlijk ook genetische predispositie (aanleg), kunnen beperkend werken op het regeneratieproces, resulterend in verstoorde leverfunctie en gecompromitteerde homeostase (balans van het inwendige milieu van een organisme). Beter begrip van de factoren die leverregeneratie reguleren/beïnvloeden draagt bij aan onderzoek naar en ontwikkeling van behandelstrategieën om dysfunctie van de lever te voorkomen en regeneratie te stimuleren, waardoor het aantal complicaties en sterfgevallen beperkt kan worden.

In dit proefschrift zijn de mechanismen betrokken bij het proces van leverregeneratie, specifiek na leverresectie en transplantatie, nader onderzocht. Verschillende aspecten die van invloed zijn op regeneratie in de setting van leverresectie en transplantatie zijn beschreven, inclusief ischemie en reperfusie schade (schade door bloed- en zuurstof tekort in de weefsels alsmede door de herstelde bloedvoorziening) alsook afstotingsonderdrukkende medicatie. Daarnaast zijn veelbelovende stam-/voorlopercel gerelateerde behandelstrategieën onderzocht, om regeneratie van de lever na chirurgische schade te moduleren en te versnellen.

Genexpressie profielen van leverregeneratie in gezondheid en ziekte

Leverregeneratie is geïdentificeerd als een meer-stappen proces, beginnend met de voorbereiding van hepatocyten (functionele levercellen) als gevolg van de uitscheiding van cytokines (boodschapper moleculen) door beschadigde levercellen en geactiveerde Kupffer cellen (lever-specifieke 'opruim'cellen). Progressie door de celdelingscyclus wordt vervolgens gestimuleerd door de activatie van transcriptie factoren (factoren betrokken bij het aflezen van de DNA-code) en de daaropvolgende productie en uitscheiding van groeifactoren. Deze groeifactoren zorgen op hun beurt voor de activatie van cascades betrokken bij de celdelingscyclus. Terwijl dit proces uitgebreid is onderzocht in proefdiermodellen, is het lastig dit in mensen te doen. In **hoofdstuk 2** hebben we, gebruik makend van de Adult-to-Adult Living Donor Liver Transplant (A2ALL) database, de vroege veranderingen in genexpressie profielen (mate van activiteit van bepaalde genen in het DNA) onderzocht in gezonde levende leverdonoren. Deze donoren ondergingen resectie van ongeveer 50%

van hun totale levermassa, wat leidde tot activatie van cascades betrokken bij acute fase en oxidatieve stress reacties, een trigger vormde voor celdeling gerelateerde signaalcascades en eveneens metabole functies gedeeltelijk uitschakelde. Deze vroege effecten op genexpressie profielen steunt de algehele hypothese dat de lever balanceert tussen zijn twee belangrijkste functionele mechanismen: regeneratieve processen om leverweefsel te herstellen in het geval van schade, en metabole processen om de homeostase te waarborgen.

Ondanks dat geen van de levende donoren klinische symptomen van beperkte regeneratie toonde, werden significante verschillen gevonden in hun geregenereerde levermassa drie maanden na chirurgie. De vergelijking tussen donoren met succesvolle en gelimiteerde regeneratie van hun resterende levermassa onthulde duidelijke verschillen in hun vroege genexpressie profielen. De bevindingen suggereren verschillende cascades en mechanismen die mogelijk bijdragen aan meer robuuste regeneratie, inclusief betere activatie van de oxidatieve stress reactie en sterkere initiatie van eiwitsynthese ten tijde van leverresectie. Succesvol regenererende donoren toonden voornamelijk verhoging van expressieniveaus na de resectie, resulterend in activatie van cascades betrokken bij stress reactie, regulatie van de celcyclus en celdeling. Daarentegen toonden donoren met gelimiteerde regeneratie voornamelijk verlaagde expressieniveaus, resulterend in remming van cascades betrokken bij vet- en koolhydraatmetabolisme. In lijn met de regeneratie-metabolisme balans hypothese, zou een mogelijke conclusie uit deze tegenstrijdige genexpressie profielen kunnen zijn dat levers met beperkte regeneratie remming danwel vertraging van het herstel alsmede regeneratie gerelateerde cascades vertonen, aangezien hun levers zich voornamelijk focussen op onderdrukking van het metabolisme.

Deze delicate balans tussen het herstel van leverweefel en het vermogen om homeostase te waarborgen is nog veel belangrijker in ontvangers van donorlevers na transplantatie. De sterke invloed van factoren zoals leeftijd, voedingsstatus, pre-operatieve klinische conditie, mate van weefselschade en bepaalde medicijnen kan resulteren in verstoorde leverfunctie of zelfs leverfalen, en uit zich klinisch door hoge leverenzymwaarden, persisterende galstuwing en verlengde bloedingsstoornissen. De analyses beschreven in **hoofdstuk 3** correleren het klinische fenotype van vroeg leverfalen (early allograft dysfunction; EAD) met de moleculaire ‘handtekening’ die in donorlevers wordt gevonden onmiddellijk na het herstellen van de bloedvoorziening in de ontvanger. De vergelijking tussen EAD en non-EAD ontvangers suggereert specifieke metabolisme, inflammatie (ontsteking) en celdeling gerelateerde genen en cascades, karakteristiek en mogelijk oorzakelijk voor EAD. Bovendien, de combinatie van verlaagde metabole activiteit en verhoogde inflammatoire activiteit steunt onze hypothese dat op elk willekeurig moment de lever een delicate energie balans tussen herstel en metabole behoeften vertoont, wat snel hersteld kan worden in goed functionerende levers. In de setting van EAD echter, kan overmatige activatie van inflammatoire processen de energie van de lever wegleiden van de metabole processen. De lever is dan mogelijk onvoldoende in staat om te voldoen aan de metabole

behoeften van het lichaam, waardoor dit leidt tot de eerder genoemde symptomen van leverfalen. Het zou daarom van significant klinisch belang zijn om de ontwikkeling van EAD op een vroeg moment te detecteren of zelfs te voorspellen. Wij hebben hiertoe een genexpressie profiel geïdentificeerd, bestaande uit 152 genen, dat onderscheid kan maken tussen EAD en non-EAD leverbiopten, welke genomen zijn ten tijde van transplantatie. We hebben deze diagnostische 'handtekening' gevalideerd, zowel in een onafhankelijk cohort van donorlever ontvangers, alsook met een andere genexpressie detectie techniek, wat het potentieel van dit genexpressie profiel voor toekomstig gebruik in de klinische setting toont.

De rol van mTOR en autophagy in leverregeneratie

Na transplantatie worden donorlever ontvangers behandeld met afweer-onderdrukkende medicatie om afstoting te voorkomen. Echter, over veel van deze medicijnen is beschreven dat ze het proces van regeneratie beïnvloeden. Met name het gebruik van de mTOR remmer rapamycine heeft bezwaren opgeroepen, omdat mTOR (mammalian target of rapamycin) betrokken is bij de regulatie van eiwitsynthese, celgrootte en celdeling. Aan de andere kant is mTOR remming een aantrekkelijk alternatief voor de huidige calcineurine remmende strategieën, aangezien de mTOR remmer rapamycine geen nierschade veroorzaakt en recentelijk veel aandacht heeft gewonnen in de behandeling van kanker.

Daarbij is gebleken dat mTOR een belangrijke rol speelt in de regulatie van autophagy, een algemene term voor processen waarbij celmateriaal, inclusief oplosbare macromoleculen en organellen, wordt aangeleverd bij lysosomen (afbraakblaasjes) voor degradatie. Men denkt dat autophagy zich ontwikkeld heeft als een stress reactie mechanisme dat organismen in staat stelt te overleven tijdens barre omstandigheden, waarschijnlijk door de energy huishouding te reguleren. Zoals beschreven in **hoofdstuk 4**, resulteert mTOR remming in ernstige beperking van leverregeneratie na chirurgische resectie. Wij beschrijven als eerste dat mTOR remming na leverresectie eveneens een significante toename van autophagy in de lever veroorzaakt. Dit effect compenseert mogelijk de beperkte levercel-deling, aangezien autophagy oxidatieve stress verbetert en cellulaire energy bespaard. De meest nieuwe bevinding van deze studie is dat het beperkende effect op regeneratie gedeeltelijk teniet kan worden gedaan door behandeling middels het cytokine interleukine 6 (IL-6) en de groeifactor hepatocyte growth factor (HGF).

Mesenchymale stromale/stamcel gebaseerde behandelstrategieën om leverregeneratie te verbeteren

Van mesenchymale stromale/stamcellen (MSCs) werd oorspronkelijk gedacht dat ze alleen een ondersteunende rol hadden voor hematopoietische stamcellen in het beenmerg. De laatste jaren is echter gerapporteerd dat ze in meerdere weefselcompartimen-

ten voorkomen, inclusief long, lever en vetweefsel. MSCs hebben het vermogen om zich te ontwikkelen tot hepatocyten en cholangiocyten (galwegcellen), en ze hebben immuun-modulerende en anti-inflammatoire eigenschappen. Daarnaast is beschreven dat ze angiogenese (vorming van bloedvaten) stimuleren door verhoging van de expressie van angiogenese stimulerende factoren. In **hoofdstuk 5** hebben we laten zien dat in de lever van volwassen personen een populatie MSCs huist, die zeer vergelijkbaar is met beenmerg MSCs, en ten tijde van transplantatie gemobiliseerd wordt uit de donorlever. Deze lever-MSCs (L-MSCs) kunnen uit de preservatievloeistof van het orgaan gehaald worden en blijken een afweer-onderdrukkende capaciteit te bezitten alsmede het vermogen om zich tot verschillende andere celtypen te ontwikkelen. Daarnaast produceren MSCs een breed spectrum aan oplosbare factoren, inclusief groeifactoren, extracellulaire matrix eiwitten en enzymen.

Er is beschreven dat MSCs aan weefselherstel en -regeneratie bijdragen, echter er is een voortdurende discussie over of MSCs hieraan bijdragen door zich tot levercellen te ontwikkelen of door de paracriene effecten van factoren die ze produceren. In **hoofdstuk 6** zetten we een kritische noot bij het gebruik van MSCs in een klinische setting, omdat we maligne transformatie van humane MSCs in kwaadaardige cellen hebben geobserveerd na langdurige celkweek, waarbij geen sprake was van besmetting door met andere cellijnen. Daarbij hebben we een aantal genen en microRNAs (miRNAs) geidentificeerd die mogelijk gebruikt kunnen worden om maligne transformatie in langdurige kweken te detecteren. Deze benadering zou van groot belang kunnen zijn in de klinische toepassing van stamcel therapieën en verminderen de angst om kwaadaardige cellen in patiënten te transplanteren.

Het gebruik van factoren geproduceerd door MSCs zou dus in de klinische setting belangrijke voordelen kunnen hebben ten opzichte van het gebruik van MSCs: er is geen risico op afstoting of mogelijke maligne transformatie en de factoren kunnen geproduceerd worden in grote 'klaar-voor-gebruik' hoeveelheden. We hebben zodoende een aantal experimentele modellen opgezet om de effecten van MSC-factoren op regeneratie van de lever na schade te kunnen onderzoeken. **Hoofdstuk 7** biedt een gedetailleerde beschrijving hoe men factoren geproduceerd door MSCs kan verkrijgen en hoe ze gebruikt kunnen worden in proefdiermodellen. Meer specifiek beschrijven we in vivo muismodellen om de paracriene effecten van MSCs op leverregeneratie na chirurgische resectie en/of ischemie en reperfusie schade te onderzoeken.

Gebruik makend van MSC-factoren in de beschreven modellen, hebben we in **hoofdstuk 8** ontdekt dat MSC-factoren effectief zijn in het stimuleren van leverregeneratie na chirurgische resectie, voornamelijk door hun invloed op expressie niveaus van cytokines en groeifactoren die relevant zijn voor celdeling, angiogenese en anti-inflammatoire reacties. **Hoofdstuk 9** onthulde soortgelijke resultaten in een model van gecombineerde chirurgische en ischemie en reperfusie schade, waarbij verminderde leverweefsel schade en stimulatie van leverregeneratie werd gezien. MSC-factoren vertegenwoordigen daarom

een veelbelovende strategie voor veilige en 'klaar-voor-gebruik' interventies om orgaanherstel en regeneratie te stimuleren in de setting van leverfalen en 'small-for-size syndrome' (ziektebeeld ten gevolge van een te kleine lever na resectie of transplantatie) na resectie en transplantatie.

Chapter 12

Appendix:
Dankwoord / Acknowledgements
PhD portfolio
List of publications
Curriculum vitae auctoris

DANKWOORD / ACKNOWLEDGEMENTS

Gedurende de intensive jaren van dit promotie-traject heb ik veel geleerd, niet alleen op wetenschappelijk en technisch gebied, maar ook op persoonlijke vlak. De groei die ik tijdens deze periode heb doorgemaakt was niet zo uitgesproken geweest zonder de begeleiding van mijn supervisoren, maar ook zeker niet zonder de steun, het geduld en de onvoorwaardelijke liefde van mijn familie en vrienden. Graag wil ik jullie hiervoor bedanken.

Professor Tilanus, beste Huug, ik kan me mijn sollicitatiegesprek bij u, Luc en Jeroen nog als de dag van gisteren herinneren. De statige indruk die u op me maakte, vertelde me meteen dat u de professor was. U stelde één vraag, over mijn stage in Botswana, en na 15 minuten hoorde ik:"We weten denk ik wel genoeg". Tegen mijn verwachting in werd ik toch deel van uw promovendi-team en al snel kwam ik erachter hoeveel humor in u schuilt. Ik sta nu aan het begin van het chirurgen-traject, u reeds aan het eind, maar weet dat de waardering en het respect niet verdwijnen.

Dr. van der Laan, beste Luc, als mijn 'lab-copromotor' was jij mijn dagelijkse begeleider. We hebben heel wat (wetenschappelijke) ups en downs samen meegemaakt en je hebt zowel mijn (soms extreme) perfectionisme als mijn nieuwsgierigheid naar de kliniek regelmatig moeten remmen. Toch denk ik dat we door de jaren heen een goede balans hebben gevonden en zeker in de laatste fase voelde onze band stevig ondanks de fysieke afstand. Jouw begeleiding, aanvullende ideeën en opbouwende kritiek hebben mij zeker gekneed tot de kritische en zelfstandige onderzoekster die ik nu ben.

Dr. de Jonge, beste Jeroen, onzeker en groen als ik aan mijn promotie begon, had ik de eerste maanden het gevoel het nooit 'goed genoeg' voor je te kunnen doen. Toen ik de moed had verzameld je dat te vertellen, begreep je meteen wat het probleem was en paste je manier van communiceren aan…wel met lachend de opmerking dat ik als Limburgs meisje een beetje steviger in mijn schoenen moest staan in Rotterdam, want ik was "nog Katholieker dan de kat van de buren"…en dan te bedenken dat ik dat niet eens ben. Daarna is veel veranderd en zijn we naar elkaar toe gegroeid. Bedankt voor je steun en motiverende woorden en last but not least de tijd in Philly.

Professor Olthoff, dear Kim, my time in Philly was the best part of my PhD-period. You welcomed me with open arms and I immediately felt at home. I admire your energy, warm-heartedness and the way you combine your work as a skilled transplant surgeon with your family life. One of our international collegues once said he saw in me a younger version of you; I don't think I have ever been more proud. I'm honoured to have you as my role model and thank you for coming all the way from the States to attend my thesis defense.

Professor Shaked, dear Avi, you gave me some unforgettable memories of my time in Philly: at the South Street Bridge when you yelled "Tsunami" and waved at me from your car while your daughter got worried on the other end of the phone line; the meeting you stopped talking because I just couldn't stop laughing about your quote that God didn't

make organs to be put in buckets; and the time you told me I was making the biggest mistake of my life by not staying, just one hour before my plane to the Netherlands left. I have to admit, I'm still in doubt whether I should have.

Professor Metselaar, beste Herold, dank voor de kritische noten, de heerlijke diners tijdens congressen op de meest spraakmakende locaties, maar zeker ook voor de mogelijkheid om langer in Philadelphia te kunnen blijven voor de laatste experimenten. U bent oprecht iemand waar we op kunnen steunen.

Professor Kazemier, beste Geert, ik vond het erg jammer dat je het Erasmus verliet voor het VUMC; het LTx-team veranderde er wel door. Ik hoop dat je je plek gevonden hebt en wens je nog heel veel succes in 020.

Professor Porte, beste Robert, je bent het tegenovergestelde van het vaak gehoorde image van een professor. De bootcongressen, Hongkong, Philly…altijd jeugdig enthousiast en met de onderzoekers swingend in de kroeg. Je reis met een vrachtschip naar de andere kant van de oceaan is benijdenswaardig. Ik waardeer dat je ook op mijn grote dag van de partij bent.

Natuurlijk was al dit werk nooit gelukt zonder de hulp van alle mede-onderzoekers en analisten op het lab, zowel het immunologie lab in Philadelphia en het Scripps Research Institute in San Diego, maar zeker het MDL-lab in Rotterdam.

The Hancock lab, it was a pleasure to work with and among you guys. Thanks for learning me new (surgical) techniques. Wayne, thank you for giving me the opportunity to work in your lab and learn from your knowledge on immunology.

Josh, thank you for helping with the ischemia and reperfusion mouse experiments. You made me a more skilled mouse surgeon. I wish you all the luck with your surgical residency at Johns Hopkins and hope to see you and your family again some time.

Shaun and Peter, I had so much fun hanging out with you, both in the lab as well as outside, visiting baseball matches, open air movies, organizing barbeques etc.. I hope we will have a chance do that again some time soon!

Professor Salomon, dear Dan, thank you for your critical questions and remarks as well as the morning Yoga lessons in your lab.

Dr. Kurian, dear Sunil, it took a while but finally we have our manuscripts submitted. Thank you for teaching me how to analyze and interpret microarray data.

Lieve MDL-lab collega's, met mijn vertrek op 2 januari 2011 naar Philly was, zonder dat ik me dat realiseerde, eigenlijk mijn tijd bij jullie afgelopen. Want na terugkomst ben ik de kliniek ingestroomd om me te kunnen bewijzen voor de opleiding Chirurgie, en voor ik het wist woonde ik niet meer in Rotterdam. Des te specialer is het dat jullie nog altijd een belangrijke rol spelen in mijn leven. De vele mailtjes en berichtjes om me te steunen met de laatste loodjes, te feliciteren met behaalde resultaten, of uit te nodiging voor het volgende feestje of kampeeruitje zijn hartverwarmend. In mijn ogen zijn jullie de beste colle-

ga's die een AIO zich kan wensen en ik dank ieder van jullie voor jullie gezelligheid en hulp. Toch wil ik een aantal collega's in het bijzonder noemen.

Anouk en Lianne, lieve kamergenootjes, wat een ups en wat een downs hebben we doorgemaakt. Jullie waren mijn grootste houvast en uitlaatklep…aan gespreksstof in ieder geval nooit een gebrek! Anouk, ik bewonder hoe je je leventje na alles weer opbouwt, op een nieuwe plek in een nieuwe situatie. Het was super je in Philly te mogen rondleiden, let's do that again. Lianne, wat hebben we genoten met onze rugzakken heel de VS door en hikend door de Grand Canyon. Zet 'm op met je nieuwe plekje in Gent! Nu ook de laatste van de Drie Musketiers klaar is, wordt het toch echt eens tijd om 'dat stukje koe te gaan knuffelen'.

Scot and Carlijn, how much I miss visiting you in New York…the sailing…the relaxed dinners…the good conversations. You are the best!

Abdullah, thanks for teaching me how to culture stem cells and for your help with the MSC projects.

Veda, it's amazing how you manage to combine two pregnancies and a family with your PhD. Good luck with the last research-bumps.

Petra, dank je voor je hulp bij de laatste experimenten! Je hebt de eindfase een stuk makkelijker gemaakt.

Waqar, lange tijd waren we de enige twee chirurgische promovendi op het MDL-lab. Dat schept toch een band. Ik mis je, gekke Pakistaan! Zet 'm op met de laatste loodjes. Ik duim voor je met de sollicitatie-ronde, maar 't komt vast goed. Veel geluk met Rhiana en jullie verwachte aanwinst.

Renee en Eelke, jullie zijn voor mij de 'nieuwe generatie' AIOS van het clubje. Veel succes met jullie onderzoek en laat je niet klein krijgen als het tegen zit. Na regen komt echt weer zonneschijn.

Aria, al onze gesprekken over liefde, relaties en geluk…en toen kwam je de ware tegen. Wat zag je er prachtig uit op je bruiloft! Veel geluk samen en ik stel voor dat we een trouwfoto-avondje organiseren.

Jan, dank je voor je aandacht, attentheid en steunende woorden, iedere keer weer.

Anthonie, Antoine, Arjan, Elvira, Eric, Gertine, Jasper, Marjolein op den Brouw, Marjolein Sikkema, Mark, Ozlem, Patrick, Paula, Shanta, Thanya, Viviana, Wendy, Werner en iedereen die ik wellicht vergeet te noemen, dank voor alle gezellige momenten, in de koffiekamer, bij Dizzy's, tijdens labuitjes, uitbundige feestjes in de L-vleugel en de fantastische kampeerweekendjes. Jullie waren een geweldig tegenwicht voor de lange werkdagen en stress-ontlokkende deadlines.

Mijn dank gaat ook uit naar mijn nieuwe collega's van het VieCuri. Een hecht team met geweldige chirurgen, A(N)IOS, verpleegkundigen en secretaresses. De steun die jullie me gegeven hebben om mijn droom te verwezenlijken, zowel voor de sollicitatie-ronde als

voor het afronden van dit boekje, was hartverwarmend. Ik voel het als een voorrecht om door en met jullie opgeleid te worden.

Mijn geduldige vrienden, Annemiek, Loes, Stephanie, Kyra, Anouk, Nard, Marco, Nico, Xander, Yvette & Roel, wat heb ik onze vriendschap de laatste jaren op de proef gesteld. De vele keren dat ik een uitnodiging moest afslaan, te laat kwam of last-minute mijn plannen moest wijzigen vanwege experimenten, congressen en deadlines…Maar nog steeds zijn jullie er voor me. Ik mag me gelukkig prijzen met zulke fijne vrienden.

Rob, ook zonder jou zou mijn boekje hier nu niet gelegen hebben. Alle keren dat je, zowel 'in person' als vanaf de andere kant van de oceaan mijn laptop weer aan de praat hebt gekregen…ik ben de tel kwijt. Dankjewel!

Philly Dutch, Elske (Tulips), Marlin (Clogs) en Emily (Windmills), ik denk dat er geen betere (of moet ik zeggen gekkere) combinatie mogelijk was! Zonder jullie was mijn tijd in Philadelphia zeker niet zo gevarieerd, spannend, feestelijk en lachen-gieren-brullen geweest. Philly hugs, Brownies.

Lieve Belinda, de laatste periode heeft heel wat geduld, teleurstellingen en frustraties gekost, maar het wachten is gedaan, een ander tijdperk breekt nu aan, eindelijk op vakantie!

Lieve paranimfen, dank dat jullie aan mijn zijde staan. Jullie hebben me enorm geholpen met het regelen van deze dag. Lieve Angela, vanaf dat je als student op het MDL-lab begon, ben je altijd een van mijn favoriete collega's geweest. Je eerlijkheid, openheid en grote hart zijn een waar voorbeeld voor iedereen. Het was altijd ontzettend gezellig met je, in het lab en op congres. Weet dat ik altijd voor je klaar zal staan als je me nodig hebt. Lieve Aurin, mijn 'kleine' zussie maar toch ook soms mijn 'grote' voorbeeld. Wat ben ik trots op je, hoe je je ontwikkeld hebt, je zorgzaamheid en hulpvaardigheid. Jij en Vahid zullen samen een gouden toekomst tegemoet gaan, Insha'Allah.

Toon Hermans zei ooit: "Geluk dat is geen zeppelin, 't is hooguit een ballonnetje." Toch ben ik dat niet helemaal met hem eens. Pap en mam, wat jullie mij door de jaren heen gegeven hebben, is voor mij geen ballonnetje maar een zeppelin vol liefde, steun en geduld. Met name het afgelopen jaar was voor jullie zeker zo intensief als voor mij: iedere ochtend een dampende kop thee voor onderweg, een lunchpakket waar op het werk al naar wordt verwezen als 'een 3-gangen diner', het avondeten bij thuiskomst warm op tafel en geen omkijken naar welke huishoudelijke taak dan ook…en dan niet te vergeten alle peptalk, knuffels, wake-up calls en spreekwoordelijke schoppen onder mijn kont om me te motiveren…allemaal zodat ik de kans kreeg om nu te mogen horen, 'Hora Est'.

PHD PORTFOLIO

Name PhD Student	Suomi M.G. Fouraschen
Erasmus MC Department	Surgery
PhD Period	January 2008 – November 2011
Promotor	Prof. dr. H.W. Tilanus
Copromotors	Dr. L.J.W. van der Laan & Dr. J. de Jonge

General courses

2007	Laboratory animal science (Proefdiercursus art. 9)	Erasmus MC Rotterdam
2009	‘Transplantation for dummies’ course	Erasmus MC Rotterdam
2010	Basic Immunology	Erasmus MC Rotterdam
2010	Basic data analysis on gene expression arrays	Erasmus MC Rotterdam
2011	LAS Safety Training	University of Pennsylvania
2011	AALAS Animal Care and Use Training	University of Pennsylvania

Presented abstracts at conferences

National conferences

- Fouraschen S., van der Laan L., Hopmans C. et al. Cytokines versus stem cell conditioned medium for stimulation of liver regeneration. Stafdag Heelkunde, Erasmus MC-University Medical Center, Rotterdam 2008 (oral)
- Fouraschen S., de Jonge J., Pan Q. et al. Acceleration of liver regeneration by mesenchymal stem cell-derived trophic factors in the context of immunosuppression. Bootcongres, NTV, Zeewolde 2009 (oral)
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- Pan Q., Fouraschen S., Ramakrishnaiah V. et al. Optimization of models for hepatic differentiation of human mesenchymal stem cells. Stafdag Heelkunde, Erasmus MC-University Medical Center, Rotterdam 2009 (oral)
- Fouraschen S., Pan Q., de Jonge J. et al. Mesenchymal stem cells: relevance in liver regeneration and transplantation, Bootcongres, NTV, Rotterdam 2010 (oral)
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- Fouraschen S., van der Laan L., Wolf J., et al. Mesenchymal stem cell-derived trophic factors promote liver regeneration after ischemia and reperfusion injury combined with liver resection, but not after ischemia and reperfusion injury alone, 6th Dutch Experimental Gastroenterology and Hepatology Meeting, NVGE, Veldhoven 2013 (poster)

International conferences

- Fouraschen S., Deelen J., Cornelis A. et al. Congenital Cystic Adenomatoid Malformation of the Lung: changes in management and outcome. European Congress of Paediatric Surgery, EUPSA, Istanbul 2008 (oral)
- Fouraschen S., de Jonge J., Pan Q. et al. Acceleration of liver regeneration by mesenchymal stem cell-derived trophic factors in the context of immunosuppression. 15th Annual International Congress, ILTS, New York 2009 (poster)
- Pan Q., Fouraschen S., Aerts-Kaya F. et al. Mobilization, immunomodulation and hepatic differentiation of human graft mesenchymal stem cells in liver transplantation. 15th Annual International Congress, ILTS, New York 2009 (poster)
- Fouraschen S., de Jonge J., Pan Q. et al. Mesenchymal stem cell-derived trophic factors accelerate liver regeneration. The Liver Meeting, AASLD, Boston 2009 (poster)
- Pan Q., Fouraschen S., Aerts-Kaya F. et al. Mobilization of hepatic mesenchymal stem cells from adult human liver grafts. 2nd Expert Meeting, MiSOT, Rotterdam 2010 (poster)
- Fouraschen S., de Jonge J., Pan Q. et al. Mesenchymal stem cell-secreted factors stimulate hepatocyte proliferation but reduce mobilization of bone marrow

stem/progenitor cells during liver regeneration. 16th Annual International Congress, ILTS, Hong Kong 2010 (oral)

- Pan Q., Fouraschen S., Kazemier G. et al. Tumor-nourishing and tumorigenic transformation of mesenchymal stem cells caution therapeutic application in liver transplantation. 16th Annual International Congress, ILTS, Hong Kong 2010 (oral)
- Fouraschen S., de Jonge J., de Bruin R. et al. Impairment of liver regeneration by rapamycin not completely explained by the role of mTOR in IL-6 and HGF pathways. The Liver Meeting, AASLD, Boston 2010 (poster)
- Pan Q., Fouraschen S., Tilanus H.W. et al. Spontaneous tumorigenic transformation of mesenchymal stem cells caution therapeutic application. The Liver Meeting, AASLD, Boston 2010 (poster)
- Fouraschen S., de Jonge J., Pan Q. et al. Factors secreted by liver-derived mesenchymal stem cells promote liver regeneration after partial hepatectomy. American Transplant Conference, Philadelphia 2011 (poster)
- Fouraschen S., de Jonge J., de Ruiter P. et al. Adverse effects of mTOR inhibition on liver regeneration. 17th Annual International Congress, ILTS, Valencia 2011 (poster)
- Fouraschen S., de Jonge J., Pan Q. et al. Factors secreted by liver-derived mesenchymal stem cells promote liver regeneration after partial hepatectomy. 17th Annual International Congress, ILTS, Valencia 2011 (poster)
- Fouraschen S., Kurian S., Emond J. et al. Living liver donors with successful liver regeneration show distinct changes in hepatic genomic profiles compared to donors with incomplete regeneration. The Liver Meeting, AASLD, San Francisco 2011 (oral)
- Fouraschen S., van der Laan L., Wolf J., et al. Mesenchymal stem cell-derived factors promote liver regeneration but do not protect against ischemia/reperfusion injury, 16th International Congress, ESOT, Vienna 2013 (oral)

Academic awards

- ILTS Travel Award 2010 ($ 1000,-)
- Novartis travel grant 2010 (€ 2050,-)
- Trustfonds travel grant 2010 (€ 750,-)
- ASTS-ESOT Exchange Grant 2010 ($ 45.000,-)
- Nel Kreeft prijs 2009
- Novartis travel grant 2009 (€ 1600,-)
- Trustfonds travel grant 2009 (€ 650,-)
- Astellas Trans(p)la(n)t(at)ionele Research Prijs 2009 (€ 5000,-)

Memberships

- European Society for Organ Transplantation (ESOT)
- Nederlandse Vereniging voor Heelkunde (NVvH)
- Nederlandse Transplantatie Vereniging (NTV)
- Nederlandse Vereniging voor Hepatologie (NVH)
- Nederlandse Vereniging voor Gastroenterologie (NVGE)
- Nederlandse Vereniging voor Gastrointestinale Chirurgie (NVGIC)

LIST OF PUBLICATIONS

1. Nijhuis J., van Dielen F., Fouraschen S. et al. Endothelial activation markers and their key regulators after restrictive bariatric surgery. Obesity 2007 Jun;15(6):1395-9
2. Pan Q., Fouraschen S., Aerts-Kaya F. et al. Mobilization of hepatic mesenchymal stem cells from human liver grafts. Liver Transpl. 2011 May;17(5):596-609
3. Fouraschen S., Pan Q., de Ruiter P. et al. Secreted factors of human liver-derived mesenchymal stem cells promote liver regeneration after partial hepatectomy. Stem Cells Dev. 2012 Sep 1;21(13):2410-9
4. Pan Q., Ramakrishnaiah V., Henry S. et al. Hepatic cell-to-cell transmission of small silencing RNA extends the therapeutic reach of RNA interference (RNAi). Gut 2012 Sep;61(9):1330-9
5. Fouraschen S., de Ruiter P., Kwekkeboom J. et al. mTOR signaling in liver regeneration: rapamycin combined with growth factor treatment. (World J Transplantation, 2013 Sept; 24;3(3):30-47
6. Pan Q., Fouraschen S., de Ruiter P. et al. Detection of spontaneous tumorigenic transformation during culture expansion of human mesenchymal stromal cells. (Exp Biol Med, conditionally accepted)
7. Fouraschen S., de Jonge J., van der Laan L. Studying paracrine effects of mesenchymal stromal/stem-derived factors in vivo on liver injury and regeneration (Methods Mol Biol, invited book chapter, conditionally accepted)
8. Fouraschen S., Kurian S., Wolf J. et al. Immediate early gene expression profiles of living donor livers show a shift in key cellular functions related to the extent of regeneration. (submitted)
9. Fouraschen S., Wolf J., van der Laan L. et al. Mesenchymal stromal cell-derived factors promote tissue repair in a small-for-size ischemic liver model, but do not protect against early effects of ischemia and reperfusion injury. (submitted)
10. Kurian S., Fouraschen S., Wolf J. et al. Genetic profiles and predictors of early allograft dysfunction after human liver transplantation. (submitted)
11. Wolf J., Bhatti T., Fouraschen S. et al. Heat shock protein-70 is required for optimal liver regeneration after partial hepatectomy in mice (submitted)

CURRICULUM VITAE AUCTORIS

Suomi Fouraschen werd geboren op 25 augustus 1981 te Maastricht. Zij groeide op in Born, een dorpje circa 25 kilometer noordelijk van haar geboortestad, waar zij als klein meisje al te kennen gaf dokter te willen worden. In 1999 rondde zij het Gymnasium af op het Serviam-College Sittard. Hierna startte zij met de opleiding Geneeskunde aan de Universiteit Maastricht, waarvoor ze tijdens haar co-schappen enkele maanden in het Maun General Hospital in Botswana werkte. Na het behalen van haar diploma vertrok ze vanuit Limburg naar Rotterdam om bij de afdeling Kinderheelkunde in het Sophia Kinderziekenhuis te gaan werken.

Haar interesse in de wetenschap achter de medische kennis en kunde deden haar besluiten om in 2008 met een promotie-onderzoek te starten bij de afdeling Heelkunde van het Erasmus Medisch Centrum in Rotterdam. Hier werkte zij met veel plezier bij het Maag-, Darm- & Leverlaboratorium, waar ze onder begeleding van prof. Hugo Tilanus, dr. Luc van der Laan en dr. Jeroen de Jonge onderzoek naar leverchirurgie en -transplantaties verrichtte. Voor het laatste jaar van haar promotie-traject verhuisde ze naar Philadelphia, waar ze een tweede thuis vond, en onder begeleiding van prof. Kim Olthoff haar onderzoek verder completeerde.

Inmiddels werkt ze als chirurg in opleiding bij het VieCuri Medisch Centrum in Venlo (dr. H.M.J. Janzing). Zij zal haar opleiding alhier en in het Maastricht Universitair Medisch Centrum (prof. dr. L.P.S. Stassen) voortzetten.