

# **On the release of small RNAs and viruses from hepatocytes**

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**On the release of small RNAs and viruses from hepatocytes**

**Over het vrijkomen van kleine RNAs en virussen uit hepatocyten**

**Thesis**

to obtain the degree of Doctor from the  
Erasmus University Rotterdam  
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by

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born in Bangalore (India)



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*Dedicated In the memory of my Grandfather  
You will always continue to live in my heart.*



# CONTENTS

<b>Chapter 1</b>	
General introduction and outline of the thesis	1
<b>Chapter 2</b>	
Hepatocyte-derived microRNAs as serum biomarkers of hepatic injury and rejection after liver transplantation. <i>Liver Transpl. 2012 Mar;18(3):290-7</i>	21
<b>Chapter 3</b>	
Bi-directional release of hepatocytes-derived microRNAs to bile and blood: Relation with liver injury and bilirubin secretion. <i>In preparation</i>	39
<b>Chapter 4</b>	
Hepatic cell-to-cell transmission of small silencing RNA can extend the therapeutic reach of RNA interference (RNAi). <i>Gut. 2012 Sep;61(9):1330-9</i>	59
<b>Chapter 5</b>	
Exosome-mediated transmission of hepatitis C virus between human hepatoma Huh7.5 cells. <i>Proc Natl Acad Sci U S A. 2013 Aug 6;110(32):13109-13</i>	85
<b>Chapter 6</b>	
Exosomes as Protective Vehicles for Feco-oral Transmission of Viral Hepatitis. <i>Hepatology. 2013 Nov 22</i>	103
<b>Chapter 7</b>	
General Discussion	111
<b>English Summary</b>	127
<b>Dutch Summary</b>	131
<b>Acknowledgements</b>	136
<b>List of Publications</b>	140
<b>Curriculum Vitae and Portfolio</b>	141





# **Chapter 1**

## **General introduction and outline of the thesis**



## **I. Liver function, liver disease and liver transplantation**

The liver is the second largest organ in the body, only after the skin. The liver is a roughly triangular organ that extends across the entire abdominal cavity just inferior to the diaphragm. It consists of 4 distinct lobes – the left, right, caudate, and quadrate lobes. It is one of the organs that can regenerate. The liver plays an active role in the process of digestion through the production of *bile*, which helps carry away waste and break down fats in the small intestine during digestion. The other well-known functions of the liver are production of cholesterol, production of certain proteins for blood plasma, conversion of excess glucose into glycogen for storage. It also regulates blood clotting and blood levels of amino acids <sup>1</sup>.

The internal structure of the liver is made of lobules. Each lobule consists of a central vein surrounded by 6 hepatic portal veins and 6 hepatic arteries. Many capillary-like tubes called sinusoids, which extend from the portal veins and arteries to meet the central vein, connect these blood vessels. Each sinusoid passes through liver tissue containing two main cell types: Kupffer cells and hepatocytes (SLECs, stellate cells and cholangiocytes are also worth mentioning).

- *Kupffer cells* are a type of macrophage that capture and break down particles like bacteria and old red blood cells passing through the sinusoids.
- *Hepatocytes* are cuboidal epithelial cells that line the sinusoids and make up the majority of cells in the liver.

A hepatocyte displays an eosinophilic cytoplasm. They are organised in plates separated by sinusoids. Hepatocytes perform most of the liver's functions – metabolism, storage, digestion, and bile production. Tiny bile collection vessels known as bile canaliculi run parallel to the sinusoids on the other side of the hepatocytes and drain into the bile ducts of the liver.

The causes of liver disease also known as hepatic disease ranges from viruses to gene mutations to environmental factors such as medications and alcohol. These diseases typically progress slowly because the organ has a large capacity and can regenerate. These conditions, however, can reach the stage of cirrhosis and in some instances, acute liver failure, which requires urgent evaluation at a transplant center. Cirrhosis is a late-stage of chronic liver diseases, a life-threatening condition in which normal liver tissue is replaced by nonfunctioning scar tissue <sup>2</sup>. Progressive scarring leads to cirrhosis. In some cases, those with cirrhosis will go on to develop liver

failure, liver cancer or life-threatening esophageal and gastric varices<sup>3</sup>.

### ***Liver transplantation***

Liver transplant is a treatment option for people who have end-stage liver failure that cannot be controlled using other treatments and for some people with liver cancer. Liver failure can occur rapidly, in a matter of weeks (acute liver failure), or it can occur slowly over months and years (chronic liver failure)<sup>4</sup>. Liver transplantation is a surgical procedure used to remove damaged or diseased liver and replace it with a healthy liver allograft. Most liver transplant operations use livers from deceased donors, though a liver may also come from a living donor.

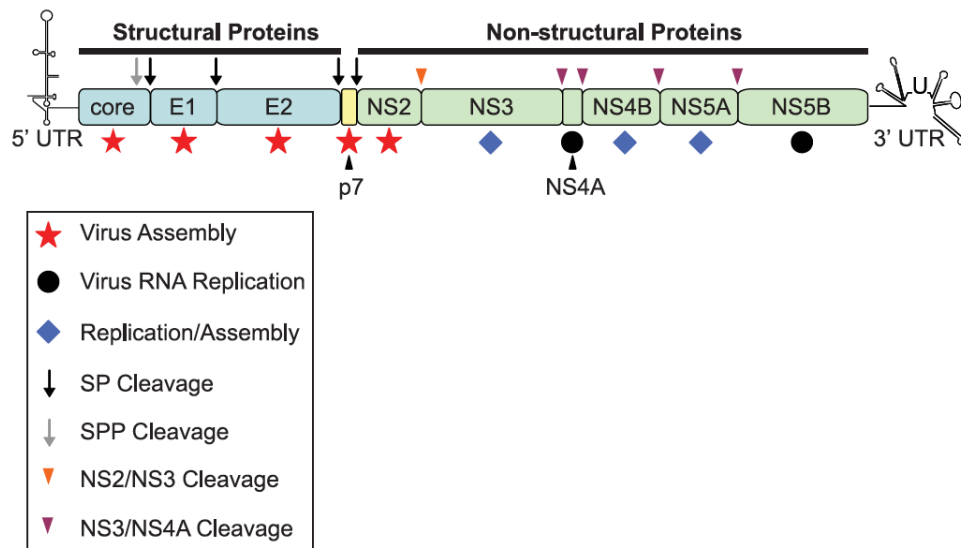
To prevent liver rejection after transplantation, recipients are treated with immune-suppressive medication. Immuno suppressive therapy after transplantation is life long and often associated with serious side effects. Side effects include nephrotoxicity, metabolic disorders and an increased risk of de novo malignancies<sup>5</sup>. Other serious complications after liver transplantation are recurrence of disease or biliary complications<sup>6, 7</sup>. Narrowing down immunosuppressive medication in transplant patients almost always results in losing the graft by immune mediated rejection.

The commonly used biomarkers for liver injury are liver transaminases. Aspartate transaminase (AST) and Alanine transaminase (ALT) are the most sensitive and widely used liver enzymes to measure dysfunction in the liver. An initial step in detecting liver damage is a simple blood test to determine the level of these enzymes in the blood. Under normal circumstances, these enzymes mostly reside within the cells of the liver. But when the liver is injured for any reason, these enzymes are spilled into the blood stream, raising the AST and ALT enzyme blood levels and signaling liver disease. The major pitfall in these tests is that the presence of AST and ALT is also released in muscle cells upon injury<sup>8</sup>. Therefore, liver biopsies become a necessity to confirm liver damage. This would not be an ideal choice after liver transplantation. This emphasizes the need to develop more sensitive and non-invasive biomarkers for liver injury and rejection. One part of our study is focused on the alternatives that can be used to detect liver injury.

## II. Hepatitis C

The hepatitis C virus (HCV) is a single stranded, enveloped, positive-sense RNA virus classified as one of the members of a distinct genus called hepacivirus in the family Flaviviridae<sup>9</sup>, which also includes viruses such as dengue and yellow fever viruses as well as the pestiviruses, such as bovine diarrhea virus<sup>10</sup>. According to the World Health Organization, approximately 170 million people, 3% of the world population, are infected with HCV. The genome of HCV encompasses a single 9.6 kb RNA molecule carrying one large open reading frame (ORF) that is flanked by non-translated regions (NTRs)<sup>11</sup>. The 3000 amino acid residue precursor polyprotein created from this ORF is cleaved co- and post-translationally by cellular and viral proteinases into at least ten different products, with structural proteins Core, E1, E2 and p7 located in the amino-terminal one-third and the non-structural replicative proteins NS2, NS3, NS4A, NS4B, NS5A, NS5B, located in the remainder<sup>11</sup> (Figure 1).

The life cycle of HCV consists of binding and entering host cells, uncoating the viral nucleocapsid, genome translation, polyprotein processing, viral replication, particle assembly and release. Viral entry involves envelope protein interaction with membrane proteins expressed on the surface of the host cells. The virus binds an hepatocyte and enters the cell by clathrin mediated endocytosis<sup>12</sup>.



**Figure 1:** The single open reading frame encodes 10 viral proteins that are divided into the structural (core, E1, and E2; shown in *blue*) and non-structural (NS2, NS3, NS4A, NS4B, NS5A, and NS5B; shown in *green*) regions.

*Adapted from Journal of biological chemistry, 2010, vol 285, no 30.*

The viral envelope proteins E1 and E2 are involved in viral binding and entry, and this is well establishing in several model systems<sup>13-15</sup>. Over several years, certain host cell surface receptors have been identified as binding partners for the envelope proteins and are considered important for viral binding and entry. These are CD81<sup>16</sup>, glycosaminoglycan<sup>17</sup>, scavenger receptor class B type-1 (SR-B1)<sup>18</sup>, low-density lipoprotein receptor<sup>19</sup>, Claudin proteins<sup>20-22</sup> and occludin (OCLN)<sup>23</sup>. Upon entry, HCV RNA is released and acts as both a template for the production of the negative-strand RNA replication intermediate and an mRNA template for the synthesis of the viral polyprotein. This is mediated by an internal ribosomal entry site (IRES) located within the highly conserved 5' non-coding region. The synthesized polyprotein is subsequently cleaved into four structural (core, E1, E2 and p7) and six non-structural (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) proteins<sup>24</sup>. HCV replication requires both viral and cellular factors. In particular the IRES sequence, plays an important function in ribosomal assembly. The non-structural proteins NS3, NS4A, NS4B, NS5A and NS5B, appear to be essential for replication<sup>25</sup>. NS5B is an RNA-dependent RNA polymerase, and responsible for the *de novo* generation of positive and negative stranded RNA<sup>26</sup>. The primary location of the assembly of HCV virions in hepatocytes are the small lipid droplets located in close proximity of the endoplasmic reticulum. The HCV core protein initiates the recruitment of non-structural proteins and replication complexes to lipid droplet-associated membranes<sup>27</sup>.

Management and treatment of recurrent HCV after liver transplantation remains a major clinical challenge. Unlike hepatitis B virus, treatment with neutralizing antibodies does not prevent HCV re-infection<sup>28</sup>. The exact mechanism of this immune deviation remains largely unknown. Therefore, one of our aims was to investigate whether exosomes can shuttle HCV in the presence of neutralizing antibodies (nAbs) *in vitro*. This could explain the ineffectiveness of prophylactic nAbs and agents targeting the entry of HCV into a cell.

Various routes of transmission of HCV such as intravenous drug use and blood transfusion have been established. However, the feco-oral route of transmission occurring due to poor sanitation and overcrowding has still not been explored well. When disease spreads through the fecal-oral route, it means that another person somehow ingests contaminated feces from an infected person. There are many

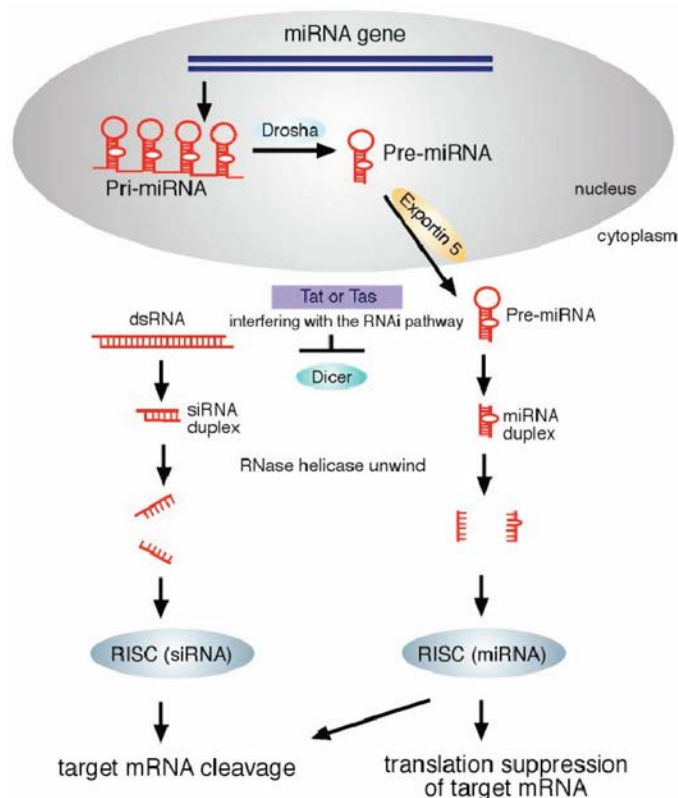
microbes that can be passed along this way and specifically there are two hepatitis viruses that spread via the fecal-oral route: Hepatitis A <sup>29</sup> and hepatitis E <sup>30</sup>.

More recently, the hepatitis A virus (HAV) has been shown to be able to escape humoral immunity by cloaking in cellular membranes upon release from host cells. HAV, an infectious disease of the liver, is well known to spread by the fecal-oral route. It is also among those viruses that lack an envelope <sup>31</sup>. By acquiring an envelope resembling exosome-like microvesicles, virions were shown to be protected from antibody-mediated neutralization <sup>31</sup>. Haruna and colleagues studied HCV <sup>32</sup> in bile and showed the presence of HCV RNA in bile, suggesting that the biliary system may be involved in the release of virus from the liver and transmission of the virus from the host. HCV, similar to HAV, is known to evade antibody-mediated neutralization but the feco-oral route of transmission has still not been explored. The presence of HCV RNA has been detected and quantified in human fecal specimens in patients chronically infected with HCV <sup>33</sup>.

### **III. microRNAs (miRNAs) and liver disease**

#### ***Regulation of liver function by miRNA***

MiRNAs are endogenous noncoding RNAs with approximately 22 nucleotides in length that can broadly regulate gene expression by using the RNAi machinery to trigger either cleavage or translational suppression of the target mRNA <sup>34</sup>(Figure 2). Before the discovery of miRNA, it had been known that a large part of the genome is not translated into proteins. This so called “junk” DNA was thought to be evolution’s debris with no function. We now realize that a portion of this non-coding DNA is highly relevant in the regulation of gene expression. The first miRNAs were characterized in the early 1990s <sup>35</sup> in *C. elegans* but their recognition as distinct class of biological regulators with conserved functions happened not until early 2000s. Since then, miRNA research has revealed multiple roles in negative regulation (transcript degradation and sequestering, translational suppression). By affecting gene regulation, miRNAs are likely to be involved in most biological processes <sup>36-38</sup>. Different sets of expressed miRNAs are found in different cell types and tissues <sup>39</sup>. MiRNAs have been shown to regulate different stages of development in *C. elegans* <sup>40</sup>.



**Figure 2:** The biogenesis of RNA silencing transcripts can be derived from either the host cell nucleus mRNA pathway to yield miRNA or the cytoplasmic double strand RNA to yield siRNA. *Adapted from Retrovirology, 2005; 2:35.*

Studies have established the role of miR-122 in maintaining the differentiation state of the liver<sup>41</sup>. MiR-122 is an important regulator of cholesterol metabolism<sup>43</sup>, iron homeostasis<sup>44</sup> and a crucial host factor for hepatitis C virus infection and replication<sup>45, 46</sup>. In addition to miR-122 several other miRNAs are involved in liver differentiation, such as, miR30a<sup>47</sup> and miR-23b<sup>48</sup>. These miRNAs are differentially expressed between embryonic and adult livers and this correlates these findings in liver development. The fast growing discoveries of miRNAs involved in liver physiology and pathophysiology will greatly strengthen the therapeutic potential of gene delivery based on miRNAs in treatment for liver disease.

### ***miRNA and hepatic injury***

Hepatocyte-derived miRNAs (HDmiRs) are detectable in serum and plasma of rodents and found to correlate with serum transaminases, aspartate transaminase (AST) and alanine transaminase (ALT)<sup>49</sup>. In humans, the HDmiR miR-122 can be detected in serum and was found to be elevated in patients with hepatocyte injury<sup>50</sup>,



<sup>51</sup>. Also a close correlation with transaminases and liver histology was observed. However, this correlation has not been evaluated in the liver transplantation setting. Recent studies in the setting of kidney transplantation have underlined the potential of mRNA and miRNA as biomarkers for assessing renal allograft status <sup>52</sup>. This would also minimize the need to take tissue biopsies to confirm the raising levels of AST and ALT. HDmiRs seemed to represent promising candidates for this due to their cell-type specific distribution, their biological stability and sensitivity of detection.

### ***miRNA Related to liver disease: HCV***

The recent advances made in identifying the miRNAs also show its deregulation in different liver diseases such as obesity<sup>53</sup>, hepatitis<sup>45</sup>, alcoholic and nonalcoholic steatohepatitis, cirrhosis and hepatocellular carcinoma<sup>41, 42</sup> which paves the way to exploit the advantages of miRNA-based therapies<sup>54</sup>. Abnormal lipid metabolism in the liver resulting from miRNA deregulation has been reported in an obese mouse model where miR-34a has been the most highly elevated hepatic miR <sup>53, 54</sup>. MiR-122 has also been the most widely studied miRNA affecting lipid metabolism. Antisense oligonucleotide inhibition of miR-122 downregulated serum cholesterol in high-fat diet mice <sup>55</sup>. Depletion of miR-122 activates AMPK which is a master regulator of metabolism in the liver <sup>56</sup>. Let-7a knockdown prevents obesity induced glucose intolerance and steatosis <sup>54, 57</sup>. MiRNAs may be the future key to fine tuning of metabolism and detect accurately metabolic disorders.

MiRNA were also found critical in the activation of hepatic stellate cells and secretion of extracellular matrix. Fibrosis is known to develop by accumulation of ECM secreted by stellate cells. Another cause of hepatic fibrosis is inflammation, where stellate cells can be activated by inflammatory cytokines. MiR-122 directly known to target hepatic CCL2, an inflammatory cytokine that activates stellate cells <sup>41, 58</sup>. Knockdown of miR-122 results in CCL2 reduction and hence switching stellate cells to quiescent status <sup>59</sup> and decrease in inflammation. Similarly, enhanced fibrosis and mortality was observed in hepatocyte-specific miR-29 knockout mice <sup>54, 60</sup>.

HCV has become a huge challenge to treat, because the virus often develops resistance to the anti-viral drugs used. There have been several findings implicating miR-122 in HCV-replication indicating the potential of miRNAs in the development of anti-viral therapy. The study performed by Jopling et.al <sup>45</sup> demonstrated the crucial

role of miR-122 in the replication of HCV RNA in hepatic cells. Two putative binding sites located in the IRES are considered from miR-122 targeting and binding to both sites was found to be necessary for viral replication. The two sites are adjacent and are separated by a short spacer, which is largely conserved between HCV genotypes. This study was instrumental in the development of Miravirsen, a modified anti-miR-122 oligonucleotide by Santaris Pharma, currently undergoing phase II clinical trials in HCV-infected patients. Liver-specific delivery of Miravirsen, a locked nucleic acid phosphorothioate modified antisense oligonucleotide (LNA-ASO), was developed to effectively targets miR-122 in hepatocytes after systemic delivery<sup>61,62</sup>. In addition to direct interaction, miR-122 can directly facilitate HCV replication by regulation of heme oxygenase-1 expression, a key cytoprotective enzyme capable of suppressing HCV replication<sup>63</sup>. More recently, miR-122 has been shown to stimulate HCV translation by enhancing the association of ribosomes with the viral RNA at an early initiation stage<sup>64</sup>. An additional miR-199a was identified as a potential inhibitor of HCV replication and hence overexpression can be a rational therapeutic strategy<sup>65</sup>.

### ***RNAi therapy for HCV***

More than a decade ago<sup>66</sup> the discovery of RNA interference (RNAi), a sequence-specific inhibition of gene expression at posttranscriptional level has emerged as a new possibility to combat viral infections. RNAi is triggered by small interfering RNA (siRNA), which can be introduced into cells directly as synthetic siRNA or indirectly as vector expressed short-hairpin RNA (shRNA) precursor<sup>67</sup>(Figure 2). Encoded shRNA can be exported to the cytoplasm and cleaved into active siRNA by a cellular enzyme, DICER. These siRNAs are assembled into a multicomponent complex, known as the RNA-induced silencing complex (RISC), which incorporates a single strand of the siRNA serving as a guide sequence to target and silence homologous messenger RNA (mRNA)<sup>68</sup>. Since HCV is a single-stranded RNA molecule, both the viral genome and host cellular factors involved in the viral life cycle, such as viral receptor CD81, can be targeted by RNAi and convey protection against infection<sup>69</sup>. A single dose administration of integrating lentiviral vectors would have long lasting therapeutic effects in treating chronic HCV or preventing recurrence in transplant patients, hence, representing it as a suitable tool for in vivo

delivery of RNAi.

#### **IV. Cell to cell transmission of RNAs and viruses: the role of Exosomes**

##### ***Intercellular communication***

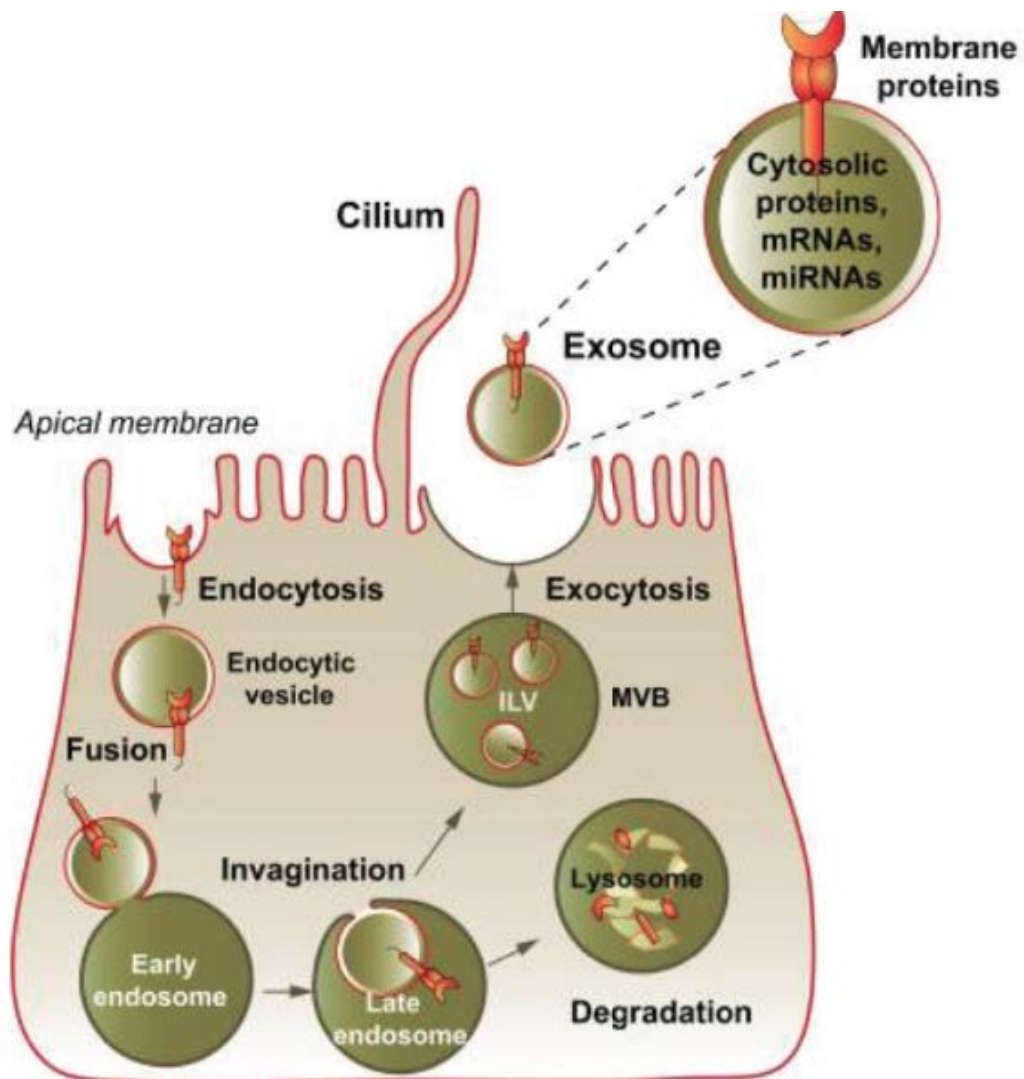
The functioning of multicellular organisms is dependent on inter-cell communication. Conventional models of cellular communication either require direct cell contact or the exchange of signaling molecules. These signaling molecules include hormones in the endocrine system, neurotransmitters of the nervous system and cytokines for the immune system. Early in the 1960's it was shown cellular communication in three epithelia is associated with the presence of certain close-junctional membrane complexes (42). It is now a well know fact that signaling molecules interact with a target cell as a ligand to cell surface receptors, like for growth factors and/or by entering into the cell through its membrane or endocytosis for signaling, like for steroid hormones. This leads to various physiological effects as a result of activation of second messengers in the cell.

The participation of mRNA in transmission of information between cells was been investigated early on. Dahl *et.al* (43) and colleague's show isolated mRNA from cells that are in the process of making new intercellular nexus, when incorporated via liposomes into communication-defective cells, that these defective cells established junctional communication <sup>44</sup>. Now it is well known that mRNAs can transfer genetic information between cells and make them functional too (32). From this study by Valadi *et.al* it is clear that exosomes can mediate thistransfer of mRNA.

##### ***Microvesicles and exosomes***

Exosomes are small vesicles now thought to play an important role in intercellular communication <sup>70, 71</sup>. They are endosomal in origin and range from 30 to 150 nm in size, appear with a characteristic cup-shaped morphology (after negative staining) or as round, well delimited vesicles as observed by transmission and cryo-electron microscopy, respectively <sup>72</sup>. Exosomes float on a sucrose gradient at a density that ranges from 1.13 to 1.19 g/ml <sup>73</sup>. It was in 1987 that Johnstone *et al.* introduced the term exosomes <sup>74</sup>. But it was only a decade later that exosomes were isolated from B lymphocytes and were demonstrated to exhibit antigen – presenting characteristics <sup>75</sup> and henceforth have been shown to be secreted by various cell types <sup>76</sup>. They

represent a specific subtype of secreted membrane vesicles <sup>70</sup> and are formed in endosomal compartments called multivesicular endosomes or bodies (MVBs), containing internal vesicles that package and store molecules in membrane-bound structures. Some MVBs are degraded in lysosomes, others fuse with the plasma membrane and release their content in an exocytic manner into biological fluids such as blood, urine, bile, saliva *in vivo*, or into culture media *in vitro* <sup>77</sup> (Figure 3).



**Figure 3:** Exosomes containing membrane and cytosolic proteins, mRNAs, and miRNAs, are derived from the multivesicular body (MVB) sorting pathway. Adapted from *Journal of Hepatology*, 2013, Sep; 59(3): 621-5.

Exosomes are important key players in intercellular communication and play potential roles in physiological and pathological processes. Exosomes can interact with target cells via receptors<sup>71,78</sup> and also directly fuse with the cell membrane to integrate the proteins into the plasma membrane or be endocytosed delivering their cargo into the cytoplasm of the recipient cells<sup>71,79</sup>. Along with mRNAs, they contain/transfer microRNAs<sup>80</sup>, facilitate viral transport<sup>81,82</sup>, spread cell damage and stimulate malignant transformation<sup>79,83</sup>.

To date, the evidence on the association of exosomes in liver diseases is restricted to the pathogenesis of hepatocellular carcinoma (HCC)<sup>84</sup>, HCV<sup>85</sup> and liver inflammation<sup>86</sup>. The role for exosomes in viral hepatitis is supported by observations that the exosomal pathway is required for release of HCV from infected cells. Also, HCV envelope proteins are associated with exosomes, and exosome-associated viral RNA is present in plasma of HCV infected patients<sup>85,87</sup>. Studies demonstrate the delivery of naturally occurring functional genetic elements to neighboring cells via exosomes, indicating that viral particles or molecules associated with viral infection can be transmitted to adjacent uninfected cells via exosomes and become functional<sup>88</sup>. It has also been shown that hepatocyte-derived exosomes can transfer viral RNA to plasmacytoid dendritic cells triggering their activation and interferon- $\alpha$  production<sup>89</sup>. However, the role of exosomes in the cell-to-cell transmission route of HCV between hepatocytes has not been demonstrated.

## **V. Aim of the thesis:**

In general the aims of this thesis are, in **Chapter 2** we aimed to study the role of hepatic miRNAs and their release during liver injury and could these miRNAs especially circulating miR122, the liver abundant miRNA, represent as potential biomarker of hepatic injury in liver transplantation patients.

The question remains for polarized cells like hepatocytes, whether the release of miRNA occurs bidirectional. In **Chapter 3** we investigated the interplay between levels of hepatic miRNAs in paired serum and bile samples during liver injury and diminished liver function after liver transplantation and provide further evidence for controlled polarized release of miRNAs from the liver.

In **Chapter 4**, we investigated whether human hepatic cells in culture, primary human B cells and mouse hepatocytes can transmit vector-derived RNAi and do these

cells have the ability to exchange small RNAs, including small silencing RNA and miRNA. We further explored the involvement of exosomes in this process. These results may be helpful for the future development of antiviral drugs for the treatment of HCV.

In **Chapter 5** we aimed to study the transmission of HCV infection via hepatocyte-derived exosomes from one cell to the other in the presence of neutralizing antibodies.

In **Chapter 6** we explored the presence of HCV in bile in the form of membrane bound viral particles by collecting bile from hepatitis C positive patients during liver transplantation and isolation of exosomes. We detected HCV RNA in these exosomes that suggests the presence of HCV in bile. This finding can open up the discussion on the possibility of feco-oral transmission of the virus.

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## Chapter 2

### **Hepatocyte-derived MicroRNAs as Serum Biomarker of Hepatic Injury and Rejection after Liver Transplantation**

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## **Abstract**

Recent animal and human studies highlight the potential of hepatocyte-derived microRNAs (HDmiRs) in serum as early, stable, sensitive, and specific biomarkers of liver injury. Their usefulness in human liver transplantation, however, has not been addressed. Aim of this study is to investigate serum HDmiRs as markers for hepatic injury and rejection in liver transplantation. Serum samples of healthy controls and liver transplant recipients (n = 107), and peri-transplant liver allograft biopsies (n = 45) were analyzed by RT-PCR quantification of HDmiRs, miR-122, miR-148a and miR-194. The expression of miR-122 and miR-148a in liver tissue was significantly reduced with prolonged graft warm ischemia times. Conversely, serum levels of these HDmiRs were elevated in patients with liver injury and positively correlated with transaminase levels. HDmiRs appears to be very sensitive, as patients with normal transaminase values (below 50 IU/L) had 6 to 17-fold higher HDmiRs levels as healthy controls ( $P < 0.005$ ). During an episode of acute rejection, serum HDmiRs were elevated up to 20-fold and appear to rise earlier than transaminase levels. HDmiRs proved stable during repeated freezing and thawing of serum. In conclusion, this study shows that liver injury is associated with release of HDmiRs into the circulation. HDmiRs represent promising candidates as early, stable and sensitive biomarkers for rejection and hepatic injury after liver transplantation.

## **Introduction**

MicroRNAs (miRNAs), a class of small non-coding RNAs, are important regulators of gene expression and they control many cellular processes by post-transcriptional suppression of gene expression (1, 2). Altered tissue expression levels of miRNAs have lately been linked to various pathologic conditions in humans, including malignant, infectious, metabolic, autoimmune, and cardiovascular diseases (3-9). These findings have led to increased interest in miRNAs as potential diagnostic markers as well as targets for therapeutic interventions.

Hepatocytes express a distinct set of miRNAs of which miR-122 is most abundant (10). MiR-122 was found to be an important regulator of cholesterol metabolism (11), iron homeostasis (12) and a crucial host factor for hepatitis C virus infection and replication (13, 14). In addition to these important cellular functions, recent studies in rodents have demonstrated that miR-122, as well as other hepatocyte-abundant miRNAs, are released from cells during drug-induced liver injury (15, 16). These hepatocyte-derived miRNAs (HDmiRs) were detectable in serum or plasma and levels increased dependent on the dose and duration of drug exposure. HDmiRs were found to correlate with serum transaminases, aspartate transaminase (AST) and alanine transaminase (ALT), as well as liver histology. Importantly, the rise in serum miRNA in these animals appeared earlier than the rise in transaminases. In addition to the diagnostic potential of miRNA, experimental animal studies have shown that miRNAs are a feasible target for therapeutic intervention to minimize and even reverse severe tissue injury caused by ischemic insults (17). In humans, it has recently been shown that the HDmiR miR-122 can also be detected in serum and was found to be elevated in patients with hepatocyte injury caused by viral, alcoholic or chemical-related hepatotoxicity (18, 19). Also in these patients, serum and plasma miR-122 showed a close correlation with transaminases and liver histology. However, this has not been evaluated in the setting of liver transplantation.

Liver transplantation has developed from a risky experimental procedure to a life-saving and effective treatment of end-stage liver failure. However, despite this success, transplant recipients can suffer from serious side effects of long-term immune suppression and remain at risk of de novo malignancies (20) or lose their allograft due to rejection, recurrent disease or biliary complications (21, 22). The potential benefit of tapering immunosuppressive medication in patients to reduce

toxicity is countered by the potential risk of losing the graft by immune mediated rejection. Therefore, there is an urgent need for better biomarkers that could provide earlier and more sensitive signs of rejection or liver graft dysfunction in a non-invasive fashion. Given their cell-type specific distribution, their biological stability and sensitivity of detection, HDmiRs could represent promising candidates for this. Indeed, several recent studies in the setting of kidney transplantation have highlighted the potential of mRNA and miRNA as biomarkers for assessing renal allograft status (23-26). Current protein-based markers for liver injury, AST and ALT, are also expressed outside the liver in muscle tissue and they can cause false elevations during muscle injury (27). Therefore, assessment of liver allograft status often still requires tissue biopsies for more definite proof of hepatic injury. Particularly after liver transplantation, taking trough-cut biopsies is a relative perilous procedure associated with pain, bleeding and infections (28-31). Alternatively, more sensitive, specific and non-invasive methods for monitoring graft injury are needed to minimize the need for liver biopsies and allow safer weaning-off of immunosuppressive medication.

The aim of the current study was to investigate the utility of serum HDmiRs as markers for hepatic injury and acute rejection after liver transplantation. We found that the expression of miR-122 and miR-148a in liver tissue was significantly diminished with prolonged graft warm ischemia times and, conversely, was elevated in serum during ischemia and reperfusion injury and acute rejection. HDmiRs were found to represent promising candidates as biomarkers for assessing allograft status after liver transplantation.

## **Patients & Methods**

### **Patient samples**

All liver transplantations were performed at Erasmus Medical Center, Rotterdam, The Netherlands. Liver graft biopsies (n = 45) were obtained during transplantation 60 minutes after portal reperfusion and directly snap frozen for storage. Serum samples were taken from 12 healthy controls and 43 recipients at different times after liver transplantation and included 13 patients with histologically proven acute rejection. All blood samples were collected using a standardized protocol and serum was processed within 2 hours and quickly stored in -80°C. Serum samples with signs of red blood cell lysis were not used. Patient demographics and clinical variables were extracted



from a prospectively filled database and summarized in Table 1. The intrinsic stability of HDmiRs in serum was determined by subjecting four individual serum samples from liver transplant recipients to five freezing and thawing cycles (-80°C / +20°C). The Medical Ethical Council of the Erasmus MC approved the use of human samples and all patients provided informed consent for the use of materials for medical research.

		Healthy controls	Pre-transplant	Post-transplant	
			End-stage liver disease	Non-rejectors	Rejectors
Serum samples (n)		12	10	34	47
Mean (UI/L)	AST	-	89 ± 17	785 ± 221	151 ± 45
Mean (UI/L)	ALT	-	95 ± 33	732 ± 166	219 ± 34
Patients (n)		12	10	20	10
Age (Years)		42 ± 3	51 ± 3	50 ± 2	40 ± 8
Gender (M:F)		7 : 5	7 : 3	14 : 6	6 : 4
Underlying disease					
	Viral	-	7	10	4
	Cholestatic	-	1	3	2
	Alcoholic	-	1	2	2
	Other	-	1	5	2

**Table 1.** Characteristics of patients and healthy controls

Serum levels of AST and ALT <50 UI/L were considered normal. Acute cellular rejection was defined by the presence of all three following criteria: a transient rise in AST and ALT levels above the upper limit of normal, a rejection activity index (RAI) of 6 or more in the consequent needle biopsy at histological examination and a decrease in transaminase levels upon treatment with methylprednisolone (32).

### RNA isolation

Total RNA was extracted from approximately 10 mg of liver tissue using the miRNeasy Mini Kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). A modified protocol was used to isolate total RNA from serum. For this, 1.5 ml of Qiazol Lysis Reagent was added to 200 µl of serum and extensively mixed

by vortexing. Chloroform (300  $\mu$ l) was added and after centrifugation (15 minutes, 16.000 RCF), 800  $\mu$ l of an aqueous RNA-containing layer was obtained, which was further processed according to the manufacturer's protocol (Qiagen). RNA extracted from liver tissue was quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA) and normalized to a concentration of 50 ng/7.5  $\mu$ l. RNA extracted from serum could not be quantified due to its low concentration and was normalized only for initial serum input.

### **Reverse transcription and real-time polymerase chain reaction (RT-PCR)**

The TaqMan microRNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, USA) was used to prepare cDNA, for multiple miRNAs in one reaction, using a modified protocol. Every multiplex cDNA reaction consisted of 0.4  $\mu$ l dNTP mix, 1.35  $\mu$ l Multiscribe RT enzyme, 2.0  $\mu$ l 10x RT Buffer, 0.25  $\mu$ l RNase Inhibitor, 1.0  $\mu$ l of each RT primer, and 7.5  $\mu$ l of diluted template RNA. The total reaction volume was adjusted to 20  $\mu$ l with nuclease free water. Based on literature, 15 miRNAs were initially tested, namely miR-30a, miR-30c, miR-30e, miR122, miR-133a, miR-148a, miR-191, miR-192, miR-194, miR-198, miR-200c, miR-222, miR-296, miR-710 and miR-711 (15, 33-36). Three highly expressed hepatocyte-rich miRNAs, miR-122, miR-148a and miR-194, were selected and further used. For analysis of liver biopsies, additional cDNA was prepared for a small nuclear RNA, RNU43, which served as reference gene for normalization of RNA input. For serum samples two additional non-liver-abundant miRNAs, miR-133a (muscle-abundant) and miR-191 (blood-abundant), served as controls. All cDNA reactions were performed according to the manufacturer's instructions. PCR reactions were carried out in duplicate and according to the manufacturer's instructions. Each reaction consisted of 10  $\mu$ l TaqMan Universal PCR Master Mix, 0.5  $\mu$ l microRNA-specific PCR primer (Applied Biosystems) and 5.0  $\mu$ l of the previously diluted (1:10 dilution) cDNA. The final volume of every PCR reaction was adjusted to 20  $\mu$ l with nuclease-free water.

### **Statistical analyses**

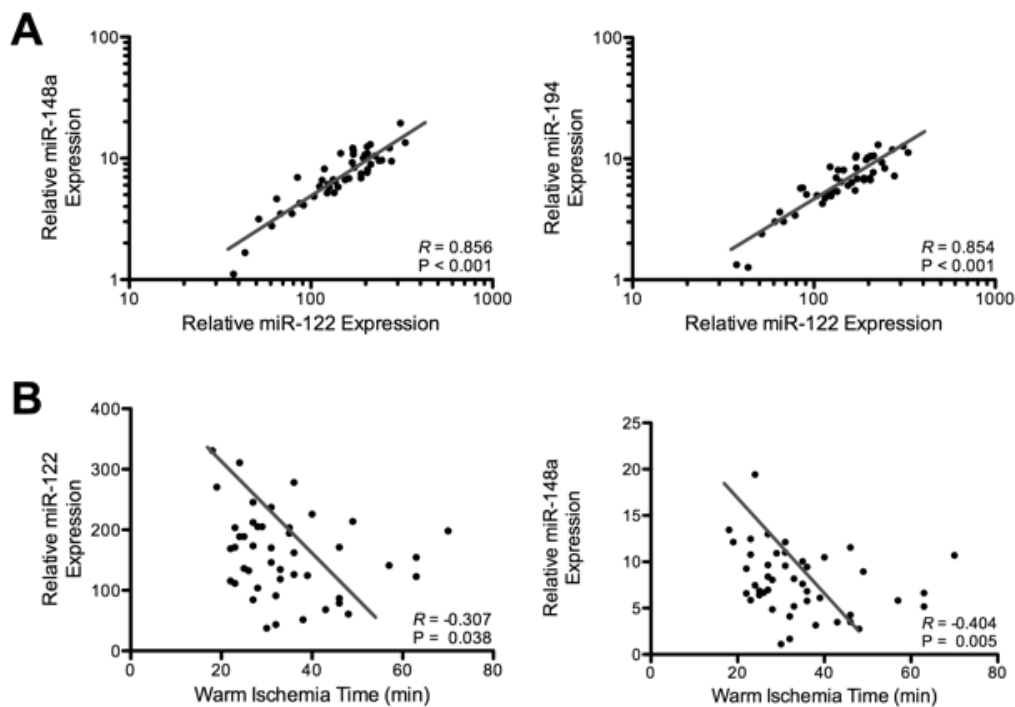
Statistics for correlation were generated using Spearman's Rank Correlation test. Comparative statistics between groups were tested using the Mann-Whitney U and the Wilcoxon matched pairs test by GraphPad Prism software (GraphPad Software Inc.,

San Diego, USA). P-values < 0.05 were considered significant.

## Results

### Reduced hepatic miRNA levels in liver grafts with long warm ischemic times

To investigate changes in intrahepatic miRNA expression in response to ischemia-reperfusion injury, 45 biopsies taken from liver grafts one hour after reperfusion were analyzed. Average cold ischemia time was  $484 \pm 25$  minutes (mean  $\pm$  SEM) and the mean warm ischemia time was  $35 \pm 2$  minutes. As shown in Figure 1A, there was a significant positive correlation between the levels of hepatocyte-abundant miRNAs. Levels of miR-122 strongly correlated with miR-148a and miR-194 ( $R \geq 0.85$ ,  $P < 0.001$ ), but were approximately 20-fold higher than those of miR-148a and miR-194. As shown in Figure 1B, the levels of miR-122 and miR-148a, but not miR-194, in these liver graft biopsies showed a significant reverse correlation with the length of warm ischemia time ( $R = -0.307$ ,  $P = 0.038$  and  $R = -0.404$ ,  $P = 0.005$  respectively). No significant correlation of miRNA levels and cold ischemia times was observed (data not shown). These findings suggest that graft injury associated with longer warm ischemia times reduced levels of specific hepatocyte-abundant miRNAs, possibly by the release of miRNAs from injured cells.

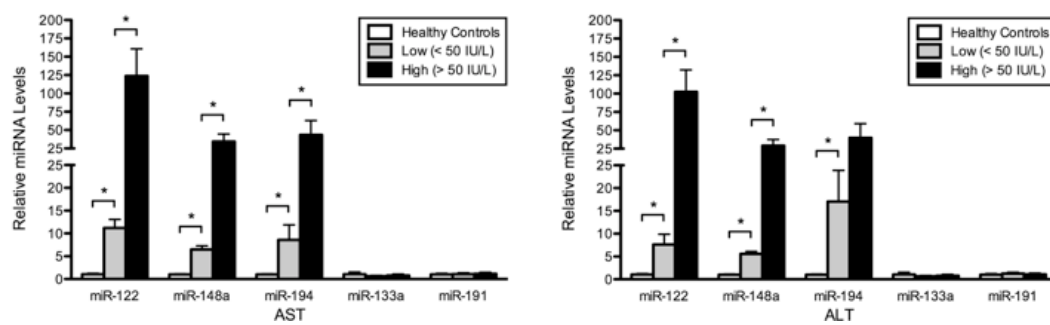


**Figure 1.** Decreased levels of hepatic miRNAs in liver grafts with extended warm ischemia times. Liver graft tissue biopsies ( $n = 45$ ) were analyzed for the hepatocyte-abundant miRNAs, miR-122, miR-148a and miR-194, by quantitative RT-PCR.

MiRNA levels were normalized to small nuclear RNA RNU43, which served as a reference gene. (A) Relative expression levels of miR-122 correlated significantly with miR-148a and miR-194 in the liver grafts ( $R \geq 0.85$ ,  $P < 0.001$ ). MiR-122 levels were approximately 20-fold higher than miR-148a and miR-194. (B) Decreased levels of miR-122 and miR-148a in liver graft biopsies correlated significantly with length of the warm ischemia time to which the graft had been exposed during liver transplantation ( $P < 0.05$ ).

### Serum HDmiRs are associated with peri-transplant ischemic liver injury

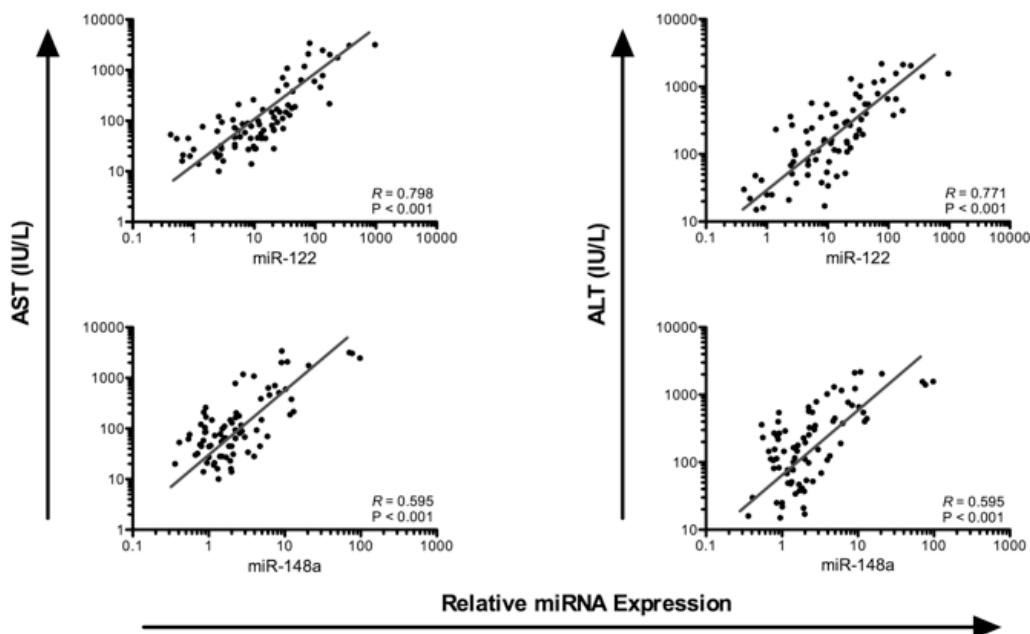
Serum samples from healthy individuals and liver allograft recipients within 2 weeks of transplantation were analyzed for the presence of HDmiRs. All three HDmiRs, miR-122, miR-148a and miR-194, and both control miRNAs, 133a and 191, were detectable in the serum from healthy individuals and patients. As shown in Figure 2, the levels of HDmiRs were significantly elevated in patients after liver transplantation as compared to healthy controls. In serum samples with high transaminase levels (AST or ALT  $> 50$  UI/L), the levels of miR-122 were respectively 124-fold and 102-fold elevated with respect to average levels in healthy ( $P < 0.0001$ ). When compared to healthy controls, levels of miR-148a and miR-194 were respectively 30-fold and 40-fold higher in the high transaminase groups ( $P < 0.0001$ ). Levels of all HDmiRs were significantly higher in the high AST and ALT groups compared to the low AST and ALT groups ( $P < 0.005$ , Fig. 2) with the exception of miR-194 in the high ALT group, which was only 2-fold elevated and not statistically significant. Levels of the control miRNAs, miR-133a and miR-191, were not significantly different between any of the groups (Fig. 2).



**Figure 2.** Hepatocyte-derived miRNAs (HDmiRs) are elevated in serum during peri-transplant ischemic liver injury. HDmiRs miR-122, miR-148a and miR-194, were quantified using RT-PCR in 92 serum samples obtained from liver transplant recipients ( $n = 40$ ) and healthy controls ( $n = 12$ ). Compared to healthy controls, levels of miR-122, miR-148a and miR-194 were significantly elevated in serum samples of patients with low AST and ALT levels by 11-, 7-, 9-, and 8-, 6- and 17-times, respectively. Levels were further elevated in serum of patients with transaminase levels above the clinical diagnostic threshold of 50 UI/L. For the high AST group miR-122, miR-148a and miR-194 were 11-, 5- and 5-fold higher and for the high

ALT group 13-, 5- and 2-fold higher compared to the low AST and ALT groups. Levels of control miRNAs, miR-133a and miR-191, were not significantly elevated in any of the serum samples compared to healthy controls. \*  $P < 0.005$

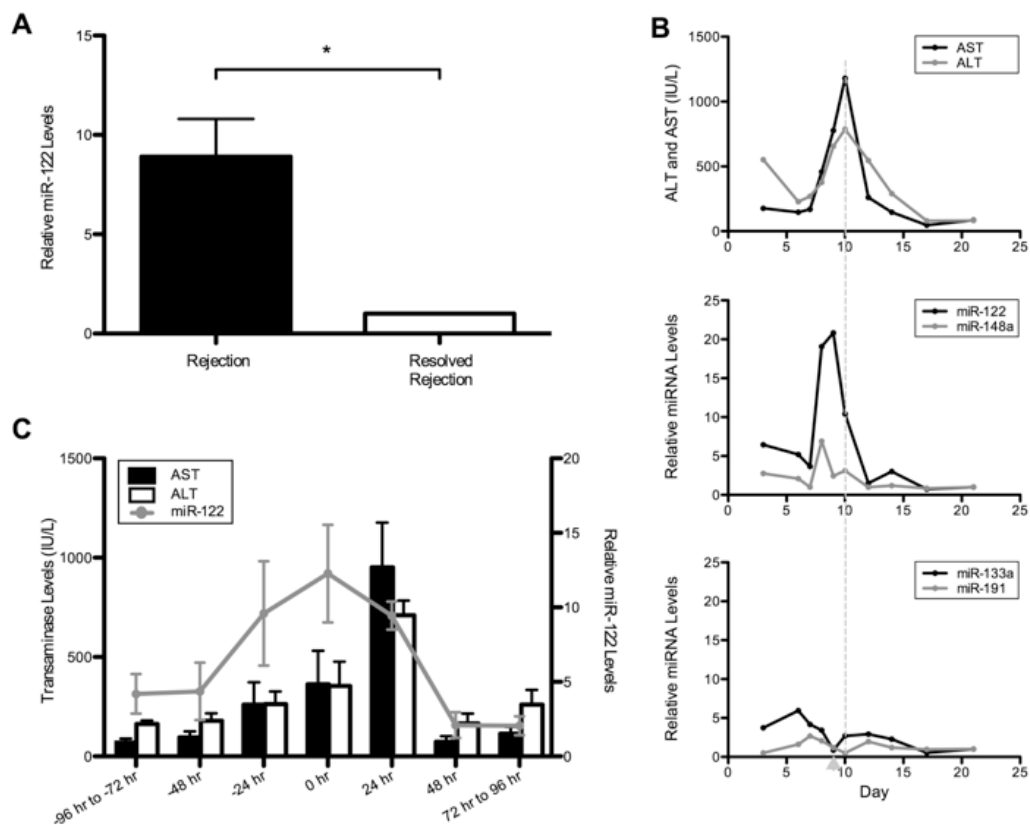
The HDmiRs appeared to be sensitive, as patients with normal transaminase values had significantly elevated levels of miR-122, miR-148a and miR-194 compared to healthy controls (respectively 11, 7, and 9-fold higher in the low AST group and respectively 8, 6 and 17-fold higher in the low ALT group,  $P < 0.005$ ). As shown in Figure 3, a positive correlation was observed between serum HDmiRs levels and transaminases in patients. The correlation with AST and ALT resulted in a coefficient  $R$  of respectively 0.80 and 0.77 for miR-122, while for miR-148a the coefficient  $R$  was 0.60 for both AST and ALT ( $P < 0.0001$ ). No significant correlations were found for miR-194 ( $R < 0.30$ ,  $P > 0.05$ ). Additional experiments to test the stability of HDmiRs in serum showed that levels of miR-122, miR-148a and miR-194 in serum were not significantly affected after five cycles of freezing ( $-80^{\circ}\text{C}$ ) and thawing to room temperature (mean  $120\% \pm 11$  SEM,  $100\% \pm 6$  and  $99\% \pm 19$  of untreated baseline values, respectively).



**Figure 3.** Levels of serum HDmiRs in liver transplant recipients correlate with AST and ALT. HDmiRs, miR-122 and miR-148a, were quantified using RT-PCR in eighty serum samples obtained from liver transplant recipients. Serum levels of miR-122 and miR-148a correlated significantly with levels of AST and ALT in the same samples.

## Elevated serum HDmiRs during acute rejection

Serum HDmiRs were analyzed in liver transplant recipients experiencing an episode of acute rejection. As shown in Figure 4A, serum HDmiR miR-122, was significantly elevated during rejection. An average 9-fold increase was observed at the time of rejection compared to levels 6 months after resolving rejection ( $P < 0.005$ ). For five patients a longitudinal series of serum samples before, during and after acute rejection was analyzed. One representative patient is shown in Figure 4B. Serum levels of miR-122 and miR-148a showed kinetics similar to those of AST and ALT and increased up to 20-fold during acute rejection. Levels of the control miRNAs, miR-133a and miR-191, did not increase during acute rejection (Fig. 4B). Although miR-122 showed similar kinetics, it appeared to rise and drop one or two days earlier than transaminase levels (Fig. 4B). As shown in Figure 4C, in pooled data of five patients a similar trend was observed. At the moment of diagnosis and start of treatment of the acute rejection (0 hr) miR-122 was already elevated to its maximum level. Levels of miR-122 dropped quickly after the start of intravenous methylprednisolone treatment, while levels of AST and ALT continued to rise even after the start of treatment and took longer to normalize.



**Figure 4.** Changes in serum HDmiRs during acute rejection. Serum samples from 13 liver transplant recipients experiencing one or more episodes of biopsy-proven acute rejection were analyzed. **(A)** Levels of serum miR-122 were significantly elevated during acute rejection by approximately 9-fold compared to levels in the same recipients 6 months after rejection was resolved ( $n = 13$ ,  $P < 0.005$ ). **(B)** From five of these patients a longitudinal series of serum samples, taken at daily intervals, was analyzed. Representative results from one patient are shown. Serum levels of miR-122 and miR-148a increased up to 20-fold during acute rejection (middle panel) and showed similar kinetics to those of AST and ALT (top panel). The peak of HDmiRs appears to precede the peak of transaminases (indicated with dashed line) and quickly normalized after starting treatment with intravenous methylprednisolone (arrow on axis). Levels of control miRNAs, miR-133a and miR-191, did not show an increase during acute rejection (lower panel). **(C)** Levels of serum transaminases and miR-122 of the 5 patients at the histologic diagnosis and start of methylprednisolone treatment ( $t = 0$  hr) and up to 96 hrs before and after are shown. Levels of miR-122 reached a maximum level at the start of treatment and quickly decreased after treatment whereas transaminase levels still continued to rise 24 hrs later.

## Discussion

Small non-coding RNAs, in particular miRNAs, have emerged as important genetic regulators of cellular processes, including tissue injury and repair responses (17). Recent studies in small animal models as well as humans have demonstrated that HDmiRs are highly stable and sensitive serum biomarkers of liver injury (15, 16, 18, 19). In both humans and rodents, HDmiRs appeared to increase earlier and more rapidly in serum than AST and ALT. In particular miR-122 was significantly elevated even in subjects with transaminases below the threshold of 50 IU/l (15, 16, 18, 19). In the current study we provide evidence that the concept of miRNAs as biomarkers of hepatic injury is also feasible in the setting of liver transplantation. Serum levels of HDmiRs were elevated in patients with liver injury after liver transplantation (Fig. 2) and during acute rejection (Fig. 4). Conversely, hepatic miRNA levels in liver graft biopsies exhibited diminished expression with prolonged warm ischemic times (Fig. 1). During acute rejection, serum HDmiRs showed similar kinetics, however, miRNA levels increased and decreased earlier than transaminases (Fig. 4B and C). As in previous studies (15, 18), miRNAs showed higher sensitivity than transaminases and miRNA stability was confirmed as proposed by earlier studies (6, 9, 37-40).

HDmiRs could provide a solution for the urgent need for better non-invasive biomarkers that could serve as earlier and more sensitive signs of rejection or liver graft dysfunction. Better markers would greatly help the management of liver transplant recipients and could allow the safer reduction of immunosuppressive

medication to achieve a better balance between effects (prevention of graft rejection) and side effects (toxicity, infection and malignancy). Long-term complications of immunosuppressive drugs, such as nephrotoxicity and *de novo* cancer, are becoming a bigger problem due to the long survival of liver transplant recipients (20). Currently, the potential benefit of tapering immunosuppressive medication in patients is countered by the potential risk of losing the graft by immune mediated rejection. Serum ALT and AST are often insufficient for the early and definitive diagnosis of acute rejection, necessitating the use of liver biopsies. Particularly in the setting of liver transplantation, liver biopsies pose a significant risk for complications such as pain, bleeding and infections (28-31). Feasibility of the concept of minimally invasive diagnosis of acute rejection, based on the detection of messenger RNA, has been demonstrated for kidney transplants (24, 25).

Currently, little is known about the mechanism and biology of release of hepatocyte-abundant miRNAs in response to liver injury. Ideally an unbiased genome-wide approach would be preferred to study release, but it is very challenging to perform gene-array analysis on serum samples because of the low yields of RNA and the relative high amounts required. In our initial analyses we tested 15 different types of hepatocyte and cholangiocyte abundant and control miRNAs selected from other studies (15, 33-36). These included miR-30a, miR-30c, miR-30e, miR122, miR-133a, miR-148a, miR-191, miR-192, miR-194, miR-198, miR-200c, miR-222, miR-296, miR-710 and miR-711, but only the three HDmiRs were found to be significantly elevated during acute rejection. Likely, many other miRNAs expressed in hepatocytes and other liver cells are released during hepatic injury, but only the most abundant and liver-specific miRNAs will be detectable in serum. Nevertheless, the hepatocyte-abundant miRNA miR-194, with expression levels in liver tissue significantly correlating with miR-122 (Fig. 1A), did not correlate with transaminase levels (data not shown). This suggests that there may be sequence specificity or selectivity regarding the release of miRNAs, rather than just a general leakage of all miRNAs from the injured cell. This hypothesis is supported by the observation that cellular miRNAs can be released from cells by secretion of microvesicles including exosomes and that only distinct sets of miRNAs are selectively packaged into microvesicles (40, 41).

This specificity in release and the distinct repertoires of miRNAs expressed by



various cell types in the liver may allow in the future distinguishing between different causes and types of liver injury, like cholangiocyte injury in bile ducts and endothelial cell injury in veins and arteries. Preliminary data from our research group indeed suggests that tissue levels of specific miRNAs expressed by biliary epithelial cells could be used to quantify biliary injury and can predict the development of long-term biliary complications and graft loss after liver transplantation (42). In addition, miRNA-based diagnostics could facilitate allograft selection, particularly of marginal donors, and potentially enlarge the pool of grafts. For example, several experimental studies demonstrated a role of hepatic miRNAs, including miR-122, in regulation of cell proliferation during liver regeneration after partial hepatectomy (43-48). Although the exact biology is not clear, it is conceivable that the decrease in miR-122 expression during graft storage may be related to hepatic cell cycle progression in response to ischemic injury. It is tempting to speculate that manipulation of miRNAs using anti-sense, anti-miRNA technology (11) could allow therapeutic manipulation for rescue of marginal grafts or allow the use of smaller size split grafts by minimizing injury and stimulating cell proliferation (17).

In summary, we demonstrate that circulating HDmiRs, miR-122, miR-148a and miR-194, are stable and detectable during hepatic injury in patients after liver transplantation. The levels of two of these HDmiRs closely correlate with AST and ALT during post-transplant liver injury and acute rejection. These data support the potential of miRNA-based diagnostic tools for various types of liver injury in liver transplant recipients.

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## Chapter 3

### **Bidirectional release of hepatocyte and cholangiocyte-derived microRNAs into bile and blood in relation to liver injury and liver function**

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*Manuscript in preparation*

## **Abstract**

MicroRNAs (miRNAs) have emerged as important regulators of cellular functions in response to injury. Recent studies demonstrated levels of hepatocyte and cholangiocyte-derived miRNAs (respectively HDmiRs and CDmiRs) in the circulation and in bile to relate to different underlying liver pathologies. However, the mechanism by which such miRNAs are released in response to injury remains largely unknown. The aim of our study was to investigate whether HDmiRs and CDmiRs are directionally released by cells into bile and serum during liver injury or impaired exocrine function in recipients after liver transplantation.

Fresh cell-free bile samples obtained from donor gall bladders were used for stability and fractionation experiments. HDmiRs and CDmiRs expression was evaluated in liver biopsies (n=10) and extrahepatic bile duct specimens (n=9). Subsequently, paired bile and serum samples (n=62) from ten liver transplant recipients were collected at different time-points after transplantation and analyzed for levels of HDmiR-122, -148a, and CDmiR-30e, -200c and 222 in the cell-free supernatant.

Fractionation experiments showed that both HDmiRs and CDmiRS in bile are mostly present in non-pelletable bile fragments, whereas only 4 to 6% were found in cell fragments or microvesicle pelletes. HDmiRs and CDmiRs in bile were protected against RNase mediated degradation partly through associated proteins. During early graft injury and after biopsy-proven acute cellular rejection, levels of HDmiRs were significantly elevated in serum, while CDmiRs were increasingly released to the bile. Besides the relation of miRNA release during injury, a strong correlation was found between miR-122 levels in bile and secretion of conjugated bilirubin ( $R=0.694$ ,  $P<0.0001$ ). Like bilirubin, miR-122 levels were lower in bile if hepatocyte exocrine function was impaired. Inversely to miR-122, CDmiR-222 was elevated in bile when bilirubin conjugation was impaired ( $P<0.05$ ).

**Conclusion** In response to injury, HDmiRs and CDmiRs are directionally released into blood and bile, respectively. Mechanisms for HDmiR and CDmiR stability in bile differ and levels of these miRNAs in bile seem a promising marker for both hepatocyte and cholangiocyte exocrine function.



## Introduction

MicroRNAs (miRNAs) are important regulators of post-transcriptional gene expression and as such control many cellular processes<sup>1</sup>. Increasing evidence shows the importance of miRNA expression levels in physiological as well as pathophysiological processes<sup>2-6</sup>. In addition, recent studies have investigated the release of specific miRNAs from cells into the circulation during tissue injury and proposed their use as highly sensitive and specific biomarkers of tissue injury<sup>7-9</sup>. Though the biological function of miRNA release is not well understood, some emerging evidence indicates that miRNAs released upon injury might serve as a danger signal that can trigger remote regenerative responses<sup>10-14</sup>.

The presence of miRNAs has been demonstrated in many bodily fluids, including amniotic fluid, breast milk, bronchial lavage, cerebrospinal and peritoneal fluid, plasma, saliva, tears, urine, pleural fluid, colostrum, and seminal fluid<sup>15</sup>. Recently, in concordance with other human and animal studies<sup>8, 9, 16</sup>, our research group has demonstrated the specific release of hepatocyte-derived miRNAs (HDmiRs) in blood during liver injury, chronic hepatitis C infection and acute rejection after liver transplantation<sup>7, 17</sup>. These miRNAs were shown to be stable, early and sensitive markers of liver injury. Besides HDmiRs, miRNAs derived from cholangiocytes (CDmiRs) were diagnostic in patients with cholangiocarcinoma<sup>18</sup>. Moreover, these CDmiRs were shown to sensitively discriminate grafts with severe biliary injury already at time of graft preservation in liver transplantation<sup>19</sup>. Whereas HDmiRs seem to increase in serum during episodes of injury, simultaneously, a decrease in expression in tissue was observed. In contrast, lower levels of CDmiRs were observed in graft perfusates at time of transplantation in grafts that later developed biliary complication. This supports the current view that miRNA release is a selective and active process rather than passive and non-specific leakage from dead or dying cells. Furthermore, it could indicate that HDmiRs and CDmiRs are released in a different direction during injury. For polarized cells like hepatocytes and cholangiocytes, the question remains whether the release of miRNA can occur bidirectional, for instance to both blood and bile. Shigehara et al.<sup>20</sup> were the first group reporting on miRNAs in bile. MiRNA-9 was found to be a potential biomarker for biliary tract cancer, and miRNAs were shown to be protected against RNase activity<sup>20</sup>. How the levels of these HDmiRs to bile related to the levels in serum was

not addressed in this study.

In this study, we investigate the interplay between levels of HDmiRs and CDmiRs in paired serum and bile samples during liver injury and diminished liver function after liver transplantation and provide further evidence for controlled polarized release of miRNAs from the liver. To our knowledge, this is the first study reporting on polarized release of miRNAs in bile during liver injury dependent on liver function, which may represent a new way of exchanging specific genetic information between cells by transfer of tissue-specific miRNAs.

## **Patients and Methods**

### **Patient samples**

Paired serum and bile samples (n = 62 each) were obtained at different time points, from 10 patients in the first two weeks after liver transplantation. Serum was withdrawn by venipuncture while bile was collected from a T-tube, which was inserted into the common bile duct during transplantation. The samples were processed within two hours of withdrawal to prevent any degradation or contamination and were stored at -80°C. Standard liver function tests (AST, ALT, AP, G-GT and bilirubin) were obtained from serum. Bilirubin levels were also obtained from bile in order to assess liver function. All these samples were obtained from patients suffering from end-stage liver disease, who required transplantation for their disease.

Additional bile material (n=4) for centrifugal fractionation and miRNA stability (n=8) was obtained from donor gallbladders co-explanted, but not transplanted, with donor livers. Large components were removed from these bile samples in a 2-step centrifugation protocol. Samples were centrifuged for 10 min at 453g at 4°C followed by 15 min of centrifugation at 3220g at 4°C. Bile samples were stored at -20°C until further use. The Medical Ethical Council of the Erasmus MC approved the use of all human samples and all patients provided informed consent for the use of materials for medical research.

### **Localization of miRNAs in bile fractions**

For fractionation of stored bile, 4 ml of bile extracted from donor gallbladders was diluted with 8 ml of sterile PBS and a baseline sample was taken (S0). Large components were pelleted by centrifugation at 20000g for 20 min at 4°C. The pellet was resuspended in 400 µl sterile PBS and mixed with 1400 µl Qiazol lysis agent (Qiagen, Hilden, Germany) and stored at -80 °C until further use (P1). The supernatant was transferred to a new tube and centrifuged at 100.000g for 1 hour at 4°C. Pellets were again prepared as described above (P2). The supernatant was then centrifuged for a third time for 2 hours at 140.000g at 4°C and the resulting pellet (P3) was dissolved and stored as mentioned. 400 µl of remaining supernatant (S3) was mixed with 1400 µl of Qiazol and stored at -80°C until further processing (S3).

### **DEPC treatment and protein degradation of stored bile**

Stored bile was treated with diethylpyrocarbonate (DEPC, Sigma-Aldrich, Zwijndrecht, The Netherlands) in a final concentration of 0.02% for 3 hours at room temperature. To completely remove traces of DEPC, samples were boiled for 15 minutes, and aliquots were stored at -20°C until further use. Immediately prior to incubation at 37°C, samples were spiked with 2 fmol each of artificial *C. elegans* miR-39 and miR-238. To degrade stabilizing proteins in stored bile samples, 400 µl samples were incubated for 1 hour at 37°C with Proteinase K (Roche Diagnostics, Almere, The Netherlands) in a final concentration of 0.1 mg/ml.

### **RNA isolation**

Total RNA was extracted using the miRNeasy mini kit (Qiagen, Hilden, Germany) using the manufacturer's protocol with minor modifications. In short, 1000µl of Qiazol lysis reagent was added to 200 µl of serum or bile and mixed extensively by vortexing. In case of supernatant or pellet from the fractioning experiment or the Proteinase K treatment, 1400 µl of Qiazol lysis agent was added to 400 µl of stored bile, supernatant (S3) or pellet (P1, P2, P3), as mentioned earlier. After 5 minutes of resting at room temperature 200µl or 280 µl, respectively, of chloroform was added and the samples were again mixed vigorously using a vortex. After centrifugation (15 minutes, 16.000 RCF at 4°C), 600 µl of aqueous RNA containing layer was obtained, which was further processed according to the

manufacturer's protocol (Qiagen). RNA content was quantified, handled and stored as described previously [7].

### **Reverse transcription and Real-Time Polymerase Chain Reaction (RT-PCR)**

RNA samples were analyzed for HDmiRs and cholangiocyte-derived miRNAs (CDmiRs), as previously reported<sup>8, 18, 21</sup>. As HDmiRs, miR-122 and miR-148a were determined and for CDmiRs, miR-30e, miR-200c and miR-222 were analyzed.

The TaqMan microRNA reverse transcription kit (Applied Biosystems, Carlsbad, CA, USA) was used to prepare cDNA, for multiple miRNAs in one reaction, using a modified protocol as reported previously<sup>7</sup>. In short, for every multiplex cDNA reaction 0.4 µl dNTP mix, 1.35 µl Multiscribe RT enzyme, 2.0 µl 10x RT Buffer, 0.25 µl RNase Inhibitor, 1.0 µl of each RT primer and 7.5 µl of template RNA were used. The total reaction volume was adjusted to 20 µl with nuclease free water. All cDNA reactions were performed according to the manufacturer's instructions (Applied Biosystems).

PCR reactions were carried out in duplo on 384 wells plates to prevent inter-plate variability and consisted of 5 µl TaqMan Universal PCR Master Mix, 0.25 µl microRNA-specific PCR primer (Applied Biosystems) and 2,5 µl of the previously diluted (1:10 dilution) cDNA. The final volume of every PCR reaction was adjusted to 10 µl with nuclease free water and the PCR reactions were run according to the manufacturer's instructions for 45 cycles. For stability and protein degradation experiments, PCR reactions were performed as described previously [7].

### **Statistical analyses**

Statistics for correlation between serum and bile were generated using the Spearman's Rank Correlation test. Comparative statistics between serum and bile were generated using the Kruskal-Wallis, Mann-Whitney U and Wilcoxon matched pair tests. *P*-values < 0.05 were considered significant, as were coefficient *R*'s  $\geq 0.70$  or  $\leq -0.70$ . Analyses were conducted using IBM SPSS Statistics for Windows, Version 20.0 (Armonk, NY: IBM Corp.) and GraphPad Prism version 5.0 (GraphPad Software, San Diego, California, USA).

## Results

### Confirmation of hepatocyte and cholangiocyte-miRNA abundance

Based on literature<sup>18</sup>, miR-122 and miR-148a were chosen to test for hepatocyte injury and/or function, and miRNAs miR-30e, miR-200c and miR-222 were tested for cholangiocyte injury/function. In order to confirm their cell-type specificity, non-paired liver biopsies (n=10) and extrahepatic bile duct specimens (n=9) that were collected during liver transplantation were analyzed for five of these presumed HDmiRs (122, 148a) and CDmiRs (30e, 222, 200c). As illustrated by Fig. 1A, median expression of HDmiR-122 was over a 1000-fold higher in liver tissue compared to bile duct tissue ( $128 \pm 20$  in liver versus  $0.02 \pm 0.80$  in choledochus,  $P=0.0021$ ). Although less abundant than HDmiR-122, also the expression of HDmiR-148a was significantly higher in liver tissue ( $5.63 \pm 0.93$  in liver versus  $1.29 \pm 0.22$  in choledochus,  $P=0.0003$ ). In choledochus tissue, expression CDmiR-222 levels were highest and 17-fold higher compared to liver tissue ( $0.52 \pm 0.45$  in liver versus  $8.47 \pm 1.56$  in choledochus ( $P=0.0002$ )). The discrepancy in expression of CDmiR-200c was even higher, up to 70-fold;  $0.04 \pm 1.56$  in liver versus  $2.78 \pm 0.50$  in choledochus.

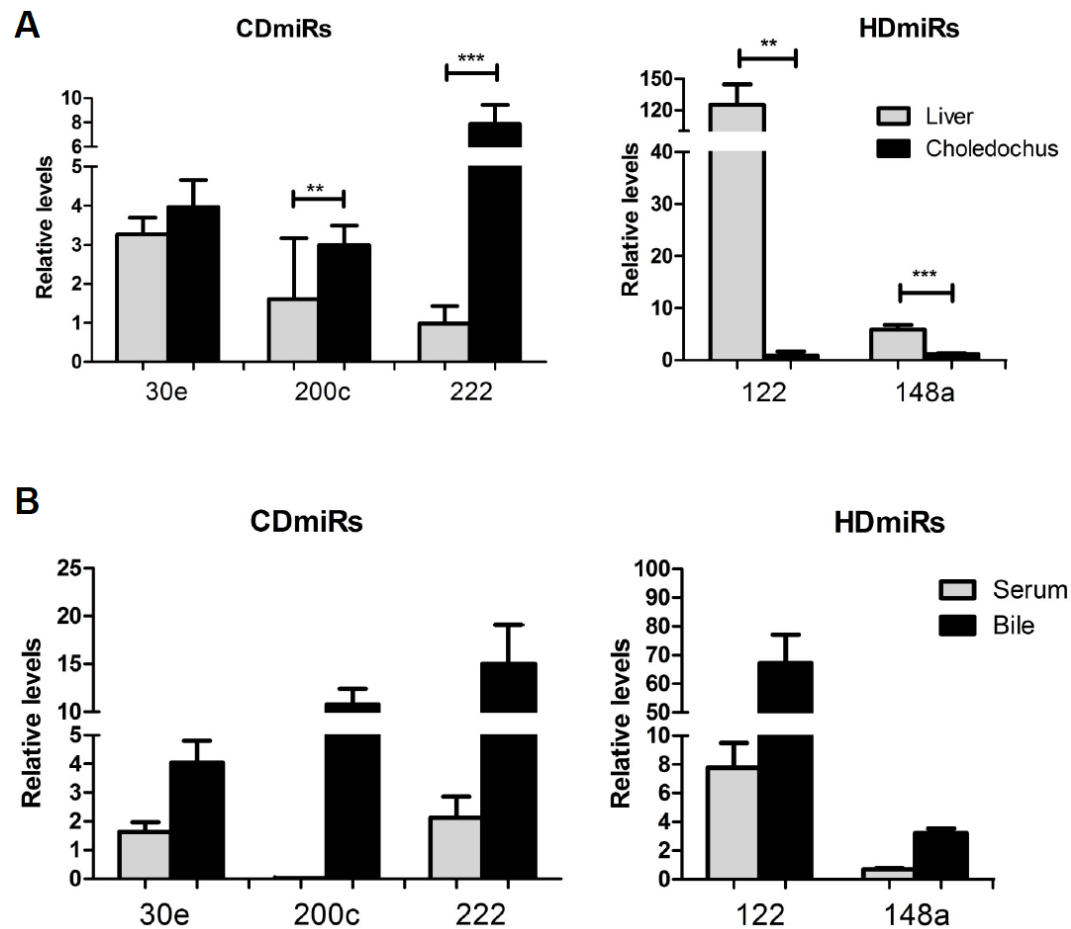
### General levels of HDmiRs in bile and serum

To investigate the release of hepatocyte and cholangiocyte-abundant miRNAs to apical and basolateral direction, sixty-two paired serum and bile samples that were simultaneously collected from patients in the first three weeks after liver transplantation were analyzed. As shown in Fig 1B, HDmiR-122 was the most abundant miRNA, in both bile and serum. Levels of CDmiR-200c in serum were significantly lower compared to other CDmiRs. CDmiR-222 was the highest abundant CDmiR in bile. Overall, relative levels of all HDmiRs and CDmiRs were significantly higher in bile compared to serum.

### HDmiRs and CDmiRs in bile are differently protected against RNase activity

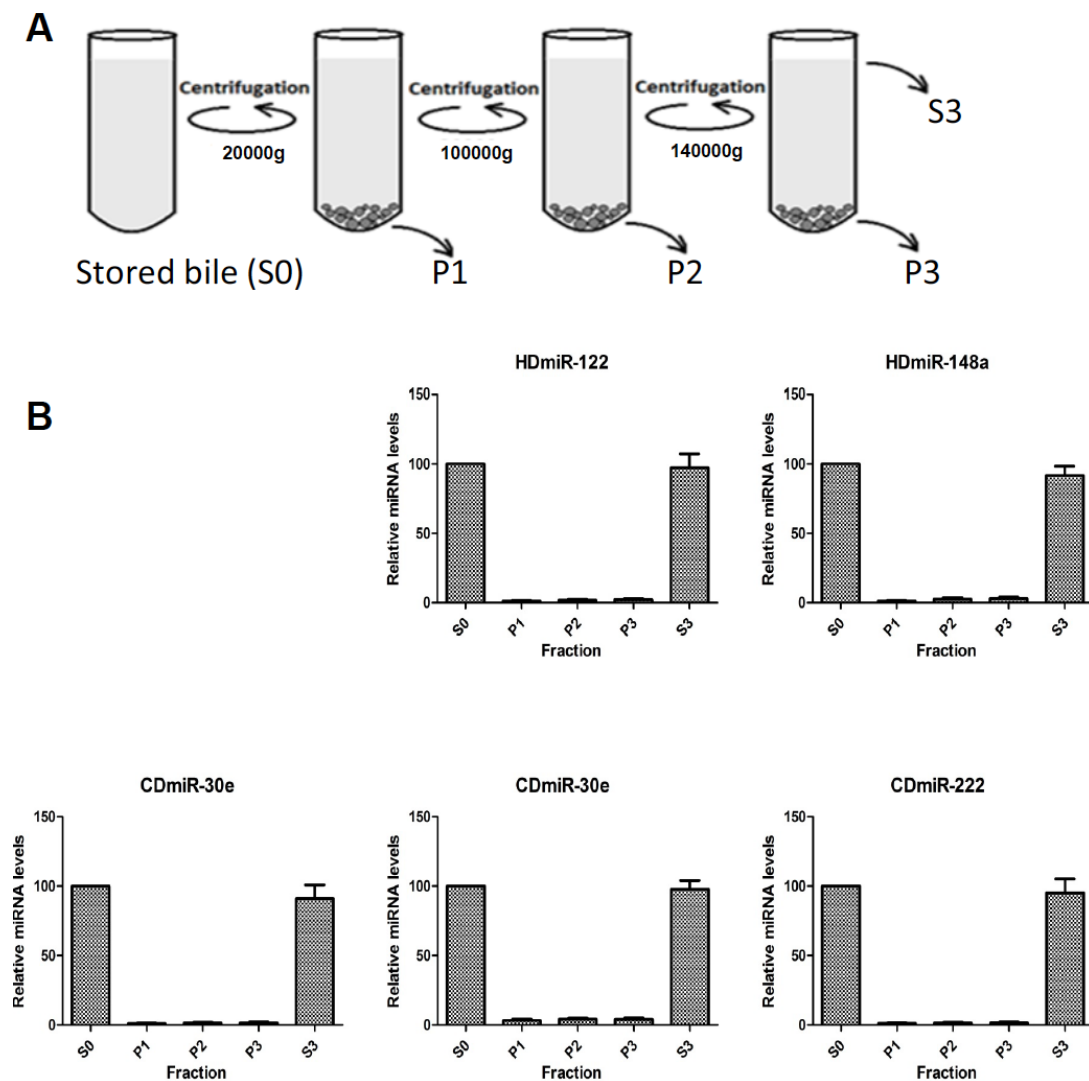
Bile samples (n=8) were fractionated, to determine the subcellular fraction in which HDmiRs and CDmiRs are released in bile. As shown in Figure 2A, fresh bile samples were sequentially centrifuged at 20000, 100000, and 140000 g to obtain pellets with a different composition (P1, P2 and P3). All pellets contained only a small percentage of the tested HDmiRs and CDmiRs compared to the baseline sample (S0). Levels in

fresh bile are comparable with stored, cell-free bile samples (data not shown).



**Figure 1.** (A) Confirmation of hepatocyte- and cholangiocyte abundance of HDmiR-122, HDmiR-148a, CDmiR-30e and CDmiR-222 in liver biopsies (n=10) and extrahepatic bile duct specimens (n=9). RNU43 levels were used for normalization. HDmiR-122 was up to 1000 fold higher in liver tissue compared to bile duct tissue. HDmiR-148a was also enriched in liver tissue, but much less pronounced than HDmiR-122. CDmiR-222 was the highest abundant miRNA in bile duct tissue and was up to 10 fold higher than in liver biopsies. CDmiR-30e was only slightly higher in bile duct tissue compared to liver tissue. (B) HDmiR and CDmiR levels in 62 paired serum and bile samples.,HD miR-122 was the most abundant miRNA in serum, followed by CDmiR-222 and CDmiR-30e. Only a small portion was accounted for by miR-148a, while miR-200c was virtually absent in serum. Also in bile, HDmiR-122 remained the most prominent of miRNA. In general, miRNA levels in bile were higher compared to miRNA levels in paired serum samples.

As shown in Figure 2B, for HDmiR-122 a mean 0.9% was in P1, 1.2%] in P2 and 1.9% in P3. Similar results were obtained for the tested CDmiRs and HDmiR-148a. For both HDmiRs in bile, over 96.4%] were in the non-pelletable fraction (Figure 2B). In order to test the stability of miRNAs, stored bile samples, spiked with artificial *C.elegans* miRs 39 and 238were incubated at room temperature up to 24h.

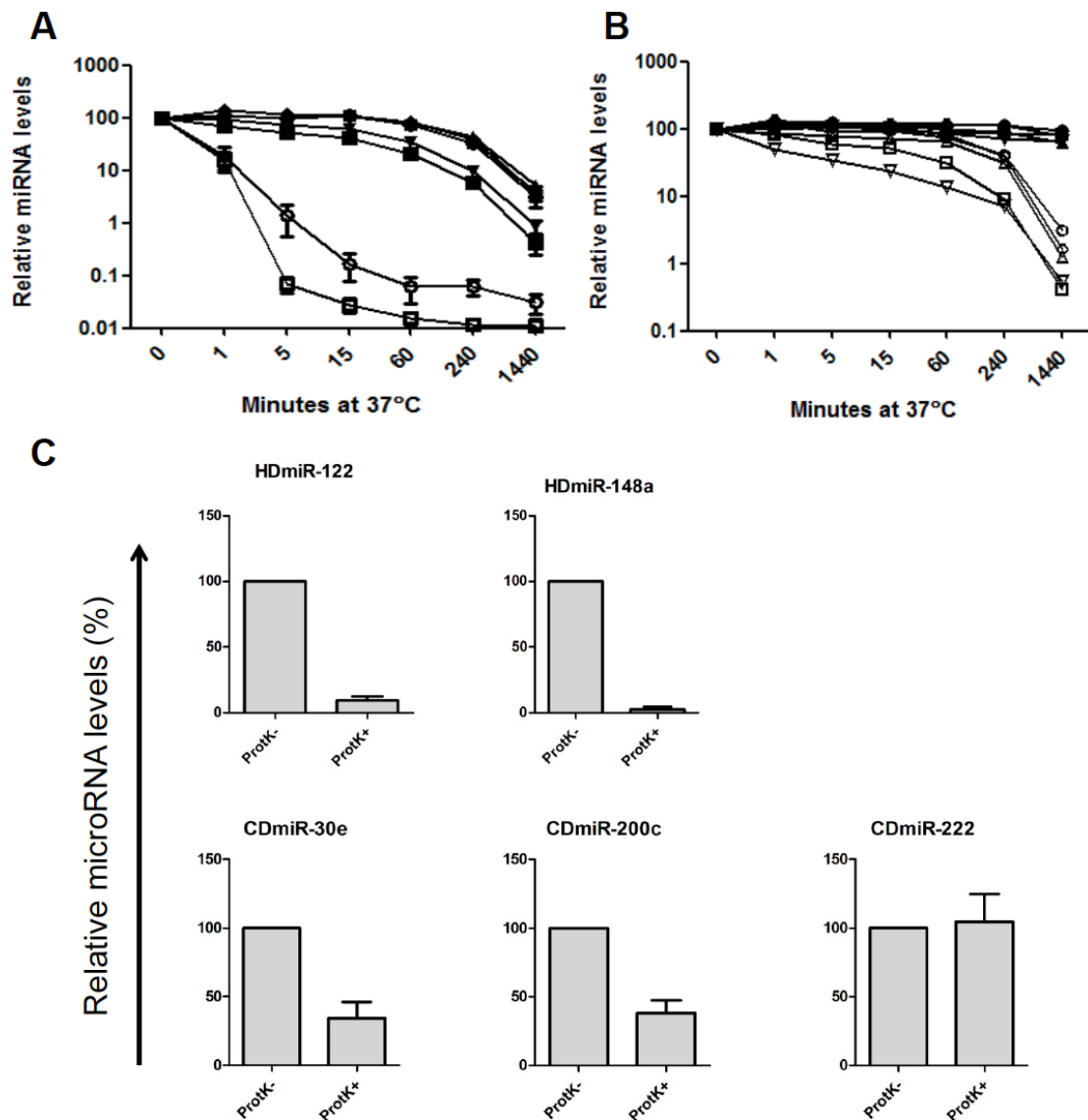


**Figure 2.** (A) Differential centrifugation procedure for stored bile samples. Pellets are enriched in mitochondria, lysosomes and peroxisomes (P<sub>1</sub>), microsomes and membrane fragment(s) (P<sub>2</sub>) and exosomes, ribosomes and viruses (P<sub>3</sub>). S<sub>3</sub> contains soluble proteins and protein complexes. (B) Experiments showed that, after differential centrifugation of bile (n = 8) at 20000, 100000 and 140000 g, all respective pellets (P<sub>1</sub>, P<sub>2</sub> and P<sub>3</sub>) only contained a very small percentage of the tested HDmiRs and CDmiRs compared to the baseline sample (S<sub>0</sub>), suggesting that the majority of the tested miRNAs in bile are protein-bound and not pelletable using the procedure presented in Fig. 2A.

As shown by Figure 3A, both HDmiRs and CDmiRs remained stable for at least one to four hours in bile. However, spiked-in control miRNAs cel-miR-39a and cel-miR-238 degraded within the first five minutes after incubation, as was also shown by other studies<sup>19, 20</sup>. To confirm that the degradation is caused by RNase activity, we also investigated stability of miRNAs in an RNase-free environment by treating bile supernatant with diethylpyrocarbonate (DEPC). As shown by Figure 3B, HDmiRs and

CDmiRs remained stable up to 24 hours in bile when the medium was treated with DEPC. The higher stability of HDmiRs and CDmiRs in normal bile supernatant, compared to exogenously spiked-in miRNAs, has been linked to the formation of miRNA-protein complexes<sup>19, 22</sup>. In order to further investigate this, an additional experiment was performed by treating the bile supernatant with Proteinase-K (ProtK) to analyze how the degradation of proteins influences the stability of separate HDmiRs and CDmiRs. As shown in Fig. 3C, HDmiR-122 and HDmiR-148a were almost completely degraded after ProtK treatment of the bile, while this degradation was less for CDmiR-30e and CDmiR-200c. In particular CDmiR-222 seemed insensitive for treatment with ProtK. These findings suggest that a substantial fraction of CDmiRs (up to 100% for CdmiR-222) is protected in a different way against RNase activity compared to HDmiRs.





**Figure 3.** (A) Levels of HDmiRs, and CDmiRs in bile remained stable up to 4 hours (closed symbols), while exogenously spiked-in cel-miR-39 (open squares) and cel-miR-238 (open circles) already degraded within 5 minutes in bile after incubation at 37°C. (B) When RNase activity in bile was inhibited by DEPC treatment, all tested miRNAs remained stable for at least 24 hours in bile (closed symbols) where the HDmiRs and CDmiRs show a strong decrease (open symbols). (C) When proteins in bile were degraded by ProtK treatment, over 90% of the HDmiRs in bile supernatant were degraded as well. In contrast, CDmiRs were less affected by ProtK treatment, suggesting that CDmiRs in bile have less conjugations with proteinase K sensitive bile components compared to HDmiRs

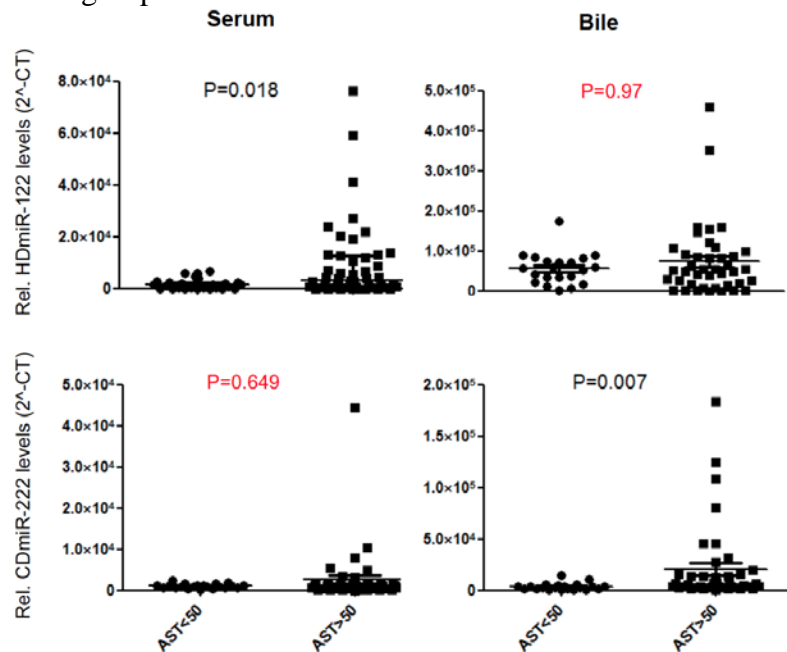
### Directional release of HDmiRs and CDmiRs into serum and bile during injury

To analyze the effect of liver injury on the levels of liver-derived miRNAs in bile and serum, samples were analyzed by dividing them into two groups: the low transaminases group with mean serum AST  $\leq 50$  IU/L (n = 21) and the group with mean transaminases  $> 50$  IU/L (n = 41). The median miRNA levels of HDmiRs and

CDmiRs in paired serum and bile samples for these groups are summarized in Table 1. As can be seen in Figure 4, levels of miR-122 were significantly higher in serum during liver injury ( $P = 0.018$ ), while its relative levels did not significantly differ in bile. This difference was not observed for the less abundant HDmiR-148a ( $P = 0.611$ ). Remarkably, all CDmiR levels were increased in bile during injury (serum AST > 50 IU) and histologically proven acute cellular rejection ( $P \leq 0.012$ , Fig. 4), though this was not observed in the paired serum samples. This suggests that during injury, hepatocytes release their HDmiRs to the circulation, while cholangiocytes release their CDmiRs to the bile.

MicroRNA	Serum microRNA levels		P-value	Bile microRNA levels		P-value
	Serum AST <50 U/L (n=21)	Serum AST >50 U/L (n=41)		Serum AST <50 U/L (n=21)	Serum AST >50 U/L (n=41)	
Median (SD) miR-122	1650 (1999)	4160 (15993)	0.018	56200 (38923)	52000 (90349)	0.970
Median (SD) miR-148a	411 (267)	408 (1049)	0.611	18600 (1258)	2490 (2972)	0.071
Median (SD) miR-30c	904 (483)	828 (3172)	0.915	1460 (1093)	2960 (7152)	0.012
Median (SD) miR-200c	7.67 (6.01)	6.30 (11.0)	0.145	3950 (3261)	7180 (14822)	0.004
Median (SD) miR-222	1030 (519)	1090 (7018)	0.649	3050 (3391)	5300 (38135)	0.007

**Table 1.** The median miRNA levels of HDmiRs and CDmiRs in paired serum and bile samples for the groups with low and mean transaminases.



**Figure 4.** Release of HDmiR-122 and CDmiR-222 to bile and blood during liver injury. Samples were analyzed by dividing them into two groups: the low transaminase group with AST  $\leq 50$  IU/L, (n = 21 paired samples) and the high transaminases group with mean serum AST > 50 IU/L (n = 41 paired samples). During injury, HDmiR-122 was significantly increased in serum ( $P = 0.018$ ), while CDmiRs were significantly higher in bile ( $P = 0.007$ ).

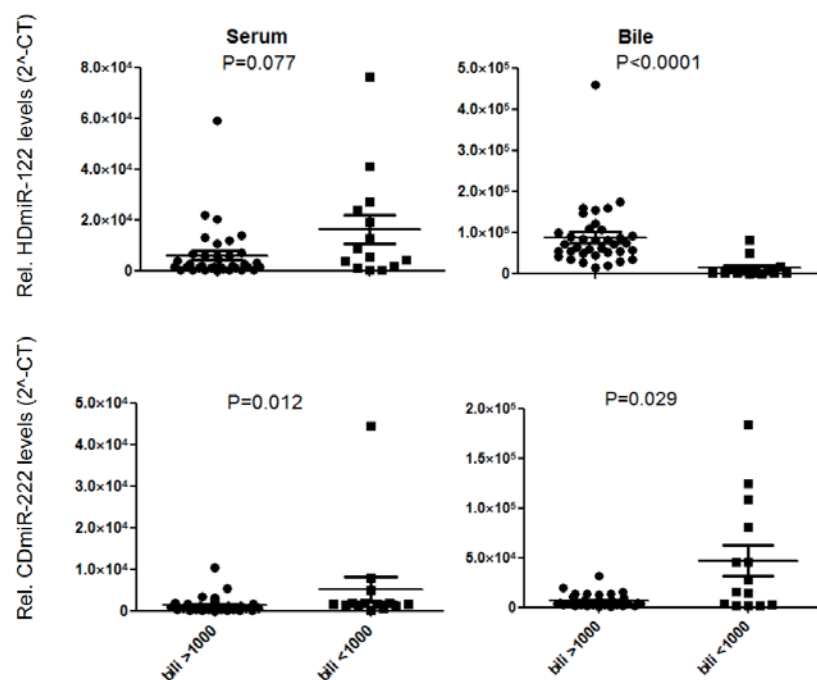
## Relation of HDmiR and CDmiR release into bile with impaired cell function

To investigate the effect of diminished liver function on HDmiR and CDmiR release, groups were stratified by their bilirubin concentration in bile (Table 2).

MicroRNA	Serum microRNA levels			Bile microRNA levels		
	Bile bili <1000 (n=14)	Bile bili >1000 (n=48)	P-value	Bile bili <1000 (n=14)	Bile bili >1000 (n=48)	P-value
Median (SD) miR-122	7065 (21153)	1820 (10638)	0.077	2410 (23856)	40200 (92402)	<0.0001
Median (SD) miR-148a	424 (1070)	386 (933)	0.757	2915 (3578)	2480 (2363)	0.364
Median (SD) miR-30e	1120 (4021)	805 (2345)	0.426	4900 (10792)	2320 (2722)	0.023
Median (SD) miR-200c	6.95 (11.34)	6.93 (10.26)	0.309	12500 (14781)	5625 (13250)	0.054
Median (SD) miR-222	1745 (11488)	1015 (1886)	0.012	21750 (56979)	3875 (6425)	0.029

**Table 2.** Groups were divided by their bilirubin concentration.

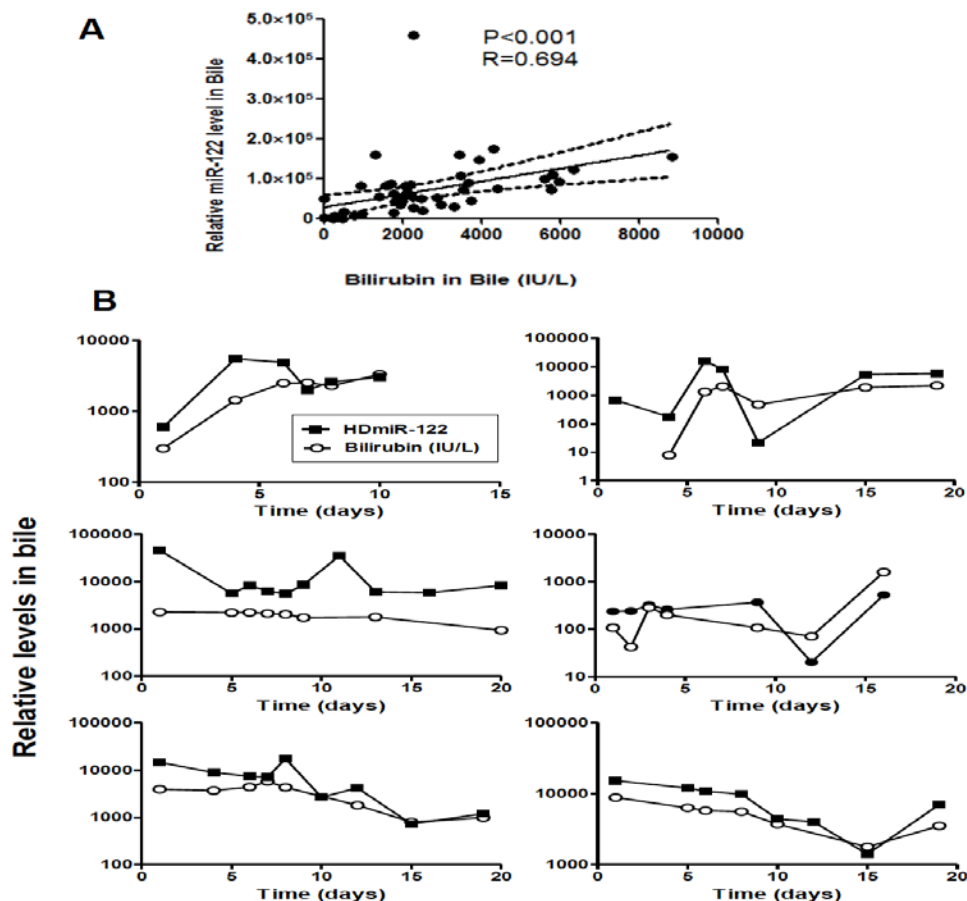
Bile with low concentrations of bilirubin ( $\leq 1000$  IU/L,  $n = 14$ ) were considered poor functioning grafts, while bile samples with bilirubin concentrations  $> 1000$  IU/L ( $n = 48$ ) were considered good functioning grafts as it meant that conjugation, and thus excretion, of bilirubin in the hepatocytes was taking place. As shown by Figure 5, serum levels of HDmiR-122 tended to increase in the serum when bilirubin secretion to bile was low and thus, cell function was impaired.



**Figure 5.** Release of HDmiRs and CDmiRs to blood and bile when cellular function was impaired. Bile with concentrations of bilirubin  $\leq 1000$  IU/L ( $n = 14$  paired samples) were considered poor functioning grafts, while bile samples with bilirubin concentrations  $> 1000$  IU/L ( $n = 48$  paired samples) were considered as good functioning grafts as it meant that conjugation, and thus excretion of bilirubin in the hepatocytes was taking place. When graft function was poor, there was a slight, but significant, increase of CDmiR-222 in serum. Effects of poor graft function were

however much more pronounced by miRNA levels in bile; when there was sufficient bilirubin secretion, HDmiR-122 levels were also secreted to the bile, while bile CDmiR-levels remained low. When secretion of bilirubin to bile was however insufficient, HDmiR-122 was also not secreted to bile. In contrast, CDmiR-levels increased in bile when graft function was poor.

Interestingly, CDmiR levels in bile were increased when bilirubin secretion was impaired. Just like during injury, CDmiRs seemed to increase in bile when levels of HDmiRs in bile were decreased and vice versa. On the other hand, CDmiR-222 also increased in serum when cellular function was impaired. Simultaneously, levels of HDmiR-122 in bile were significantly lower when bilirubin secretion was impaired. As shown by Figure 6, a strong correlation existed between HDmiR-122 and bilirubin levels in bile ( $P < 0.001$ ,  $R = 0.694$ ); dynamics of bile HDmiR-122 levels were similar to bilirubin levels in bile. These results suggest that HDmiRs and CDmiRs are released bi-directionally, dependent on the physiological condition of the liver.



**Figure 6.** (A) Correlation between HDmiR-122 and bilirubin levels in bile. (B) During the first weeks of recipient follow-up after liver transplantation, dynamics between bilirubin levels in bile and HDmiR-122 levels in bile were similar, suggesting a relation between HDmiR-122 and hepatocyte cellular function. Each graph represents one patient.

## Discussion

In this study, we show that miRNAs are bidirectionally released from hepatocytes and cholangiocytes into bile and blood in response to cellular injury and impaired cellular function. During cellular stress and injury, hepatocytes mainly direct the release of HDmiRs into the circulation, whereas cholangiocytes seem to direct CDmiR release into bile. We also observed a strong correlation between bilirubin and HDmiR-122 secretion to bile. This not only suggests that HDmiR-122 could be useful marker for hepatocyte function, but also that this miRNA might be involved in the conjugation and exocrine function of hepatocytes. Beside inverse directions of release, also the protection of HDmiRs and CDmiRs against RNase activity seems to differ; while HDmiRs in bile supernatant were degraded by ProtK-treatment, CDmiR levels remained detectable, ranging from ~25% (CDmiR-30e) to 100% (CDmiR-222), suggesting that these miRNAs have, at least in part, less conjunctions with protein complexes.

Several studies confirmed that serum levels of HDmiR-122 are sensitive for the detection of liver injury<sup>7, 17, 23</sup>. The finding that HDmiR-122 is also secreted to bile and the strongly correlates with cellular function however is new. The first study reporting on the presence of specific miRNAs in bile, identified miR-9 as a potential biomarker for biliary tract cancer. Moreover, the investigators of this study already verified the presence of HDmiR-122, CDmiR-200c and CDmiR-222 in bile. Despite the RNA hostile environment of human bile, in general, biliary miRNAs were found to be highly stable and protected from degradation<sup>20</sup>. A recent paper by Li et al. reports on biliary miRNAs located in extracellular vesicles as potential diagnostic markers for cholangiocarcinoma<sup>24</sup>. The results of this study suggest that patients suffering from cholangiocarcinoma have higher miRNA contents in bile extracellular vesicles compared to patients with non-malignant biliary obstructions. The authors plea for the analyses of extracellular vesicles rather than whole bile, in order to have a better discrimination between pathologies. Evidence that using whole bile is inferior for designing biomarker assays was however not provided. Furthermore, based on the results from previous studies as well as in the current one, the percentage of miRNAs present in vesicles like exosomes appears to be very low<sup>20</sup>. By only looking at miRNAs in the vesicle fraction, over 90% of the miRNA signal in bile would be overlooked and ignored for analyses.

The studies from Shigehara et al. and Li et al. both report on CDmiR-222 as one of the enriched miRNAs in cholangiocarcinoma<sup>24</sup>, confirming that this miRNA is a potentially relevant marker for various cholangiopathies. Earlier work from our group found CDmiR-222 release to be lower in preservation solutions that were used to flush grafts which later developed ischemic-type biliary lesions after liver transplantation<sup>19</sup>. Based on this observation, we hypothesized that cholangiocytes release their miRNA content to the bile rather than to the blood. The results from the current study further support this hypothesis by the inverse release between HDmiRs and CDmiRs to bile and blood. Furthermore, in esophageal adenocarcinoma cells, bile acid has been shown to increase CDmiR-222 expression in tissue, along with the farnesoid X receptor (FXR) activity<sup>25</sup>. Together with the finding that HDmiR-122 is strongly correlated with bilirubin secretion into bile, this supports a role of bile miRNAs in the enterohepatic circulation, though this hypothesis should be further investigated<sup>26, 27</sup>.

A remarkable finding from our study was the difference in ProtK-treated sensitivity between HDmiRs and CDmiRs. As mentioned before, CDmiR levels were less influenced when protein was degraded, while HDmiR levels drastically decreased. This implies that cholangiocytes release their miRNAs in a different manner to the bile than hepatocytes. Previous studies suggested that miRNAs can be released and bound to lipoproteins as HDL<sup>28, 29</sup>, which perhaps could explain the protein-independence of CDmiRs, though no evidence for this hypothesis is provided in this study. The results from our study not only confirm that HDmiR-122 is a suitable injury marker in serum, but also that its levels in bile are strongly correlated with hepatocyte function. This provides insight in the mechanism and direction of miRNA, which is relevant for the development of diagnostic assays. In particular for cholestatic diseases, the miRNA composition of the bile in different fragments could be helpful in distinguishing different pathologies. But also in the setting of liver transplantation, analysis of miRNAs in bile which is produced during graft machine preservation might be informative on the degree of biliary injury which is currently the second cause of graft failure after liver transplantation<sup>30</sup>. In conclusion, this study demonstrates the polarized release of hepatocyte and cholangiocyte abundant miRNAs into bile and blood during liver injury and impaired cellular function. The difference in HDmiR and CDmiR release is further underlined by the disparity in

protein dependent stability in bile. MicroRNAs in bile are potential markers for assessing liver function and as a marker for biliary injury in liver transplantation and cholestatic diseases.

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## Chapter 4

### **Hepatic cell-to-cell transmission of small silencing RNA can extend the therapeutic reach of RNAi**

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## **Abstract**

RNA interference (RNAi), a sequence-specific gene silencing technology triggered by small interfering RNA (siRNA), represents promising new avenues for treatment of various liver diseases, including hepatitis C virus (HCV) infection. In plants and invertebrate, RNAi provides an important mechanism of cellular defense against viral pathogens and is dependent on the spread of siRNA to neighboring cells. In this study, we investigated whether vector-delivered RNAi can transfer between hepatic cells in vitro and in mice and whether this exchange could extend the therapeutic effect of RNAi against HCV infection. Transmission of RNAi was investigated in culture by assessing silencing of HCV replication and expression of viral entry receptor, CD81, using a human hepatic cell line and primary B lymphocytes transduced with siRNA-expressing vectors. In vivo transmission between hepatic cells was investigated in NOD/SCID mice. Involvement of exosomes was demonstrated by purification, uptake and mass spectrometric analysis. We found that human and mouse liver cells as well as primary human B cells have the ability to exchange small RNAs, including cellular endogenous microRNA and delivered siRNA targeting HCV or CD81. The transmission of RNAi was largely cell-contact independent and partially mediated by exosomes. Evidence of RNAi transmission in vivo was observed in NOD/SCID mice engrafted with human hepatoma cells producing CD81 siRNA, causing suppression of CD81 expression in mouse hepatocytes. In conclusion, both human and mouse hepatic cells exchange small silencing RNAs, partially mediated by shuttling of exosomes. Transmission of siRNA potentially extends the therapeutic reach of RNAi-based therapies against HCV as well as other liver diseases.

## Introduction

The translation of molecular biology research has recently fuelled a rapid progress in the drug development for hepatitis C virus (HCV) infection. The directly acting antivirals, including a range of protease and polymerase inhibitors, are at various stages of clinical development.<sup>1</sup> These compounds have potent antiviral activity but also dramatically potentiate the efficacy of the current standard of care, based on pegylated interferon-alpha (IFN- $\alpha$ ) combined with ribavirin<sup>2-3</sup> However, given the large infected population (approx. 170 million carriers), accumulated non-responders, poor tolerability to interferon or the directly acting antivirals, and special populations (e.g. human immunodeficiency virus (HIV) co-infected patients and transplanted patients), novel antivirals remain urgently required, which ideally should act on distinct mechanisms and be applicable in the current non-responders and special populations with less side effect.

RNA interference (RNAi) is a sequence-specific inhibition of gene expression at posttranscriptional level. It is triggered by small interfering RNA (siRNA), which can be introduced into cells as synthetic siRNA or synthesized from a transgene in the cells as the short-hairpin RNA (shRNA) precursor.<sup>4</sup> By using the cellular gene silencing/microRNA (miRNA) biogenesis machinery, these delivered siRNA induces degradation of mRNA by targeting the complementary sequences.<sup>5</sup> This technology has now emerged as a new avenue to combat viral infections and recent developments in the field of gene therapy have increased the feasibility of clinical applications with dozens of RNAi clinical trails currently underway ([www.ClinicalTrials.gov](http://www.ClinicalTrials.gov)). Both the viral genome and host cellular factors involved in the viral life cycle, such as viral receptor CD81, can be targeted by RNAi and convey protection against infection.<sup>6-7</sup> In the context of treating chronic HCV or preventing recurrence in HCV-positive transplant, a single dose administration with long-lasting therapeutic effects would be ideal. Therefore, integrating lentiviral vector expressing shRNA represents a suitable strategy.<sup>8</sup>

In plants and invertebrates, RNAi naturally provides an important defense mechanism against pathogens. Pathogen-derived siRNA, formed by processing of double stranded RNA (replication) intermediates during infection, spread to

neighboring cells and even propagate throughout the entire organism.<sup>9-11</sup> This transmission of RNAi was shown to be of critical importance for plant and insect resistance against infections.<sup>9, 11-12</sup> RNAi transmissions are also able to direct epigenetic modification in recipient cells in plants and conveys protection against pathogenic challenges.<sup>13-14</sup> Mammalian cells, like mouse or human mast cell lines,<sup>15</sup> the African green monkey kidney fibroblast-like cell line,<sup>16</sup> and human glioma, embryonic kidney, Epstein–Barr virus positive nasopharyngeal carcinoma and B lymphocyte cell lines,<sup>16-19</sup> were shown to be able to transfer cellular or viral encoded miRNAs in culture via secreted exosomes in cell-contact independent manner. In contrast, transmission of endogenous miRNA, viral miRNA or delivered small RNA between B and T cell lines in culture occurs in a cell contact-dependent.<sup>20</sup>

In this study, we investigated transmission of vector-derived RNAi in culture of human hepatic cells and primary human B cells and in mice liver. We found that human and mouse liver cells and primary human B cells have the ability to exchange small RNAs, including small silencing RNA as well as miRNA. We further demonstrated that transmission of gene silencing is cell-cell contact independent and, like reported for miRNA, can be partially mediated by exchange of secreted exosomes. The property of hepatic cells to exchange small silencing RNAs can significantly extends the therapeutic reach of RNAi-based therapy against HCV infection and other liver diseases.

## **Materials & Methods**

### **Cell culture**

Cell monolayers of the human embryonic kidney epithelial cell line 293T and human hepatoma cell line Huh7, Huh6 and HepG2 were maintained in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen–Gibco, Breda, The Netherlands) supplemented with 10% v/v fetal calf serum (Hyclone, Logan, Utah, USA), 100 IU/ml penicillin, 100 ~~U/ml streptomycin~~ <sup>U/ml gentamicin</sup> (Invitrogen–Gibco) on Huh7 cells containing a subgenomic HCV bicistronic replicon (I389/NS3-3V/LucUbiNeo-ET, Huh7-ET) were maintained with 250 µg/ml G418 (Sigma, Zwijndrecht, The Netherlands).

Primary human B cells were expanded from liver transplant donor splenocytes

using a mouse fibroblast cell line stably transfected with human CD40L. The detailed protocol was described in our previous study.<sup>21</sup>

### **Luciferase assay**

Effects on HCV replication were determined based on luciferase activity. 100 mM luciferin potassium salt (Sigma) was added to Huh-7 ET cells and incubate for 30 min at 37°C. Luciferase activity was quantified using a LumiStar Optima luminescence counter (BMG LabTech, Offenburg, Germany).

### **miR-122 reporter assay**

pMiR-Luc reporter vector expressing firefly luciferase gene incorporated with a unique miR-122 target site at its 3'UTR was purchased from Signosis, Inc. (Sunnyvale, CA, USA). 293T cells were transfected with the plasmid and treated with concentrated Huh7-CM or control medium for 24h. Luciferase activity was measured as described above.

### **Lentiviral vectors, conditioned medium (CM) and RNAi transfer experiments**

Lentiviral vectors LV-shCD81 and LV-shNS5b, were constructed and produced as previously reported.<sup>8</sup> LV-shNS5b contains expression cassettes of shRNA targets the viral NS5b region (GACACUGAGACACCAAUUGAC 6367-6388). LV-shCD81 targets human and mouse CD81 mRNA (GGAUGUGAAGCAGUUCUAU). Lentiviral vector expressing miR-122 (LV-miR-122) was constructed by cloning of the precursor sequence of mature miR-122 amplified by PCR from human genomic DNA. A third-generation lentiviral packaging system pND-CAG/GFP/WPRE was used to produce high-titer VSV-G-pseudotyped lentiviral vectors in 293T cells. Vector supernatants were removed 36 and 48 hr post transfection, passed through a 0.45  $\mu$ m filter and concentrated by ultracentrifugation. Concentrated virus stocks were titrated using 293T cells 24h after infection, with transduction efficiency based on the number of GFP-positive cells as determined by flowcytometry (FACSCalibur; BD BioSciences, Mountain View, CA, USA) after 72 hours. Vector concentration was determined in 293T cells based on the number of GFP-positive cells as determined by flowcytometry. CD81 expression was determined using flowcytometry by staining with phycoerythrin (PE) conjugated mouse anti-human

CD81 monoclonal antibody (BD Pharmingen, San Diego, USA). Mouse IgG1 was used as isotype-matched control antibody (BD Pharmingen). Effects of RNAi on CD81 expression were determined by flow cytometry.

Huh7 cells were cultured with normal culture medium. When cultures reaching 60-70% confluence, cells were un-transduced or transduced by LV-shCD81, LV-shNS5b or LV-shCon for 6 hrs, washed three times with PBS and subcultured in normal medium for more than 8 days. Conditioned medium (CM) was collected after the second refreshment of the culture medium. To generate CM specifically containing miR-122, 293T cells were transduced with LV-miR-122 or control lentiviral vector (LV-CTR). After overnight transduction, 293T cells were washed three times and cultured for up to 8 days. The CM from 293T was prepared using fresh culture medium and collected after 48 hrs. All CM were centrifuged at 4 000 rpm for 30 min to remove cell contaminants. Concentrated CM (approx. 25- to 100-fold) was prepared using ultrafiltration units with a 3-kDa cutoff membrane (Millipore, Bedford, MA, USA). Huh7 cells were treated with conditioned medium for 48 hrs at 1:1 dilution.

### **Cell co-culture experiments**

To generate stable shRNA integrated cell lines, naïve Huh7 cells were transduced with the lentiviral vectors and were expanded in culture for at least 8 days before using in experiments. Co-culture experiments were performed for 48 hrs in 96-well culture plates, with 20 000 Huh7-ET HCV replicon cells per well mixed with 20 000, 10 000 or 2 000 control LV-shRNA or LV-shNS5b transduced Huh7 cells. HCV replication was determined by luciferase activity. Co-culture with control (parental) Huh7 cells had no effect on HCV replication/luciferase activity and did not affect Huh7-ET cell proliferation at any condition, as measured by CFSE dilution assays.

Similarly, primary human B cells were also transduced with LV-shRNA or LV-shNS5b vector to generate stable shRNA donor cells. Co-culture experiments were performed by mixing with Huh7-ET cells.

### **RNA transfer experiments in mice**

Immunodeficient NOD/SCID mice (Charles River Laboratories, Wilmington, MA, USA) aged 3-4 weeks were used. The use of animals was approved by the



institutional animal ethics committee at Erasmus Medical Center Rotterdam. Mice were engrafted with  $0.5 \times 10^6$  Huh7-shCD81 (four mice) or Huh7-shCon (seven mice) cells injected intrasplenic. Cell transplantations and surgical procedures were performed under 1.5% isoflurane inhalation anaesthesia and a prophylactic antibiotic was given. Two and half weeks after engraftment, mice were sacrificed and liver tissue obtained for analysis. To demonstrate cell-free transfer of small RNA, NOD/SCID mice were intravenous injected with 200  $\mu$ L of 100-fold concentrated shCD81-CM or shCon-CM every two days for three times (four animals per group). After 6 days, mice livers were procured, dissociated by collagenase digestion<sup>22</sup> and analyzed for CD81 expression by flowcytometry.

### **Exosome purification and electron microscopy imaging**

Exosomes were prepared from the supernatant of Huh7 cells by differential centrifugation. Briefly, supernatant was centrifuged at 3 000 g for 20 min to eliminate cells and at 10 000 g for 30 min to remove cell debris. Exosomes were pelleted by ultracentrifugation (Beckman SW28) at 64,047 g for 110 min followed by a sucrose gradient isolation at 100 000 g (Beckman SWTi60). For uptake experiments, 0.1% Rhodamine C18 solution was added to the sucrose before centrifugation. For electron microscopy, exosomes were visualized by negatively staining using uranyl acetate.

### **Exosome uptake and RNAi transfer**

For visualization of exosome uptake, Huh7 cells were seeded on glass cover slips. Rhodamine labeled exosomes were added to live cells on coverslips in heated-chamber (37°C) and uptake was measured real-time using confocal microscopy (Zeiss LSM510META). To determine the kinetics of exosome uptake, images were taken every minute for 45 minutes. Paraformaldehyde (PFA)-fixed cells served as control to exclude passive transfer of Rhodamine by exosome cell-fusion. In order to specify the subcellular localization of exosomes, nuclear staining using the Hoechst dye were performed. In these experiments, only two time points were measured, 1 and 30 min after adding exosomes. This is to avoid cytotoxicity of Hoechst induction by the laser and decay of the nuclear staining.

Treating Huh7-ET cells with shNS5 containing exosomes for 48h tested RNAi transfer by purified exosomes and viral replication was measured based on luciferase

activity. Similarly, Huh7 cells were treated with shCD81-containing exosomes for 48h and CD81 cell surface expression was quantified by flowcytometry.

### **Mass spectrometric analysis**

Two batches of purified exosomes were subjected to mass spectrometry at the Erasmus MC Proteomics Center. Briefly, 1D SDS-PAGE gel lanes were cut into 2-mm slices using an automatic gel slicer and subjected to in-gel reduction with dithiothreitol, alkylation with iodoacetamide and digestion with trypsin (Promega, sequencing grade), essentially as described by Wilm et al.<sup>23</sup> Nanoflow LC-MS/MS was performed on an 1100 series capillary LC system (Agilent Technologies) coupled to an LTQ-Orbitrap mass spectrometer (Thermo) operating in positive mode and equipped with a nanospray source. Peptide mixtures were trapped on a ReproSil C18 reversed phase column (Dr Maisch GmbH; column dimensions 1.5 cm × 100 µm, packed in-house) at a flow rate of 8 µl/min. Peptide separation was performed on ReproSil C18 reversed phase column (Dr Maisch GmbH; column dimensions 15 cm × 50 µm, packed in-house) using a linear gradient from 0 to 80% B (A = 0.1 % formic acid; B = 80% (v/v) acetonitrile, 0.1 % formic acid) in 70 min and at a constant flow rate of 200 nl/min using a splitter. The column eluent was directly sprayed into the ESI source of the mass spectrometer. Mass spectra were acquired in continuum mode; fragmentation of the peptides was performed in data-dependent mode. Peak lists were automatically created from raw data files using the Mascot Distiller software (version 2.3; MatrixScience). The Mascot search algorithm (version 2.2, MatrixScience) was used for searching against a customized database containing all IPI\_human protein sequences (release 2010\_09). The peptide tolerance was typically set to 10 ppm and the fragment ion tolerance was set to 0.8 Da. A maximum number of two missed cleavages by trypsin were allowed and carbamidomethylated cysteine and oxidized methionine were set as fixed and variable modifications, respectively. The Mascot score cut-off value for a positive protein hit was set to 65. Individual peptide MS/MS spectra with Mascot scores below 40 were checked manually and either interpreted as valid identifications or discarded. Typical contaminants, also present in immunopurifications using beads coated with pre-immune serum or antibodies directed against irrelevant proteins were omitted from the table.

### **RNA isolation and real-time RT-PCR analysis**

Total RNA was extracted using the miRNeasy mini kit according to manufacturer's instructions (Qiagen, Hilden, Germany). Mouse liver tissues were mechanically disrupted and lysed using Trizol (Invitrogen–Gibco). RNA was quantified using a Nanodrop ND-1000 (Wilmington, DE, USA). cDNA was prepared from 1 µg total RNA using a iScript cDNA Synthesis Kit from Bio-Rad (Bio-Rad Laboratories, Stanford, CA, USA). The cDNA of mouse CD81, TBP, CyB, and GAPDH were quantified using real-time PCR (MJ Research Opticon, Hercules, CA, USA) performed with SybrGreen (Sigma-Aldrich) according to manufacturer's instructions. CD81 mRNA levels were normalized to the average level of the three independent reference genes using the ddCT method. TaqMan-based real-time PCR kit for detection of miR-122 was purchased from Applied Biosystems and analysis was performed according to manufacturer's guideline. A customized kit for quantification of small silencing RNA was designed by amplification of the antisense sequence of shCD81 (UAGAACUGCUUCACAUC) using TaqMan-based real-time PCR technique ordered from Applied Biosystems. The assay supposes to preferentially amplify the mature miR-122 or siCD81, but possibly also detect the precursors.

### **Fluorescent immunohistochemistry**

Mouse liver tissue was dissected and cryoprotected in 30% sucrose for generation of frozen sections. Serial 6 µm cryosections were air-dried for 48 h at room temperature followed by a washing step with PBS. Sections were fixed with 50% acetone in PBS for 10 min on ice and blocked in PBS containing 4% fat free milk for 1 h at room temperature. Subsequently, sections were incubated with Alexa Fluor647 labeled anti-mouse CD81 antibody (AbD Serotec, Oxford, UK) at the dilution of 1:100 for 30 min. After three washes, nuclear staining was achieved by incubating with DAPI (Sigma-Aldrich) at the dilution of 1:50 for 5 min. Multiple areas from the mouse liver tissue surrounding nodules of engrafted Huh7 cells were analyzed by confocal microscopy. The Huh7 nodules were distinguished from liver parenchyma based on GFP-positivity and tumor morphology.

## Statistical analysis

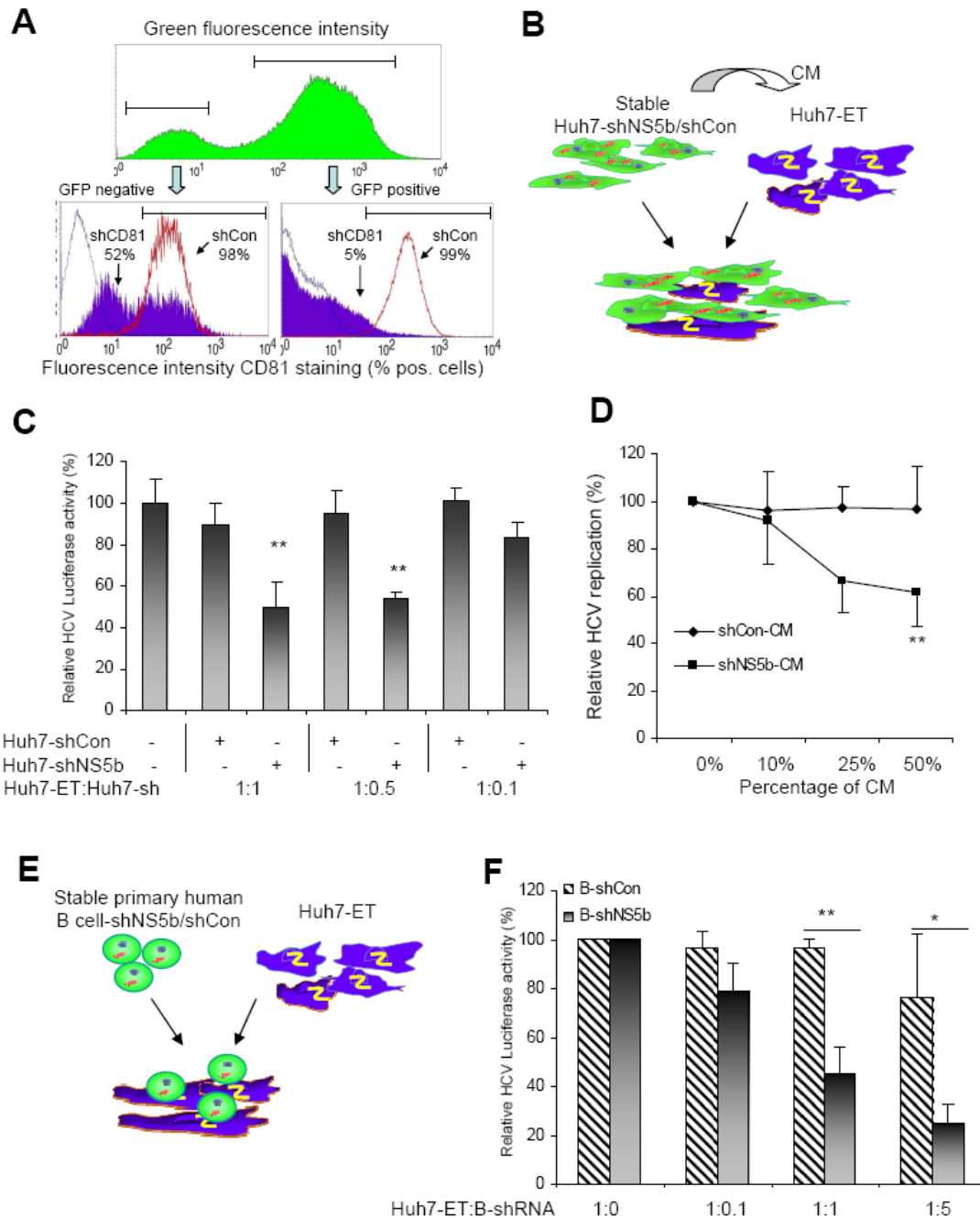
Statistical analysis was performed by using either matched-pair nonparametric test (Wilcoxon signed-rank test) or the non-paired, nonparametric test (Mann–Whitney test) using GraphPad Prism software. P-values less than 0.05 were considered as statistically significant.

## Results

### Transmission of lentiviral vector-delivered RNAi targeting HCV receptor or viral genome

We have constructed a lentiviral vector, LV-shNS5b, which contains both the green fluorescent protein (GFP) reporter gene and a shRNA targeting the HCV NS5b region, which encodes the viral RNA-dependent RNA polymerase. We used a subgenomic HCV replication model, based on a Huh7 hepatoma cell line containing the non-structural sequence of HCV genome with a luciferase reporter gene (Huh7-ET), mimicking viral replication without virus particle production.<sup>24</sup> As reported, LV-shNS5b resulted in a maximum inhibition of HCV replication of  $98\% \pm 0.5$  (mean  $\pm$  standard deviation,  $n = 8$ ,  $P < 0.001$ ) at highest transduction efficiency.<sup>8, 25</sup> However at suboptimal transduction efficiency, the percentage inhibition of viral replication, as measured by luciferase activity, significantly exceeded the percentage of transduced cells, as measured by GFP expression. For instance, with a transduction efficiency of 45% GFP the observed inhibition of HCV replication was 58%, suggesting possible extension of RNAi to non-transduced cells. Similar results were observed with the LV-shCD81, a vector containing GFP and shRNA targeting the HCV receptor CD81. LV-shCD81 profoundly reduced CD81 cell surface expression in transduced Huh7 cells (mean inhibition  $92.9\% \pm 5.9$  SD,  $n=8$ ,  $P < 0.001$ ), but also significantly reduced CD81 expression in, the non-transduced, GFP-negative cells ( $30.1\% \pm 12.9$  inhibition,  $P < 0.001$ ) (Fig. 1A). CD81 reduction was not related to loss of cell viability as dead/permeable cells were excluded from analysis. To ensure that the gene silencing effect on GFP negative cells was not due to insensitivity of GFP detection or silencing of transgenic expression, additional co-culture experiments were performed (Fig. 1B). A significant inhibition of HCV replication was observed when Huh7-ET HCV replicon cells were co-cultured with naïve Huh7 cells stably expressing shNS5b at a 1:1 ratio ( $51\% \pm 12$  SD,  $n=6$ ,  $P < 0.01$ ) as compared to Huh7-shCon co-cultures and

untreated Huh7 cells (Fig. 1C). A similar effect was observed at a lower ratio of 1:0.5 of Huh7-ET and Huh7-shNS5b cells, but lost significance when co-culturing at very low ratios (Fig. 1C). To confirm in primary human cells, B cells were generated from splenocytes and stably transduced with shRNA vectors (Fig. 1E).



**Figure 1.** Evidence for intercellular functional transmission of small silencing RNAs. (A) Silencing of CD81 expression by LV-shCD81 extended to, GFP-negative, non-transduced cells. Huh7 cells were transduced by LV-GFP containing either a CD81 targeting shRNA (LV-shCD81) or a scrambled control shRNA (LV-shCon). Shown in the upper panel is representative histogram of GFP fluorescence intensity. Lower

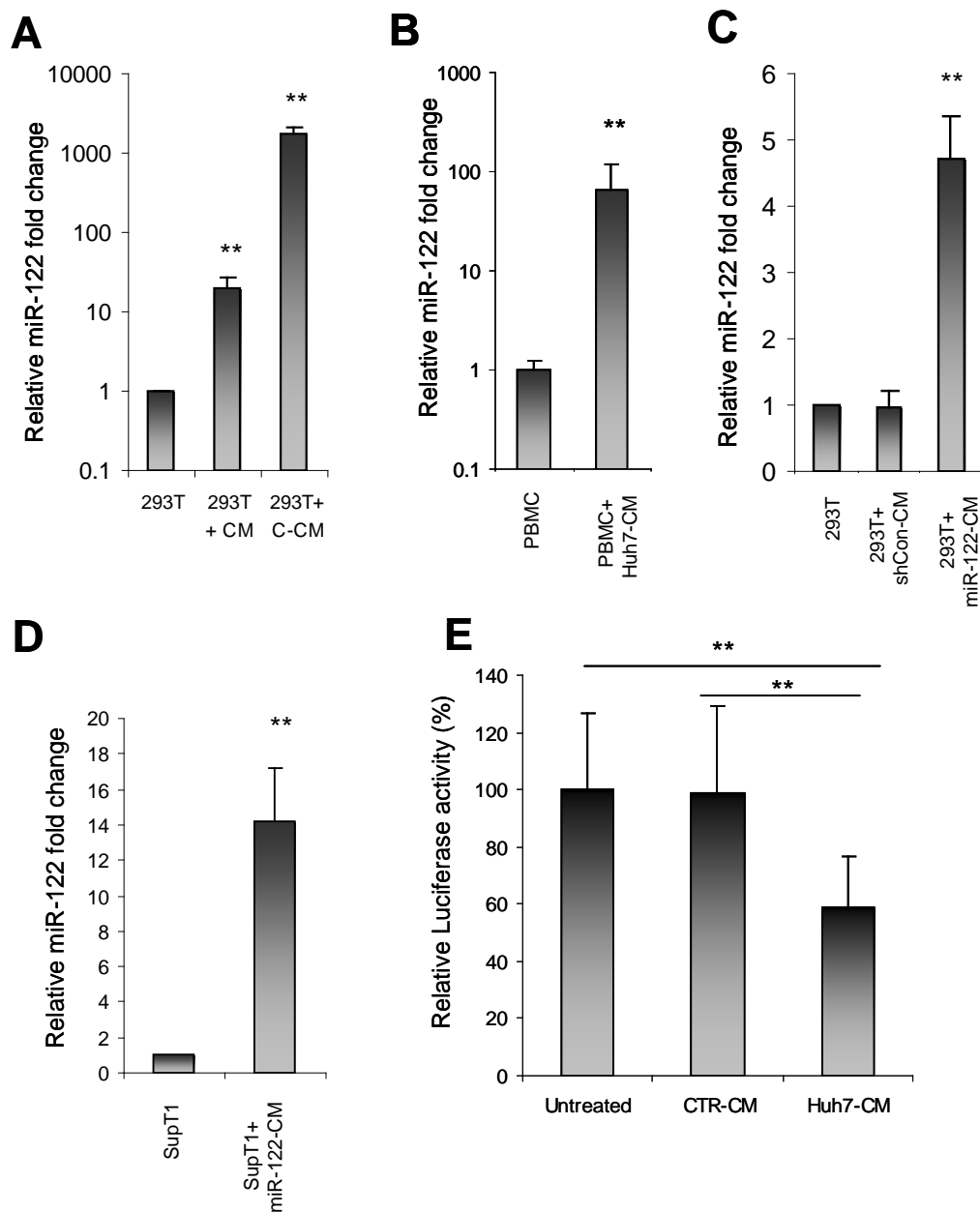
panels show flowcytometric analysis of CD81 staining in gated GFP negative (left panel) and GFP positive (right panel) cells transduced with LV-shCon (Red line) or LV-shCD81 (Purple area). Blue lines show isotype-matched control staining. The percentages of CD81 positive cells are indicated. (B) HCV replicon cells (Huh7-ET) were directly co-cultured with cells stably expressing shNS5b (Huh7-shNS5b) or control shRNA (Huh7-shCon) or treated with conditioned culture medium (CM) of these cells. (C) Significant inhibition of HCV replication was observed at a 1:1 and 1:0.5 ratio of Huh7-ET with Huh7-shNS5b as compared to co-cultures with Huh7-shCon or untreated cells. Shown is the mean  $\pm$  SD of six independent experiments. \*\* $P < 0.01$  (D) Huh7-ET replicon cells treated with shNS5b-CM (at final concentration 50%), but not shCon-CM, showed a significantly reduced HCV replication of  $39\% \pm 12$  ( $n=9$ , \*\* $P < 0.01$ ) compared to untreated controls. (E) Huh7-ET cells were co-cultured with primary human B cells stably expressing shNS5b or shCon. (F) Significant reduction of viral replication was observed when co-cultured with B cells expressing shNS5b at 1:1 ratio ( $n=4$ , \*\* $P < 0.01$ ). Such an effect was also confirmed at 1:5 ratio ( $n=3$ , \* $P < 0.05$ ), although high density of B cells appears to cause some non-specific effects.

Similarly, a robust inhibition of viral replication was observed in replicon cells co-cultured with B cells stably expressing shNS5b at 1:1 ( $n=4$ ,  $P < 0.01$ ) or 1:5 ratio ( $n=3$ ,  $P < 0.05$ ) as compared to B cell-shCon co-cultures (Fig. 1F).

RNAi transmission has been reported as acting via cell contact-dependent<sup>20</sup> or independent<sup>15</sup> fashions depending on the models used, although the exact mechanisms remain largely elusive. Using immunofluorescence microscopy we observed that LV-shCD81-dependent knockdown of CD81 expression in GFP negative cells was not restricted to cells in direct contact with GFP positive cells, but rather a general pattern of CD81 reduction was seen (data not shown). To further investigate whether RNAi can be transmitted in the absence of direct cell-cell contact, conditioned medium (CM) was prepared from stably transduced Huh7 cells expressing shCon, shCD81 or shNS5b (Fig. 1B). As shown in Figure 1D, exposure of Huh7-ET cells to shNS5b-CM (at final concentration of 50%) specifically reduced HCV replication by  $39\% \pm 12$  SD ( $n=9$ ,  $P < 0.01$ ), without transfer of GFP positivity. Treatment with shCD81-CM also significantly reduced CD81 expression in Huh7 cells ( $23.5\% \pm 5.1$  inhibition,  $n=7$ ,  $P < 0.01$ ). These results suggest that transmission of RNAi is cell-contact independent but rather seem to involve the uptake of released silencing RNA components.

### **Functional transmission of liver abundant miRNA**

We further investigated whether such a cell-contact independent manner of small RNA transmission also exist for endogenous miRNA. Huh7 cells highly express miR-122, a liver abundant miRNA that has been reported to be a crucial positive regulator of HCV replication and translation.<sup>26</sup> We found that cell-free conditioned medium of Huh7 cells (Huh7-CM) contained high levels of miR-122 (data not shown). Concentration of Huh7-CM (Huh7-C-CM) using ultrafiltration resulted in a 10-fold increase of miR-122 levels. The miR-122 level of Huh7 cells is more than 200-fold higher than another hepatoma cell line HepG2 and over 50,000-fold higher than the embryonic kidney epithelial cell line 293T (data not shown). Treatment of HepG2 cells with Huh7-CM or Huh7-C-CM significantly increased intracellular miR-122 levels by 3- to 4-fold ( $p < 0.01$ ), indicating uptake of miR-122 from the medium. An even more pronounced miRNA uptake was observed in 293T cells, leading to about a 20- or 1750-fold elevation of cellular miR-122 levels after exposure to Huh7-CM and Huh7-C-CM, respectively (Fig. 2A). The miRNA transfer was also observed in freshly isolated human peripheral blood mononuclear cells incubation with Huh7-CM, resulted in approx. 100-fold increase in cellular miR-122 levels (Fig. 2B). To more specifically demonstrate the transfer of miRNA and to exclude possible induction of miRNA gene expression by other factors present in conditioned medium, we generated a lentiviral vector specifically expressing the precursor of miR-122 (LV-miR-122). Conditioned medium were produced from LV-miR-122 or control vector (LV-shCon) transduced 293T cells. 293T cells naturally expressed very low levels of miR-122 and transduction with LV-miR-122 (~5% transduction efficiency) resulted in approx. 10-fold increase of cellular miR-122 levels. As shown in Figure 2C, miR-122-CM but not shCon-CM specifically increased the cellular miR-122 levels in 293T cells by approx. 5-fold. Similarly, incubation with miR-122-CM increased the cellular miR-122 levels of the T cell line, SupT1 cells, by approx. 15-fold (Fig. 2D). To evaluate the functional consequence of miRNA transmission, a reporter plasmid expressing luciferase gene coupled with miR-122 complementary sequence was used to transfect 293T cells. Treatment of concentrated Huh7-CM significantly reduced miR-122 associated luciferase activity compared with either untreated or control medium treated group ( $P < 0.01$ ) (Fig. 2E), suggesting functional regulation of target reporter gene expression by transferred miRNA.

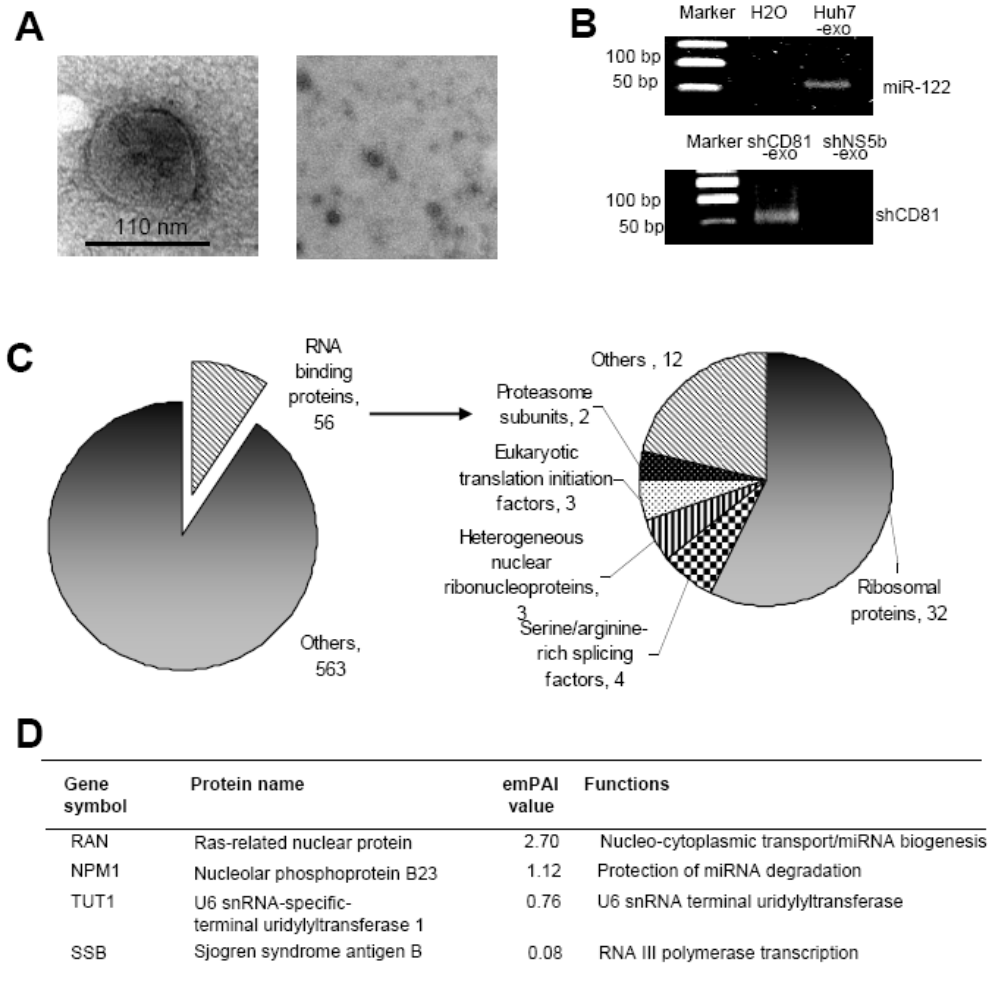


**Figure 2.** Evidence for intercellular functional transmission of liver abundant miRNA. (A) Uptake of miR-122 by 293T cells after exposure to Huh7-CM or Huh7-C-CM. (B) PBMCs from healthy controls, showing about 100-fold increase in cellular miR-122 level after 6 hours of incubation with Huh7-CM. (C) To confirm miRNA transfer and rule out the induction of miRNA gene expression by other factors present in CM, we generated CM of 293T cells either transduced LV-miR-122 or LV-shCon. Treatment of naïve 293T cells with miR-122-CM but not shCon-CM increased the cellular miR-122 level by approx. 5-fold. (D) Incubation with miR-122-CM resulted in about 15-fold increase of cellular miR-122 levels in the T cell line, SupT1 cells. Data shown above is the mean  $\pm$  SD of three or four independent experiments. (E) Treatment of concentrated Huh7-CM resulted in significant reduction of miR-122 related luciferase activity in 293T cells transfected with miR-122 reporter plasmid, compared with control medium treated or untreated group. Shown is mean  $\pm$  SD of three independent experiments (n=11 replicates in total). \*\*P<0.01.

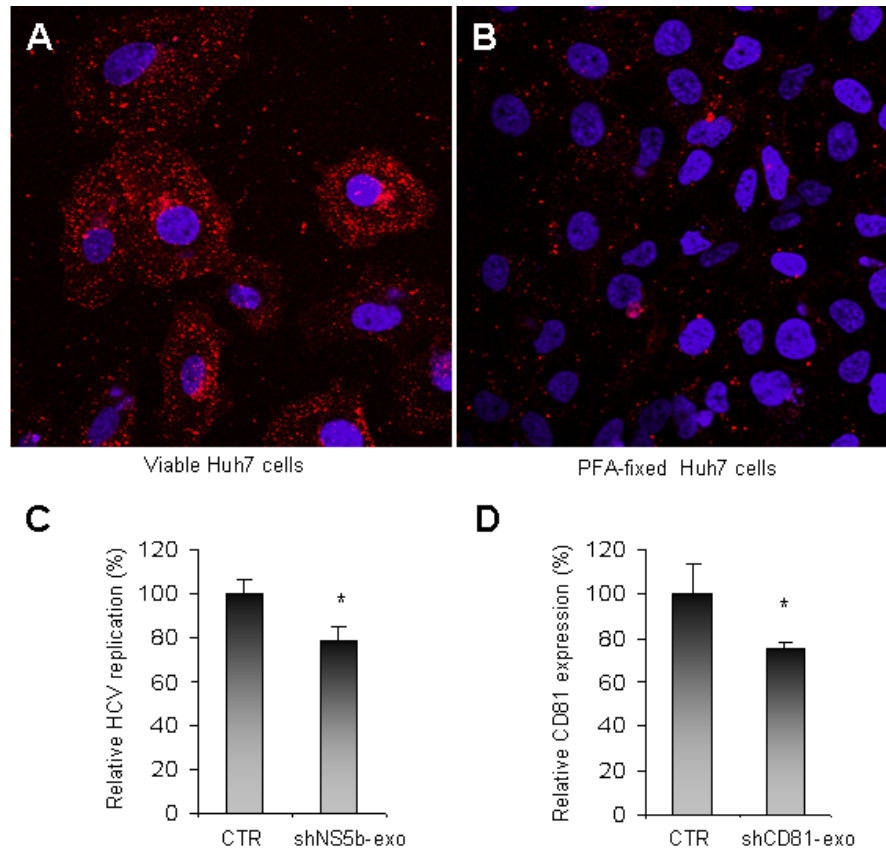


### **Secreted exosomes contain small RNAs and RNA binding proteins**

Previous studies have shown that cellular miRNA can be released from cells by secretion of microvesicles/exosomes.<sup>15, 17</sup> To further investigate whether exosomes are involved in the transfer of small silencing RNA, we purified secreted exosomes from Huh7-CM or CM of stably transduced Huh7 cells expressing shCon, shCD81 or shNS5b using density gradient ultracentrifugation. Figure 3A shows an electronmicrograph of a purified exosome. RT-PCR analysis of shCD81-CM exosomes showed the presence of both miRNA (miR-122) and shCD81 (Fig. 3B). Huh7-CM derived exosomes were analyzed by mass spectrometry to characterize the protein content. From two independent preparations of exosomes, over 600 common proteins were detected, including the established exosome markers Tsg101, CD63, CD9, Alix, Flotillin and RAB5.<sup>27</sup> Importantly, 56 distinct RNA binding proteins were present, including ribosomal proteins, serine/arginine-rich splicing factors, heterogeneous nuclear ribonucleoproteins, eukaryotic translation initiation factors and proteasome subunits (Fig. 3C). The presence of RNA binding proteins is consistent with a previous study, showing exosomes derived of primary rat hepatocytes are highly enriched for nucleotide binding proteins.<sup>28</sup> Relevant to the content of miRNA and siRNA, we identified four proteins in exosomes which are known to be important for the miRNA pathway and which are potential binding partners of the small silencing RNA cargo in hepatic exosomes (Fig. 3D). In particular interesting is the nucleolar phosphoprotein B23, NPM1, which has been recently shown to specifically protect the degradation of miRNAs.<sup>29</sup> RAN, the Ras-related nuclear protein, is known for its involvement in nucleo-cytoplasmic transport. Interestingly, recent studies have shown that Exportin-5 mediated nuclear export of pre-miRNA or shRNA acts in a Ran-GTP dependent manner.<sup>30-32</sup> Further studies will be required to identify the exact molecular machinery, which is involved in the sorting and packaging of small silencing RNA into exosomes.



**Figure 3.** Exosomes contain small RNAs and RNA binding proteins. Secreted exosomes were purified from conditioned medium (CM) from Huh7 cells using density gradient ultracentrifugation. (A) Electronmicrograph imaging shows presence of exosomes in purified fraction. (B) RT-PCR analysis of purified exosomes from shCD81-CM showed the presence of both miRNA and shCD81. Markers indicate the anticipated amplicon size for miR-122 and shCD81 No-template (H2O) and purified exosomes from shNS5b-CM served as negative controls. This assay supposes to preferentially amplify the mature miR-122 or siCD81, but possibly also detect the precursors. (C) Mass spectrometry was performed to analyze the protein content of two independent batches of Huh7-CM derived exosomes. Using a Mascot cutoff for specificity (Mascot >40), in total over 600 common proteins were identified including many exosome-specific proteins. There are 56 proteins are known RNA binding proteins, including 32 ribosomal proteins. (D) Of the RNA binding proteins, four are known to be involved in the miRNA pathway and are potentially involved in the selection, sorting and packaging of small silencing RNA in hepatic exosomes. Shown are protein name, main function and relative abundance in exosomes indicated by the amPAI value (mean of two samples).



**Figure 4.** Exosome-mediate functional transmission of small silencing RNAs. (A) Dynamic visualization of rhodamine-labeled exosome uptake by live Huh7 cells shows intracellular accumulation in viable cells but not (B) in PFA-fixed cells. Red staining represents exosomes and blue staining marks the nucleus. Shown is one of three independent experiments. 800 x magnification. (C) Treatment of Huh7-ET replicon cells with purified exosomes derived from shNS5b-CM significantly reduced viral replication by  $21.6\% \pm 6.4$ . (D) Treatment of normal Huh7 cells with purified exosomes derived from shCD81-CM resulted in a significant down regulation of CD81 cell surface expression by  $24.5\% \pm 3.1$ . Shown is the mean inhibition  $\pm$  SD of four independent experiments. \* $P < 0.05$ .

### Transmission of gene silencing is partially mediated by exosomes

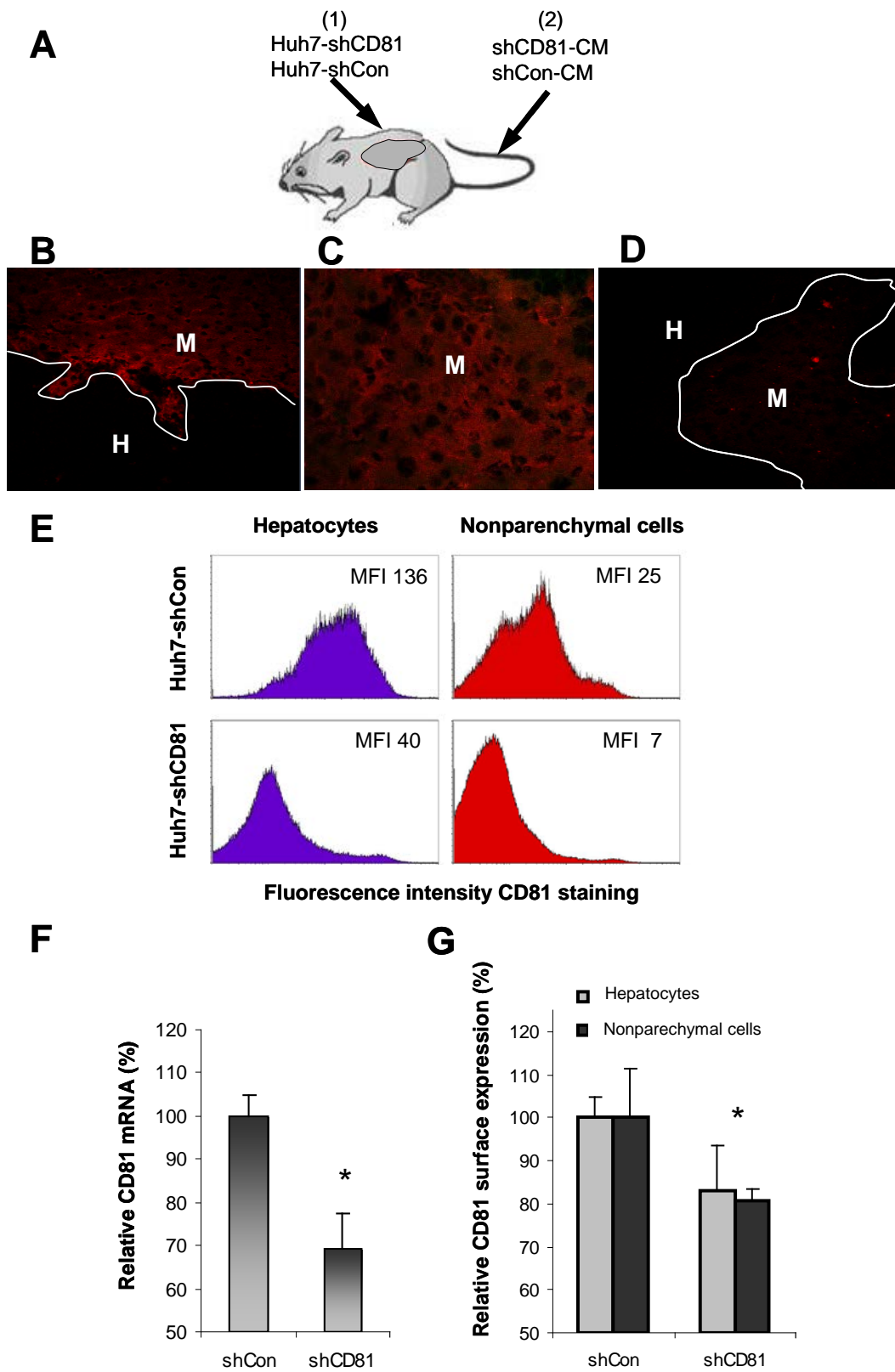
To investigate the involvement of exosomes in small RNA transfer, real-time live cell imaging was performed with Huh7 cells exposed to fluorescent-labeled exosomes using confocal microscopy. Real-time analysis showed that exosome uptake is rapid and occurs within 45 minute (data not shown). As shown in Figure 4A, ingested exosomes predominantly accumulate in the cytoplasm or other intracellular compartments but not in the nucleus. Exosome uptake was observed in most of the living cells (>80%), but hardly uptake was observed in PFA-fixed cells (Fig. 4B), confirming that uptake is an active process. Treatment of HCV replicon cells with

purified exosomes derived from shNS5b-CM resulted in a significant reduction of viral replication (mean inhibition  $21.6\% \pm 6.4$  SD,  $n=4$ ,  $P<0.01$ ) (Fig. 4C). Similarly, treatment of Huh7 cells with exosomes derived from shCD81-CM resulted in a significant down regulation of CD81 cell surface expression ( $24.5\% \pm 3.1$  reduction,  $n=4$ ,  $P<0.05$ ) (Fig. 4D). These findings confirm that secreted exosomes contain small RNAs, including miRNA and small silencing RNA, can mediate transmission of functional gene silencing. In addition, recent studies have suggested the co-existence of exosome dependent and independent pathways of small RNA release and transfer.<sup>20, 32, 33</sup>

### **Transmission of gene silencing in mouse liver**

To explore the evidence for small RNA exchange *in vivo*, we engrafted Huh7-shCD81 cells, stably expressing shRNA targeting mouse CD81, or Huh7-shCon cells, containing irrelevant shRNA, in the liver of immunodeficient NOD/SCID mice by intrasplenic injection (Fig. 5A). Human hepatomas in the mouse liver tissue were visualized based on green fluorescent protein (GFP) positivity. Mouse liver tissue surrounding nodules of Huh7-shCon cells showed comparable CD81 expression (Fig. 5B) as untreated mice (Fig. 5C). Contrary, liver tissue adjacent to Huh7-shCD81 nodules showed a marked reduction in CD81 expression (Fig. 5D). Flowcytometric quantification of mouse-specific CD81 expression on dissociated liver cells showed an average reduction of 71.3% on both hepatocytes and non-parenchymal cells in Huh7-shCD81 versus Huh7-shCon engrafted mice ( $P=0.002$ , Fig. 5E). This finding suggests transfer of RNAi from the human cells to the primary mouse cells *in vivo*. In order to determine whether RNAi transfer *in vivo* is cell-contact dependent, NOD/SCID mice were intravenously treated with shCD81-C-CM or shCon-C-CM (Fig. 5A). At day six, a significant reduction of CD81 mRNA level was observed in mouse livers by shCD81-CM treatment (mean reduction of  $31.6\% \pm 15.6$ ,  $n=4$ ) as compared to the shCon-CM controls ( $n=4$ ,  $P<0.05$ ) (Fig. 5F). Consistent with the gene expression levels, an approximate 20% reduction of CD81 cell surface expression was observed in both hepatocytes and non-parenchymal cell populations by flowcytometry (Fig. 5G). The gene silencing by shCD81-CM was comparable to that of liposome or nanoparticle delivery of siRNA observed in a transgenic mouse model of HCV or in human tumors.<sup>35-36</sup> Despite earlier reports of hepatotoxicity by

adeno-associated vector mediated RNAi,<sup>37</sup> we observed no evidence of liver injury by histology or serum transaminases as a result of shCD81-CM treatment.



**Figure 5.** *In vivo* evidence for transmission of RNAi in mice. (A) Schematic representation of *in vivo* experiments with immunodeficient mice. (1) NOD/SCID were either engrafted with control Huh7 cells expressing irrelevant shRNA targeting NS5b (Huh7-shCon) or Huh7 cells expressing shRNA targeting murine CD81 mRNA (Huh7-shCD81) in the liver. (2) Alternatively, NOD/SCID we injected intravenously with 200  $\mu$ l of 100-fold concentrated cell-free conditioned medium (CM) from Huh7-shCD81 or Huh7-shCon cells, three times with 48 hours intervals. All groups had four animals. Confocal immunofluorescence staining using an anti-mouse CD81-specific antibody showed normal CD81 expression (Red fluorescence) in the mouse liver tissue (M) surrounding nodules of Huh7-shCon cells (H) (B), comparable to expression in untreated mice (C). (D) Contrary, CD81 expression in mouse liver tissue (M) surrounding Huh7-shCD81 cells (H) was markedly reduced. (E) Flowcytometric quantification of dissociated liver cells showed a significant reduction of CD81 expression in mouse hepatocytes and non-parenchymal cells (average reduction of 71.3%,  $P=0.002$ ) in mice engrafted with Huh7-shCD81 (bottom panels) as compared to mice engrafted with Huh7-shCon (top panels). GFP positive human cells were gated out and a mouse specific anti-CD81 antibody was used to specifically determine mouse CD81 expression. Number indicated the average geometric mean fluorescence intensity (F) Analysis of liver mRNA showed a significant knockdown of CD81 expression in mice treated with shCD81-CM as compared to shCon-CM treatment. (G) Knockdown of CD81 surface expression was confirmed by flowcytometry in both hepatocyte and non-parenchymal cell populations (Approx. 20%). \*  $P<0.05$ .

## Discussion

From the discovery of RNAi in 1998<sup>38</sup> to the approval of RNAi therapeutics or RNAi-based gene therapy by FDA, the face of its application has been dramatically changing. Much attention has been received for developing antiviral RNAi against such as HIV<sup>39</sup>, HBV<sup>40</sup> or HCV<sup>6</sup> infection. If RNAi therapies are to be utilized as an effective treatment or prevention of HCV infection, long-term, stable siRNA expression needs to be achieved. Raw synthetic siRNA or plasmid-encoded shRNA transfections elicit only short-term silencing, whereas viral vectors that encode for shRNA can potentially induce long-term and continuous gene-silencing.<sup>6</sup> Adeno-associated viral (AAV) vectors are currently considered the prime candidate for clinical gene therapy applications, including for the treatment of various liver diseases. Biotech companies, such as Tacere therapeutics, have pioneered the development of an AAV-based anti-HCV RNAi regimen, termed “TT-033” (<http://www.tacerebio.com>). However, AAV-mediated expression of shRNA was shown to evoke liver toxicity in mice ultimately causing death.<sup>37</sup> It was suggested that the saturation of endogenous miRNA processing machinery by overexpressed shRNA is the potential cause,<sup>41</sup> but the exact mechanism remains unclear. Lentiviral vector

represents another promising candidate for clinical RNAi delivery. Although certain lentiviral RNAi systems, such as some commercial RNAi libraries, express high levels of shRNA and cause disturbance of cellular miRNA machinery, no significant cell toxicity was observed.<sup>42</sup> The lethal toxicity observed by Grimm et al.<sup>37</sup> could be caused by the combination of AAV vector and overexpressed shRNA. Of note, the lentiviral RNAi vectors used in this study express moderate levels of shRNA without clear effect on miRNA pathway.<sup>42</sup> To overcome the potential toxicity and off-target issues, liver-specific promoters<sup>43</sup> or miRNA-based RNAi constructs<sup>44</sup> have been used to generate safer vectors.

Although studies have demonstrated the feasibility of combating HIV infection via ex vivo delivery of lentiviral RNAi<sup>45</sup>, it remains challenge to produce sufficient vectors targeting the entire liver organ. Virtually for any type of vectors, it is not possible to achieve 100% transduction efficacy in patients. The phenomenon we described in the current study that gene silencing could transfer to neighboring non-transduced cells could indeed potentially overcome the issue of suboptimal vector transduction to certain extent. Whether it would be sufficient to silence the virus in the non-transduced cells solely via the RNAi transmission route remains questionable. Like HIV<sup>46</sup>, HCV is prone to develop resistant mutants, if the antiviral potency is suboptimal. Vector simultaneous delivery of multiple shRNAs targeting different regions of the virus or combination of targeting host factors could be one solution to prevent mutagenesis<sup>47</sup>, since the non-transduced cells could receive multiple antiviral shRNAs even though the levels are not so abundant. Like other new antivirals,<sup>2-3</sup> combining interferon is likely required for RNAi-based therapy to achieve ultimate success in chronically infected HCV patients.<sup>48</sup>

The mechanism of RNAi transmission in plants and invertebrates has been proposed via direct cell-to-cell contact or systemic spreading, although the exact mechanism remains largely elusive. Rechavi et al. has reported transmission of small RNA between B and T cell lines in culture occurring in a cell contact-dependent,<sup>20</sup> whereas many others<sup>15-19</sup> described the secretory transmission pathway involving exosomes in different mammalian cell culture systems. In this study, we also observed the release and uptake of small RNA-packed exosomes by hepato-like cells. We further performed mass spectrometric analysis to characterize the protein content of these exosomes. Along with the previous studies charactering exosomes derived

from monocytes<sup>49-50</sup> or hepatocytes<sup>28</sup> there appears to be some cell type specificity. For instance, AGO2, a protein involved in the RNAi machinery, is detectable in monocytes<sup>49-50</sup> but not hepatocytes-derived<sup>28</sup> exosomes. The differential enrichment of nucleotide and nucleic acid binding proteins was observed between Huh7 and primary hepatocytes-derived exosomes<sup>28</sup>. Interestingly, we found several proteins present in our exosomes that potentially contribute to the functional transmission of small RNAs. RAN, the known nucleo-cytoplasmic transport, was demonstrated to be involved in Exportin-5 mediated nuclear export of pre-miRNA or shRNA.<sup>30-32</sup> The co-presence of NPM1 that can specifically protect the degradation of miRNAs<sup>29</sup> and TUTase that can potentially edit miRNAs or shRNAs<sup>51</sup> suggests that the process of degradation and modification of small RNAs can be potentially regulated within the exosomes. Conceivably, both the protein and RNA content deters the function of transferred exosomes.

In this study, a comprehensive *in vitro* and *in vivo* demonstration of RNAi transfer was achieved by using shRNA donor cells, conditioned medium and purified exosomes. We assumed that exosomes only partially mediated the transmission of gene silencing and the other part would be contributed by the secreted small RNAs independent of exosomes. Consistently, recent study showed that substantial amount of extracellular miRNAs are associated with Argonautes but not with exosomes.<sup>33</sup> Further studies will be required to identify the exact molecular machinery which regulates the release and uptake functional small RNAs.

In summary, this study provided *in vitro* and *in vivo* evidence that small RNA could be exchanged between hepatic cells and that this property extended RNAi-mediated gene silencing against HCV receptor or viral genome. Exchange of small RNAs, in our models, was independent of direct cell-to-cell contact and appeared to be mediated by the secretory pathway partially involving exosomes. Cells stably expressing shRNA, like stem cells, may represent an effective way for the therapeutic delivery of RNAi *in vivo*. These findings might bear relevance for clinical application of RNAi-based therapy in the treatment of chronic hepatitis C as well as metabolic and immunomediated liver diseases.<sup>52</sup>



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## Chapter 5

### **Exosome-mediated transmission of hepatitis C virus between Huh7.5 cells**

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## Abstract

Recent evidence indicates a role for small membrane vesicles, including exosomes, as vehicles for intercellular communication. Exosomes secreted by most cell types can mediate transfer of proteins, mRNAs and microRNAs but their role in transmission of infectious agents is less established. Recent studies showed that hepatocyte-derived exosomes containing hepatitis C virus (HCV) RNA can activate innate immune cells, but the role of exosomes in transmission of HCV between hepatocytes remains unknown. In this study, we investigated whether exosomes transfer HCV in the presence of neutralizing antibodies (nAB). Purified exosomes isolated from HCV-infected Huh7.5.1 cells were shown to contain full length viral RNA, viral protein and particles as determined by RT-PCR, mass spectrometry and transmission electron microscopy. Exosomes from HCV infected cells were capable of transmitting infection to naive Huh7.5.1 cells and establishing a productive infection. Even with subgenomic replicons, lacking structural viral proteins, exosome-mediated transmission of HCV RNA was observed. Treatment with patient-derived IgGs showed a variable degree of neutralization of exosome-mediated infection compared to free virus. In conclusion, this study showed that hepatic exosomes can transmit productive HCV infection *in vitro* and are partially resistant to antibody neutralization. This sheds new light on nAbs resistant HCV transmission by exosomes as a potential immune evasion mechanism.

## Introduction

Most tissue and cell types produce and release 'exosomes', a distinct population of microvesicles ranging from about 30 to 150 nm in size. Exosomes are formed in the endocytic compartment of multivesicular bodies<sup>1</sup> and are secreted in various body fluids under normal and pathological conditions<sup>2, 3</sup>. Extensive studies have now implicated exosomes in many biological processes like tissue injury and immune responses by transfer of antigens, antigen presentation<sup>2, 4</sup> and the shuttling of proteins, mRNAs and microRNAs (miRNA) between cells<sup>5</sup>. As such, it has been postulated that exosomes play a crucial role in cell communication and transfer of genetic information between cells<sup>5</sup>.

The role of exosomes and other secretory vesicles in the transfer of pathogen-derived antigens and virulence factors is emerging<sup>6, 7</sup>. Whether release of vesicles from infected cells contributes to immune control and clearance of infection by the host is still not clear. For example, the HIV Gag protein recruits the outward vesicle budding machinery of exosomes to form free virions<sup>8</sup>. Recently, it has been shown that exosomes released from HIV infected cells contain Nef, which induces apoptosis of uninfected cells<sup>9</sup>. Epstein-Barr Virus-infected B cells also secrete exosomes that contain virally encoded miRNA<sup>10</sup>. This study further demonstrates the delivery of naturally occurring functional genetic elements to neighboring cells via exosomes, indicating that viral particles or molecules associated with viral infection can be transmitted to adjacent uninfected cells via exosomes and become functional. More recently, the hepatitis A virus has shown to be able to escape humoral immunity by cloaking in cellular membranes upon release from host cells. These virus-containing microvesicles, resembling exosomes, were shown to protect virions from antibody-mediated neutralization.<sup>11</sup>

Hepatitis C virus (HCV) infection, known to be one of the leading liver diseases, has been shown to have multiple routes of transmission. Apart from the classical transmission by free viral particles, also an antibody resistant cell-to-cell transmission route has been described<sup>12</sup>. Indeed, HCV is known to evade humoral immune responses as indicated by a lack of resistance to HCV re-infection in intravenous drug users<sup>13</sup>, HCV re-infection during liver transplantation<sup>14</sup>, and ongoing difficulty to develop effective vaccines. The role of exosomes in HCV infection is still largely unknown. One earlier paper reported the presence of viral

RNA in exosomes isolated from plasma of HCV-infected patients<sup>15</sup> but did not show exosome-mediated transmission of infection. More recent studies suggest that HCV virus assembly and release in hepatocytes maybe linked to the exosome secretory pathway<sup>16</sup> and that hepatocyte-derived exosomes can transfer viral RNA to plasmacytoid dendritic cells triggering their activation and interferon- $\alpha$  production<sup>17</sup>. However, the role of exosomes in the cell-to-cell transmission route of HCV between hepatocytes has not been demonstrated. The aim of our study was to investigate transmission of HCV infection by hepatocyte-derived exosomes in the presence of neutralizing antibodies (nAbs) *in vitro* that could explain the ineffectiveness of prophylactic nAbs and agents targeting the entry of HCV into a cell. We further observe that this route of infection can generate productive viral infection.

## **Materials and Methods**

### **Cell Culture and Isolation of Exosomes**

The following human hepatoma cell lines were used: Huh7.5.1 and Huh7-ET, containing a HCV subgenomic replicon linked to a luciferase reporter gene. Cells were cultured in Dulbecco's Eagle's medium (Invitrogen-Gibco, Breda, the Netherlands) supplemented with 10% FCS (Hyclone, Logan, UT), 100 IU/mL penicillin, 100 mg/mL streptomycin, and 2 mM L-glutamine (Invitrogen-Gibco) to 80 % confluency, as previously described<sup>18</sup> Huh7.5.1 cells were primarily infected with HCV strain Jc1 genotype 2a or cell culture derived HCV Luc-Jc1 and medium was refreshed after 24 hours after inoculation. Prior to exosome-isolation, Huh 7-ET or infected Huh7.5.1 cells were cultured for 48 hours. Exosomes were isolated as described before<sup>19</sup>. In brief, cell culture supernatants were subjected to successive centrifugations of 3042 X g (20 min), and 10,000 X g (30 min). Exosomes were then pelleted at 64,000 X g for 110 min using an SW28 rotor (Beckman Coulter Instruments, Fullerton, CA). Exosome pellets were resuspended in 0.32 M sucrose and centrifuged at 100,000 X g for 1 hour. (SW60Ti rotor, Beckman Coulter Instruments). The exosome pellet was then resuspended in PBS.

### **Electron Microscopy of Isolated Exosomes**

Exosomes from Huh7.5.1 and Huh7.5.1 Jc1 obtained after ultracentrifugation of cell culture supernatants were resuspended in 10 $\mu$ l of PBS and spotted onto



Formvarcoated grids (200 mesh). Adsorbed exosomes were fixed in 2 % paraformaldehyde for 5 min at room temperature. After fixation the exosomes were either directly negatively stained using uranyl acetate or immunolabeled with antibody C1 (The Scripps Research Institute, La Jolla ) against E2 protein of Hepatitis C virus. Antigen-antibody complexes were visualized with protein A conjugated with 6-nm colloidal gold particles (1:20 dilution; Aurion, Wageningen. The Netherlands). Omitting the primary antibody tested the specificity of the labelling procedure. Grids were examined by a Philips CM100 electron microscope (EM) at 80 kV.

### **Mass Spectrometry data analysis**

1D SDS-PAGE gel lanes were cut into 2-mm slices using an automatic gel slicer and subjected to in-gel reduction with dithiothreitol, alkylation with iodoacetamide and digestion with trypsin (Promega, sequencing grade), essentially as described by Wilm *et al.*<sup>20</sup>, Nanoflow LC-MS/MS was performed on an 1100 series capillary LC system (Agilent Technologies) coupled to an LTQ-Orbitrap mass spectrometer (Thermo) operating in positive mode and equipped with a nanospray source. Peptide mixtures were trapped on a ReproSil C18 reversed phase column (Dr Maisch GmbH; column dimensions 1.5 cm × 100 µm, packed in-house) at a flow rate of 8 µl/min. Peptide separation was performed on ReproSil C18 reversed phase column (Dr Maisch GmbH; column dimensions 15 cm × 50 µm, packed in-house) using a linear gradient from 0 to 80% B (A = 0.1 % formic acid; B = 80% (v/v) acetonitrile, 0.1 % formic acid) in 70 min and at a constant flow rate of 200 nl/min using a splitter. The column eluent was directly sprayed into the ESI source of the mass spectrometer. Mass spectra were acquired in continuum mode; fragmentation of the peptides was performed in data-dependent mode. Peak lists were automatically created from raw data files using the Mascot Distiller software (version 2.3; MatrixScience). The Mascot search algorithm (version 2.2, MatrixScience) was used for searching against a customized database containing all IPI\_human protein sequences (release 2010\_09) plus all Hepatitis C virus protein sequences from Uniprot (release 2010\_09). The peptide tolerance was typically set to 10 ppm and the fragment ion tolerance was set to 0.8 Da. A maximum number of 2 missed cleavages by trypsin were allowed and carbamidomethylated cysteine and oxidized methionine were set as fixed and variable modifications, respectively. The Mascot score cut-off value for a positive protein hit

was set to 65. Individual peptide MS/MS spectra with Mascot scores below 40 were checked manually and either interpreted as valid identifications or discarded. Typical contaminants, also present in immunopurifications using beads coated with pre-immune serum or antibodies directed against irrelevant proteins were omitted from the table.

### **Real-time Confocal Imaging**

For imaging the cellular uptake of labeled exosomes, Huh7.5.1 cells were seeded on 24-mm-diameter coverslips. Imaging was performed using a confocal laser-scanning microscope (LSM510; Carl Zeiss MicroImaging, Inc.), equipped with a Plan-Neofluar 40X/1.3 NA oil objective (Carl Zeiss MicroImaging, Inc.) and a heated stage. A 543 nm HeNe laser was used for the excitation of rhodamine. At the same time a transmitted light image was made. The cells were kept at 37°C and scanned continuously (zoom 2x, averaging 8x) for 30 minutes.

### **Transmission of infection via exosomes**

Naive Huh7.5.1 cells were infected with exosomes isolated from Huh7.5.1 cells infected with HCVcc strain Jc1 genotype 2a or from Huh7-ET. Conditioned medium (CM) containing free virus from the same cell culture was used as positive control for Jc1 virus. The exosomes and free virus batches were normalized based on the HCV viral genome content as determined by quantitative RT-PCR. 24 hours after addition of samples to naive cells the medium was refreshed. The cells were analysed at day 5-post infection by quantitative RT-PCR and at day 2 for immunohistochemistry. Huh7.5.1 cells exposed to Huh7-ET exosomes were analysed at day 2 by quantitative RT-PCR.

### **Antibody-mediated neutralization of HCV**

Naive Huh7.5.1 cells were infected with exosomes isolated from Huh7.5.1 cells infected with HCV strain Jc1 and HCV Luc-Jc1. Conditioned medium (CM) containing free virus from the same cell culture was used as positive control. Naïve Huh7.5.1 cells were also exposed to Huh7-ET exosomes. Nine different purified IgGs (100µg/ml) from chronically infected HCV patient serum (approval from the Strasbourg University Hospital IRB ClinicalTrial.gov Identifiers NCT00638144)

were obtained. Three purified control IgGs derived from anti-HCV-negative individuals were used as control. Neutralizing antibodies (nAbs) were added to the Jc1/Luc-Jc1 infected exosomes and Jc1/Luc-Jc1 CM one to two hours prior to infection. nAbs were also added to exosomes isolated from Huh7-ET cells. The cells infected with HCV Jc1 were lysed 5 days after infection and quantified for viral infection by real time RT-PCR. For cells infected with HCV Luc-Jc1, luciferase activity was measured 3 days after infection. For cells exposed to Huh7-ET exosomes, cells were lysed 2 days later and quantified for viral RNA by real time RT-PCR.

### **Inhibition of HCV entry receptors CD81, SR-BI, Claudin-1 for Huh7-ET exosome transmission.**

To study the involvement of HCV entry receptors in exosome route of infection, naive Huh7.5.1 cells were incubated with rat anti-CD81 (1/100), rat anti-Claudin-1 (1/100), rat anti-SR-BI (1/100) serum and control rat serum (1/100) for 2 hours at 37°C <sup>21</sup>. Exosomes isolated from Huh7-ET cultured cells were added to these cells and analysed after 48 hours for viral transmission by RT-PCR.

### **Immunocytochemistry**

The infectivity assay was performed in an 8-well chamber slide. After treatment with exosomes, the cells were permeabilized and endogenous peroxidase was blocked with PBS containing 0.3% hydrogen peroxide. The cells were then labelled with an HCV-Core antibody (Affinity Bioreagents; MA1-080; Clone C7-50). Secondary antibody binding and amplification of signal was accomplished with Envision horseradish peroxidase (DAKO Corporation, Carpinteria, CA) then visualized with 3'-Amino-ethyl-carbozole (Sigma). Blinded scoring of nine optical fields by two independent observers was performed for quantification at 200-fold magnification.

### **Viral RNA isolation and direct Sanger sequencing**

Viral RNA was extracted from 140µl of sucrose purified exosome suspension using the QIAamp viral RNA mini kit (Qiagen) and RNA was eluted with 40 µl of buffer AVE according to the manufacturer's instructions. Ten microliters of this RNA was reverse transcribed with the superscript III first strand synthesis system (Invitrogen

Corp) using HCV specific primer HCV 2a cDNA (5'-GCTCTACCGAGCGGGGAG-3'). Partial NS5b sequences and partial core sequences spanning the region from positions 426 to 904 and 8322 to 8713 of the HCV genome (Gene bank accession no AB047639) were amplified by PCR using HotStart Hifidelity Taq DNA polymerase (Qiagen) with the 2a Core primers (forward, 5'-AGATCGTTGGCGGAGTATAC-3' and reverse 5'-CGGAACGGTGATGCAGGACA-3') and 2aNS5b primers (5'-ATGATACCCGATGCTTCGAC-3' and 5'-AGGGGCAGAGTACCTGGTCA-3' ). The PCR cycling conditions were as follows: 95° C for 5 min, followed by 35 cycles of 95° C for 30 s, 48° C for 30 s, 72° C for 40 s and a final extension of 72° C for 10 min. The PCR amplified products were gel purified using gel extraction kit (Qiagen) and directly sequenced on both strands using the BigDye Terminator version 3.1 Cycle sequencing kit on an ABI PRISM 3100 genetic analyzer (Applied Biosystems). The obtained sequences were compared with the reference HCV 2a sequence and the analysis was performed using the CLC genomics work bench (CLC bio, Aarhus, Denmark).

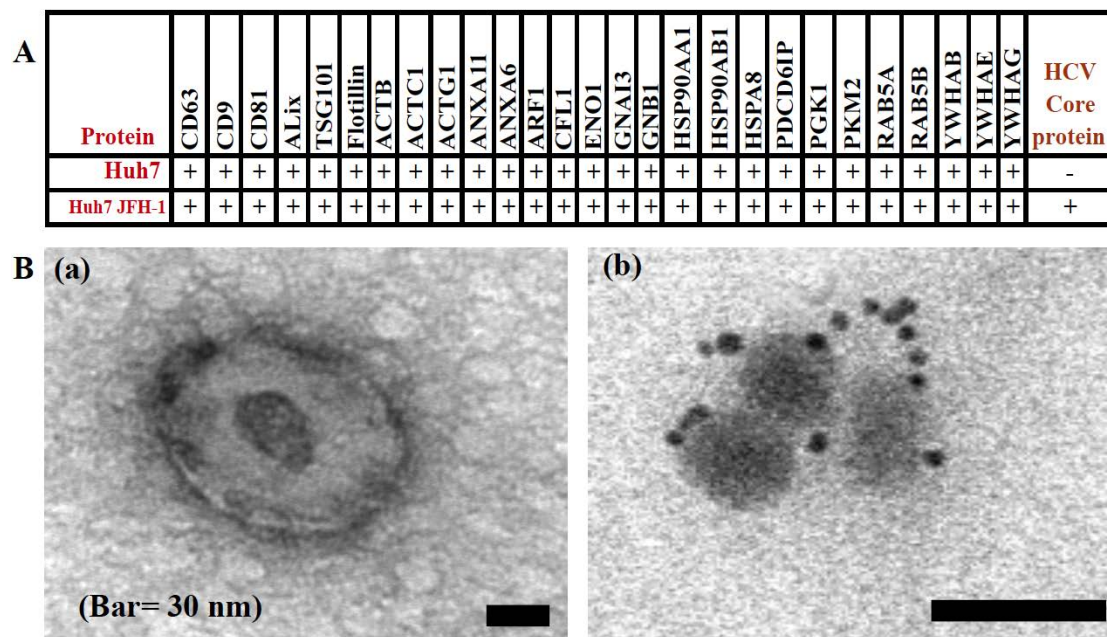
## Results

### **Exosomes derived from HCV infected hepatoma cells contain virus particles**

To establish the role of exosomes in shuttling HCV between cells, exosomes were isolated from Huh7.5.1 hepatoma cells using an established sucrose-gradient ultracentrifugation procedure. As shown in Fig.1A, mass spectrometric analysis of exosomes confirmed the presence of common exosome markers <sup>3</sup>, such as Tsg101, CD63, CD9, and Alix in these preparations. Moreover, exosomes isolated from Huh7.5.1 cells infected with HCV Jc1 contained detectable levels of HCV core protein (Fig.1A). Two unique HCV peptides were detected with mascot scores higher than 50 in three independent batches. As reported in a previous study using a comprehensive proteome profiling of exosomes secreted by hepatocytes <sup>2, 3</sup>, also apolipoproteins ApoE and ApoB were detectable. These lipoproteins were present in both control and HCV positive exosomes, suggesting that ApoE and ApoB are associated with hepatocyte-derived exosomes rather than contamination by HCV-associated LDL particles.

Electron microscopic imaging confirmed the purity of the exosome preparations using negative staining with uranyl acetate showing a lipid bilayer

structure (Fig.1B). Immunogold labeling with an anti-E2 antibody showed the presence of viral envelope proteins in exosomes isolated from HCV infected Huh7.5.1 but not in exosomes from naive Huh7.5.1 cells. In these preparations, no electron dense particles were observed outside the exosomes. RT-PCR analysis showed crude exosomes isolated from Jc1 infected cells and HCV subgenomic replicon cells did contain HCV RNA (Fig. S1). Full-length PCR analysis showed that these exosomes contained complete HCV genomes. As determined by direct Sanger sequencing, the amplified products from exosomes were identical to the HCV genotype 2a of Jc1. Consistent with our earlier report <sup>22</sup>, both control and HCV-positive exosomes contained high levels of miRNAs, including miR-122.

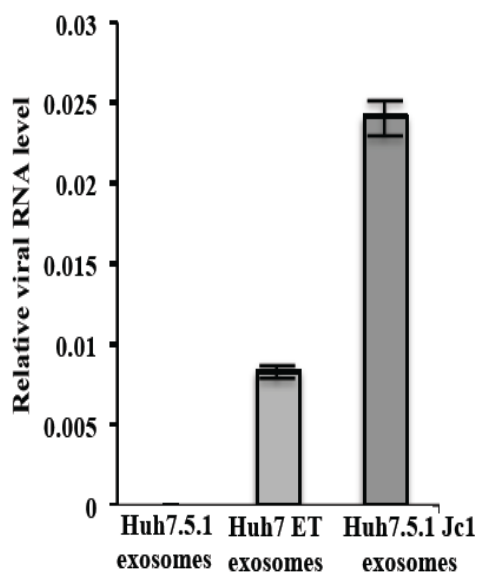


**Figure 1.** Purified exosomes of HCV infected Huh7.5.1 cells harbor viral protein and RNA. (A) Mass spectrometric analysis of Huh7.5.1 and Huh7.5.1 Jc1 derived exosomes contain exosome-related proteins and HCV core (n=3). (B) Electron microscopic images of purified exosomes isolated from infected Huh7.5.1 cells (a) using uranyl acetate staining. Immunogold labeling of HCV E2 envelope protein shows the presence of viral protein in exosomes derived from infected Huh7.5.1 cells (b). Immunogold and uranyl acetate staining could not be combined. Shown is one representative experiment of three.

### Exosomes can transmit HCV and establish a productive infection

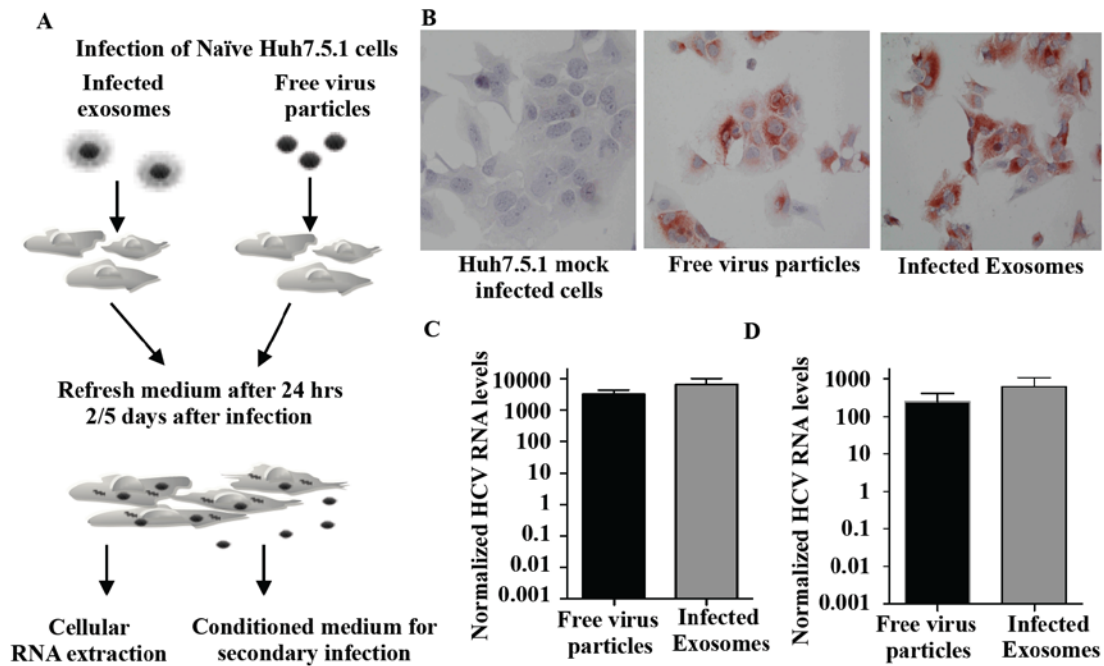
To investigate the functional role of exosomes in transmission of infection, exosomes were isolated from Jc1-infected cells and incubated with naive Huh7.5.1 cells as outlined in Figure 2A. As shown in Figure 2B, two days after exposure to HCV-positive exosomes, Huh7.5.1 cells stained highly positive for HCV core

protein by immunohistochemistry. The level of HCV core staining was comparable to cells infected with free virus particles. Huh7.5.1 uninfected cells were used for mock infection. The percentage of HCV positive cells when infected with free virus was mean  $93.0 \pm 3.7$  SD compared to  $91.0 \pm 5.4$  in exosome-treated cells with no significant differences among the treatments.

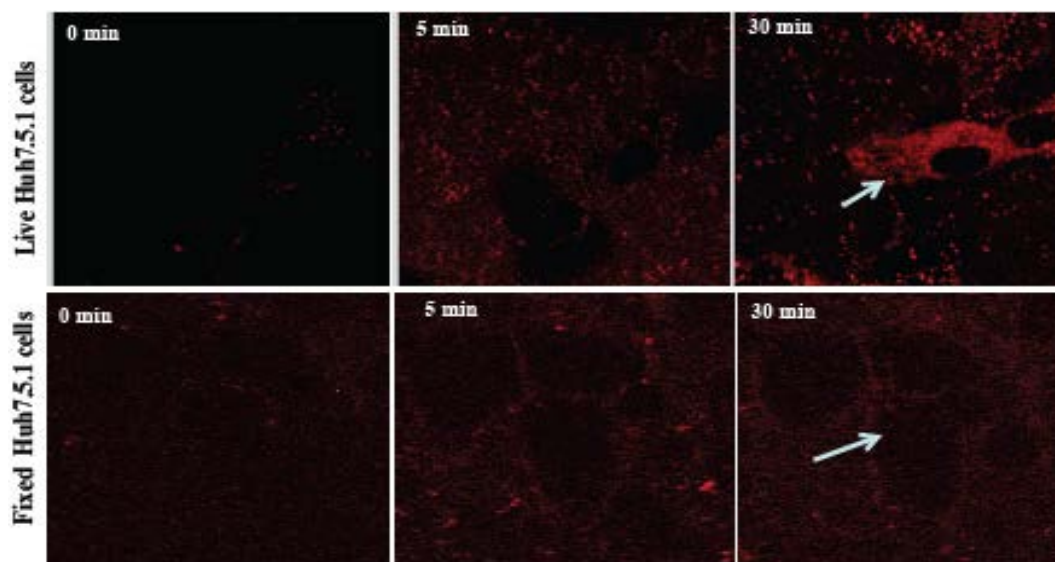


**Supporting Figure S1.** Crude exosomes isolated from Huh7.5.1 cells, Huh7-ET subgenomic replicon cells or Jc1 infected Huh7.5.1 cells, contain HCV genomic RNA as detected by qPCR. Exosomes from uninfected Huh7.5.1 cells do not contain viral RNA. Shown are the results in duplicates of three independent experiments

As HCV RNA input was normalized, exosomes appear as efficient as free virus to transmit infection. As shown in Figure 2C, cellular RNA of exosome-treated cells contained high levels of HCV viral RNA, comparable to cells infected by free infectious particles. Importantly, conditioned medium of cells infected by HCV-positive exosomes was able to establish a secondary infection of naive cells, confirming that the exosome pathway results in productive infection (Fig. 2D). The level of this secondary infection was comparable to that established by free infectious particles. The uptake of exosomes by Huh7.5.1 cells was confirmed by real-time confocal microscopy using fluorescently labeled exosomes. As shown in Figure S2 over a time period of 30 minutes cells gradually take up exosomes. Paraformaldehyde fixed cells remained negative over that time period confirming exosome-uptake is an active process and excluding passive transfer of fluorescent label.



**Figure 2.** Infected exosomes transmit infection to naive Huh7.5.1 cells and cause productive infection. (A) Schematic representation of infection experiments. (B) Immunocytochemical staining of HCV core protein Huh7.5.1 cells infected with infectious virus or exosomes (n=4). (C) Quantitative RT-PCR analysis of HCV RNA after primary infection and (D) secondary infection. The levels are normalized HCV RNA multiplied by 1000. Shown are the mean  $\pm$  SEM of four independent experiments in triplicates.



**Supporting Figure S2.** Uptake of exosomes is an active process. Exosomes isolated from hepatoma cells were labeled with Rhodamine C18. They were added to cells seeded on cover slips at time 0 and real-time confocal imaging for 30 minutes was performed on live Huh7.5.1 cells and PFA fixed Huh7.5.1 cells. Live Huh7.5.1 cells took up Rhodamine labeled exosomes compared to fixed Huh7.5.1 cells that did not take up any exosomes. The arrows indicate the cells and the red dots represent the exosomes labeled with Rhodamine.

**Exosome-mediated transmission is partly resistant to neutralizing antibodies and independent of structural viral proteins.**

Next we investigated exosome-mediated transmission of infection in the presence of nAbs. For this, HCV-specific immunoglobulins (IgGs) were purified from serum of ten chronic hepatitis C patients, as reported earlier<sup>23</sup>. Infectivity levels of Jc1-virus containing the luciferase reporter gene were tested in the presence of a pool of these nAbs. As shown in Figure 3A, both the infection by free virus and HCV-positive exosomes were significantly inhibited by pooled nAbs. Of these ten patients, nAbs were present in eight (Supporting Table 1).

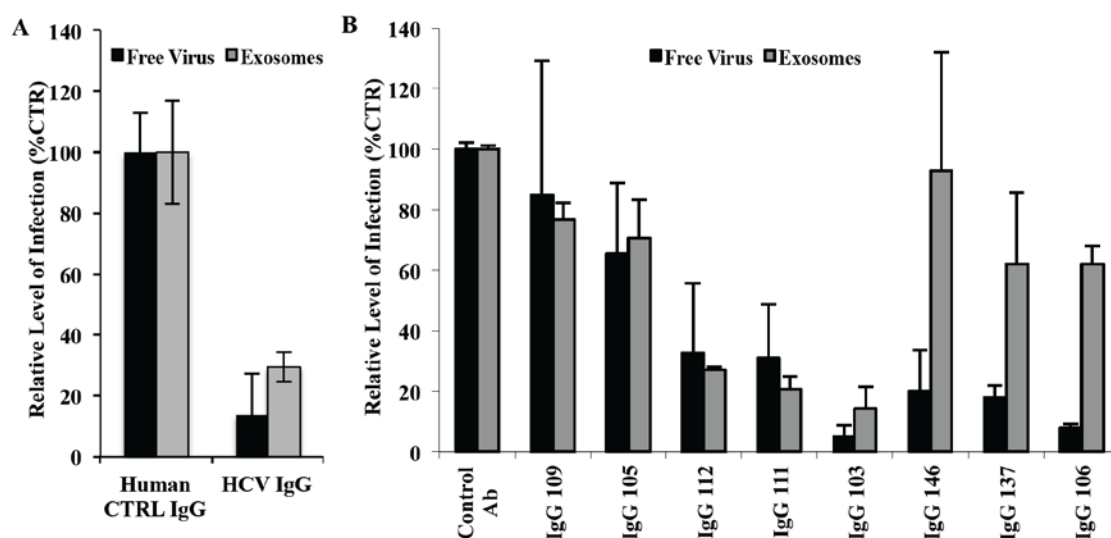
<b>Patient ID</b>	<b>HCV genotype</b>	<b>Viral load IU/ml</b>	<b>Cryoglobulinemia</b>	<b>Exosome neutralization (% of free virus neutralization)</b>
103	1a	9.77 X 10 <sup>6</sup>	weak positive	90%
105	3a	8.51 X 10 <sup>5</sup>	weak positive	85%
106	1a	9.77 X 10 <sup>5</sup>	negative	41%
109	1	1.12 X 10 <sup>6</sup>	negative	>100%
111	3a	6.17 X 10 <sup>5</sup>	weak positive	>100%
112	1b	5.62 X 10 <sup>4</sup>	negative	>100%
137	1b	1.51 X 10 <sup>5</sup>	negative	46%
146	3a	ND	negative	9%

**Supporting Table 1.** Characterization of Patients Used in Neutralization Studies  
ND, not detectable

As shown in Figure 3B, a comparative analysis of neutralization by HCV-specific IgGs, from these eight patients showed variable levels of neutralization of both free virus and exosomes. For five patients, IgGs showed similar neutralization of exosome- and free virus-mediated infection ( $p > 0.05$ ). In three patients, however, IgGs showed limited inhibition of exosome-mediated infection compared to free virus (Mean % inhibition  $\pm$  SEM of exosomes  $27.6 \pm 13.5$  versus free virus  $84.6 \pm 4.4$ ,  $p = 0.002$  Mann Whitney test). No link was found between patient's characteristics or virus genotypes with the inability of IgGs to inhibit exosome-mediated infection (Supporting Table 1). To determine the involvement of the structural proteins in the transmission of viral particles through exosomes, we exposed naive Huh7.5.1 cells to exosomes isolated from HCV subgenomic replicon cells (Huh7-ET). This bistrionic HCV replication model lacks the coding sequence for the structural viral proteins E1, E2 and core, which are replaced by the luciferase reporter gene, and therefore cannot



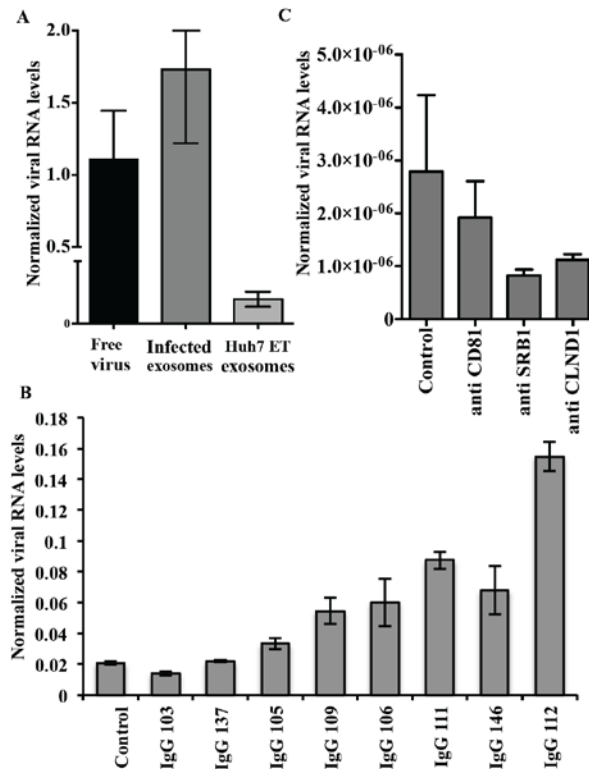
result in assembly of infectious virus particles.



**Figure 3.** Exosome-mediated transmission of HCV in the presence of primary neutralizing immunoglobulin's (IgGs) varies between patients (A) Huh7.5.1 cells were infected with Luc-Jc1 particles or purified exosomes from Luc-Jc1 infected cells in the presence of pooled patient-derived anti-HCV-specific IgGs from ten HCV patients. Shown are the mean  $\pm$  SEM of four (control IgG) or five (HCV IgGs) independent experiments in duplicates. (B) Infectious Jc1 particles or exosomes were incubated with Huh7.5.1 cells in the presence of patient-derived anti-HCV-specific IgGs (n=8, Supporting Table 1) or control IgGs pooled from three healthy controls. Shown are mean  $\pm$  SEM of four independent experiments in duplicates.

As shown in Figure 4A, exosomes from subgenomic replicon cells can transmit viral RNA to naive cells. However, transmission was less efficient than observed with exosomes derived from Jc1 infected Huh7.5.1 cells. These results indicate that virus could be transmitted by exosomes independent of structural proteins, be it at a lower level of infection. As shown in Figure 4B, no neutralization of subgenomic replicon-derived exosomes by HCV-specific IgGs from the eight patients was observed.

To study the role of viral entry receptors, CD81, SR-BI and Claudin-1 on exosome-mediated HCV transmission, naive Huh7.5.1 cells were treated with specific rat anti-sera prior to adding the Huh7-ET-derived exosomes. After 48 hours, RNA levels were detected as shown in Figure 4C. Some inhibition of HCV RNA transfer was observed for all three-entry receptors but none reached statistical significance. This suggests that entry receptors may also partly contribute to, E1/E2 envelope-independent, exosome uptake. Further research is required to specifically address this.



**Figure 4.** Transfer of HCV RNA via exosomes independent of structural proteins. (A) Naive Huh7.5.1 cells were incubated with Jc1 containing medium or with exosomes isolated from HCV subgenomic replicon cells (Huh7-ET) or exosomes isolated from Jc1 infected Huh7.5.1 cells. Naive cells exposed to Huh7-ET exosomes have clearly detectable amount of HCV RNA, but at a lower level than exosomes from Jc1 infected cells. Shown are the mean  $\pm$  SEM of three independent experiments in triplicates. (B) Huh7-ET derived exosomes were incubated with Huh7.5.1 cells in the presence of patient-derived anti-HCV-specific IgGs (n=8, Supporting Table 1) or control IgG from healthy controls. No neutralization was observed, rather enhanced exosome-mediate transmission with some IgGs. Shown is the mean of triplicates  $\pm$  SEM of one representative experiment of three. (C) Naive Huh7.5.1 cells were blocked with anti-CD81, anti-SRBI and anti-Caudin-1 antibody and infected with Huh7-ET derived exosomes. Some inhibition of HCV RNA transfer was observed for all three entry receptors compared to control antibody, but did not reach statistical significance. Shown is the mean  $\pm$  SEM of three independent experiments in duplicates.

## Discussion

Exosomes are established vehicles for the shuttling of proteins, mRNAs and miRNA between cells<sup>5</sup> and as such play an important role in many biological processes<sup>2,4</sup>. Though a role of exosomes in the shuttling of infectious agents between cells has been postulated, this has still not been extensively demonstrated. For HCV, a recent study showed that hepatocyte-derived exosomes containing viral RNA can elicit interferon- $\gamma$  production in plasmacytoid dendritic cells<sup>17</sup>. In the current study, we showed that HCV infection can be transmitted by exosomes between hepatocyte-

like cells and establish a productive infection (Fig. 2). Electron microscopic analysis confirmed the purity of exosomes and indicated the presence of intact E2 envelope protein positive viral particles inside the vesicles (Fig. 1). This is consistent with the earlier observation that approximately 1-2% of viral particles released from infected cells are actually associated with multivesicular particles that may represent exosomes<sup>24</sup>. Moreover, we found that exosome-mediated transmission of HCV was partly resistant to neutralization by antibodies (Fig. 3). This latter finding suggests that the exosome route of HCV transmission is variably inhibited by nAbs. The exosomes derived from Huh7-ET cells were not inhibited by neutralization (Fig. 4B). This is consistent with the fact that neutralizing antibodies are directed against virus envelope proteins which are lacking in the subgenomic replication model. Surprisingly, several of the IgGs enhanced the transmission of Huh7-ET derived exosomes, but the reason for this effect is unknown. In general, the mechanism of uptake of exosomes by cells is not fully understood. Several uptake pathways have been postulated, including receptor mediated endocytosis, pinocytosis and plasma membrane fusion, but none of these pathways have been convincingly established for hepatocytes or other epithelial cells. It is unknown whether HCV entry receptors, SR-BI, CD81, claudin-1 and occludin, are involved in exosome uptake by hepatocytes. The experiments shown in Fig. 4C however, suggest that entry receptors may partly contribute to exosome uptake even in the absence of viral envelope or core proteins. Indeed a very recent study on endocytosis of exosomes identified an important role for lipid rafts and caveolins as important factors for uptake<sup>25</sup>. Both these pathways are also known to be involved in virus uptake and lipid rafts support HCV replication<sup>26,27</sup>. As a matter of fact, HCV entry receptors CD81 and SR-BI are known to localize in lipid rafts<sup>28,29</sup> supporting a hypothetical role of these receptors in exosome uptake by hepatocytes. Further research is required to determine different pathways involved in uptake of exosomes and HCV virions.

There are several possibilities why the exosome-mediated transmission of HCV was not completely resistant to neutralizing antibodies. Firstly, though free virus particles were not observed under EM, this does not fully rule out the presence of free virus particles in the isolated exosome samples. The density of HCV in sucrose gradients has been measured between 1.08 and 1.11 g/ml, very close to the buoyant density of exosomes, which is between 1.11 and 1.21g/ml<sup>2,5</sup>. Secondly, viral

envelope proteins may be packaged into the outer membrane of exosomes, making it susceptible to neutralization. No evidence for this scenario was found in our immunoelectron microscopy (Fig. 1) and it is unlikely, as it would require different post-translational modifications of envelope between free virus and exosomes. Mass spectrometric analysis of exosomes only detected HCV core protein, but may lack sensitivity to detect low quantities of envelope proteins. This analysis did confirm the presence of various proteins known to be exosome markers both in exosome preparations of infected or uninfected Huh7.5.1 cells (Fig. 1).

In conclusion, although two previous studies have shown the association of HCV virus with exosomes, in the present study we are the first to demonstrate that exosomes can shuttle virus to hepatocyte-like cells and establish a productive infection. Indeed, Sanger sequencing confirmed that hepatocyte-derived exosomes contained full-length HCV genomes. Taken together, these data suggest that viral transmission through exosomes contributes to the known immune evasive properties of the virus.

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## Chapter 6

### **Exosomes as Protective Vehicles for Feco-oral Transmission of Viral Hepatitis**

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## **Abstract**

**Background & Aim:** One of the worldwide health hazards is caused via the hepatitis viruses and is caused by various routes of infection. For hepatitis C virus (HCV), blood-blood contact such as blood transfusion and needle sharing intravenous drug use are well-established routes of transmission. Infections occurring in poor sanitation and overcrowding, via a feco-oral route of transmission is well established for the hepatitis A virus (HAV) and hepatitis E virus (HEV) but has not been established for HCV. The aim was to examine the possibility of HCV fecal transmission by release in bile and protection of virus in biliary exosomes.

**Methods:** Human bile from gallbladders obtained from chronic HCV positive and negative patients were collected at the time of liver transplantation. Serial ultracentrifugation steps isolated bile exosomes. RNA was obtained from total bile and exosome fractions and analyzed for presence of HCV genomic RNA by real time RT-PCR.

**Results & Discussion:** A recent article in nature considers the possibility of HAV virus hijacking the cellular membrane resembling an exosomes. This encapsulation in exosome-like vesicles promotes virus spread in the liver and is relative resistant to neutralizing antibodies. We observed that HCV in bile is enriched and protected in the exosome-fraction. Similar to HAV, our preliminary results indicate a potential for fecal transmission of HCV by release in bile in protective exosome-vesicles.



## Introduction

Hepatitis viruses have a tremendous global health impact and spread of infection occurs at a high rate and via different routes of administration. For the hepatitis A virus (HAV) and hepatitis E virus (HEV) the predominant route of infection is by feco-oral transmission. For hepatitis C virus (HCV), direct blood-blood transmission is most prominent but the rate of feco-oral transmission is still largely unknown.

Viruses come in many forms and shapes, which are highly selected by evolution for optimal transmission between hosts and effective infection of host-cells. Now emerging evidence suggests that some viruses also can make use of the microvesicle system of host cells for their transmission. Most mammalian cell types are thought to constitutively release small membrane particle microvesicles, including so called exosomes. Since their discovery 30 years ago<sup>1</sup>, these 50 to 100 nm-sized extracellular signaling vesicles are now known to contribute to many (patho)physiological processes, including immunity, coagulation and bone mineralization<sup>2</sup>. Importantly, exosomes can mediate cell-to-cell transmission of genetic information and transfer of mRNAs and microRNAs<sup>3</sup> as well as transfer of proteins including interferon-induced antiviral molecules<sup>4</sup>. In addition, also their role in the transfer of pathogens, pathogen-derived antigens and virulence factors is emerging<sup>5</sup>. A recent study revealed that the Hepatitis A virus (HAV) exploits exosomes for their transmission<sup>6</sup>.

HAV belong to the group of non-enveloped viruses and is the most common form of acute hepatitis that spreads through the fecal-oral transmission. Feng *et al.*<sup>6</sup> identified two distinct populations of viral particles (virions) in HAV-infected hepatoma cell culture supernatant, using a density-gradient: the known capsid virions and a second low-density HAV population that is not detected in a capsid antigen ELISA. Electron microscopy confirms that this second population of HAV particles is surrounded by membrane structures. The particle size ranges from 50 to 110 nm in diameter, similar to that of exosomes. The authors termed these exosome-like virus particles 'enveloped HAV' (eHAV). The exosome-encapsulation of HAV capsid might exert protection of antibody neutralization. To confirm this hypothesis, the authors show that these eHAV are indeed resistant to antibody neutralization and are infectious with infectivity equivalent to that of non-enveloped virions. The authors showed that *in vitro* most of the virus released in culture medium is actually eHAV,

since the majority of virions contain unprocessed capsid protein VP1pX and mature VP2, whereas the non-enveloped virions contain only fully processed VP1.

Confirming the importance of the exosome-pathway in the HAV biogenesis, the authors demonstrated an important role for ESCRT-associated proteins in virus transmission. The ESCRT system is known to be involved in exosome biogenesis in a cellular compartment called the multivesicular body<sup>7, 8</sup>. Two ESCRT-III binding proteins, VPS4B and ALIX, were shown to function in the release of eHAV from cells, but were not involved in viral replication or encapsidation of viral RNA. HAV capsid protein VP2 was found to contain YPX1/3L motifs, (a motif through which structural proteins interact with ESCRT proteins) which mediate interactions with ALIX. However, it should be noted that the role of ESCRT-III associated proteins maybe more complex than just exosome-biogenesis, as knockdown of VPS4b and ALIX also inhibited the release of non-enveloped HAV.

Non-enveloped HAV as well as eHAV required entry receptor TIM-1 for viral entry. Interestingly, only eHAV infection was dependent on endosomal acidification as shown by chloroquine-mediated inhibition, suggesting different post-entry steps for the enveloped and non-enveloped HAV. Antibodies directed against the viral capsid effectively neutralize non-enveloped HAV but did not affect eHAV infection. However, IgG and IgA anti-capsid antibodies did affect eHAV infection when given 6 hours post-inoculation. This was not seen with an IgM anti-capsid antibody, suggesting eHAV may be neutralized intracellular after endocytosis of monomeric or dimeric but not pentameric antibodies. The exact mechanism of this observation still needs to be demonstrated, but to some extent it is consistent with the clinical observation that antibody prophylaxis given several days after HAV replication in the liver has been established, still effectively protects against hepatitis A.

One of the conclusions of the paper is that the classic distinction between enveloped and non-enveloped viruses is blurry, as both category viruses can be cloaked in exosome or other host derived-membranes. As a wolf in sheep's clothing, encapsulated viruses can bypass host immune responses. Whether the cloaking of HAV protects viruses from degradation in the gastrointestinal tract and the exterior environment after defecation in contaminated food and drinking water, remains to be demonstrated, but is interesting to postulate. Do exosomes act as protective vehicles

for virions and thereby facilitate fecal-oral transmission? HAV is known to be excreted from the liver both via blood and bile, and exit the host via the stool<sup>9</sup>. Though in feces of infected chimpanzees the non-enveloped virus dominates, the authors showed that exosome-cloaked HAV survives the toxic environment of bile. Moreover, recent data suggest that RNA packaged in exosomes, is protected against degradation in feces<sup>10</sup>. Li.et.al further establishes the presence of exosome-like vesicles in human bile contains microRNAs that can be used for cholangiocarcinoma diagnosis<sup>11</sup>. Therefore the aim of the present study is to examine the possibility of HCV packaging in biliary exosomes as a potential protection for the virus during fecal transmission.

## **Materials and Methods**

### **Isolation of exosomes from bile**

Human bile from gallbladders obtained from chronic HCV positive and negative patients were collected at the time of liver transplantation. The bile was diluted with equal amount of sterile PBS. Large components were pelleted by centrifugation at 10.600 rpm for 20 min in a SW40 rotor (Beckman Coulter) at 4°C. The pellet was resuspended in 400 µl sterile. The supernatant was transferred to a new tube and centrifuged at 24.000 rpm using a SW40 rotor for 1 hour at 4°C. Pellets were again prepared as described above. The supernatant was then centrifuged for a third time for 2 hours at 28.000 rpm at 4°C using a SW40 rotor and the resulting pellet (exosome) was dissolved with sterile PBS again and stored at -80°C until further processing.

### **RNA isolation and quantitative PCR**

Total RNA was extracted from 200µl of bile and exosome pellet isolated from bile using the miRNeasy mini kit (Qiagen, Hilden, Germany). A modified protocol derived from the manufacturer was used to isolate total RNA. For this, 1000µl of Qiazol lysis reagent was added to 200 µl of bile and mixed extensively by vortexing. In case of pellet, 1400 µl of Qiazol lysis agent was added to 400 µl of resuspended pellet. After 5 minutes of resting at room temperature 200µl of ice cold chloroform was added and the samples were again mixed vigorously using a vortex. After centrifugation (15 minutes, 16.000 RCF at 4°C), 600 µl of aqueous RNA containing layer was obtained, which was further processed according to the manufacturer's

protocol (Qiagen). RNA samples were quantified for presence of HCV by real time RT-PCR.

## Results

For HEV, also a non-enveloped virus, it is well known to spread through the fecal contamination of water or food. Like HAV, HEV has been shown that viral particles in the blood of infected persons are associated with membranes<sup>12</sup> Moreover, HEV was shown to utilize ESCRT components for release from infected cells<sup>13</sup>. Whether HEV in feces is encapsulated in exosome-like particles, which are protective against degradation in the gastrointestinal tract and beyond as depicted in Figure 1, is interesting to postulate but again remains to be demonstrated.

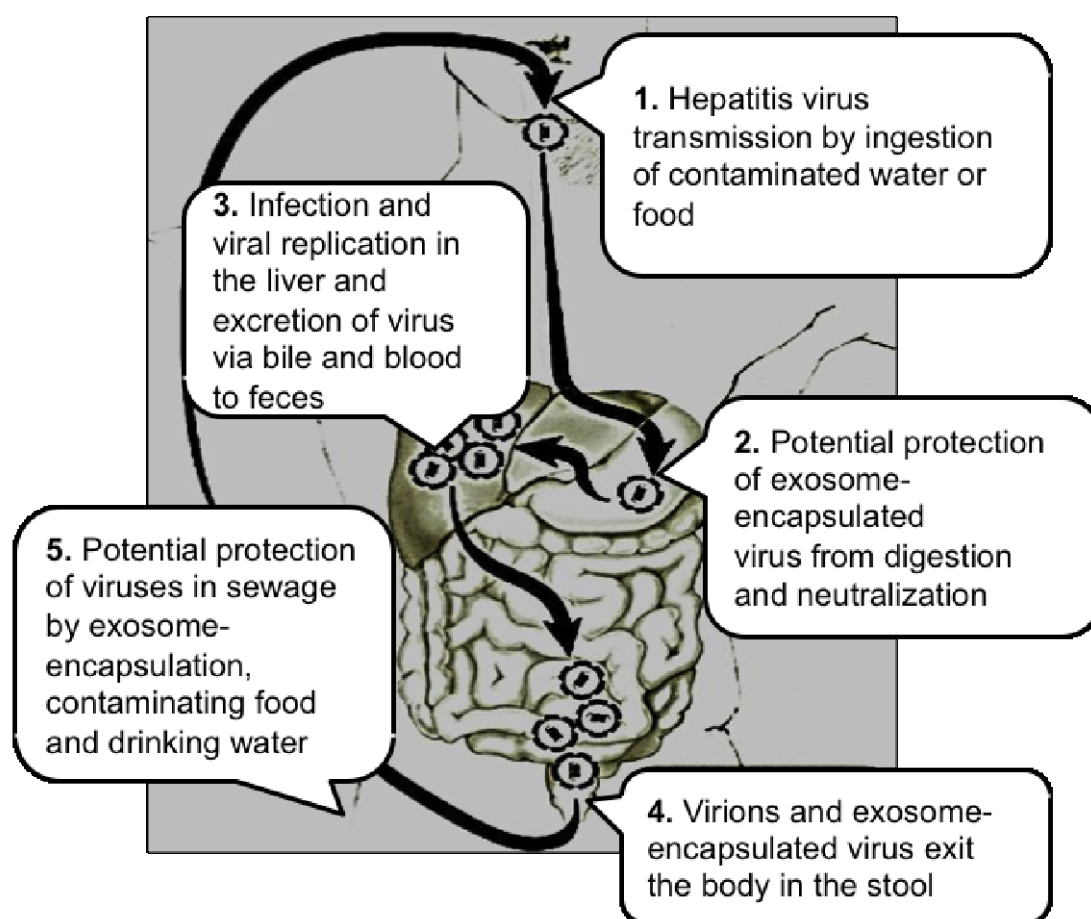


Figure 1: Model of exosome-mediated fecal-oral transmission of Hepatitis A Virus (HAV) and possible other hepatitis viruses like Hepatitis E Virus (HEV) and Hepatitis C Virus (HCV). Preliminary data suggests HCV in bile is enriched and protected in the exosome fraction. Modified after image source: <http://yourstdhelp.com/hepatitis>.

In contrast to HEV, for HCV our group recently published a study showing that hepatocyte-derived exosomes, isolated from infected hepatoma cells, can contain

and transmit virus<sup>14</sup>. We showed that exosome-mediated transmission of HCV was partly resistant to antibody neutralization. Earlier studies showed the presence of HCV RNA in bile and fecal samples of HCV-infected individuals<sup>12, 15</sup>, but the question remained if exosome-encapsulated HCV can act as a protective vehicle for fecal transmission of HCV? To explore this hypothesis, we purified exosomes from human bile from gallbladders obtained from chronic HCV patients at time of liver transplantation. Exosome-isolation was performed by ultracentrifugation, as published previously<sup>13</sup>. Our preliminary results show that in isolated bile exosomes of three patients, HCV genomic RNA could be detected by quantitative PCR, in contrast to total bile (Table 1) and non-HCV patients.

Patient ID	Ct-values Quantitative RT-PCR HCV IRES		
	Serum	Purified Bile Exosomes	Total Bile
Hep C # 1	6.8 x 10 <sup>5</sup>	36.5	>40
Hep C # 2	5.7 x 10 <sup>3</sup>	35.1	>40
Hep C # 3	5.6 x 10 <sup>5</sup>	36.6	>40
Non-Hep # 1	ND	>40	>40
Non-Hep # 2	ND	>40	>40
Non-Hep # 3	ND	>40	>40

**Table 1:** Positive detection of HCV genomic RNA in purified biliary exosomes. Detection of HCV genomic RNA in purified exosomes from bile of end-stage liver disease patients with (Hep C) but not without (Non-Hep controls) chronic hepatitis C.

## Discussion

The field of exosome research is truly captivating and opens up a completely new way of thinking in relation to diseases in general and now also for viral hepatitis. As recently shown by us exosome is involved in transmitting viral HCV RNA from one cell to another<sup>14</sup>. Other evidence revealed the involvement of exosome-like vesicles in protecting HAV from neutralizing antibodies<sup>6</sup>. The presence of exosomes in human bile has now been well established<sup>11</sup> and shown to be protected from the toxic environment of the bile<sup>10</sup>. We isolated exosomes from whole bile obtained from HCV positive and negative patients at the time of transplantation. The detection of HCV RNA in these exosomes, like for HAV, could open up a discussion on the possibility of fecal related transmission of HCV in light of the recent hepatitis C epidemic in men who have sex with men in Europe, North America and Australia<sup>16</sup>.

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## **Chapter 7**

### **General Discussion**

According to the first-ever World Health Organization (WHO) study of liver disease mortality, total deaths worldwide from cirrhosis and liver cancer rose by 50 million per year over the last two decades. Medical research on liver disease has now become critically important to bring under control the toll of liver disease on human health and well-being.

The cytoplasmic mass of the liver constitutes of about 75%-85% of hepatocytes. These cells carry out the various functions of the liver. Hepatocytes are the main cells for protein synthesis in the liver and are responsible for breakdown of lipids from triglycerides<sup>1</sup>. Nowadays we are able to understand hepatocytes better and realize their importance is huge in maintaining the homeostasis of the liver and remaining healthy. During liver damage or disease the hepatocytes are damaged and there is formation of scar matrix, so-called liver fibrosis. Various diseases affecting the liver such as hepatitis, cancer, and alcohol related and acute liver failure, when cannot be controlled by medication lead to cirrhosis and ultimately end-stage liver failure. End-stage liver failure requires liver transplantation as the treatment option. However, the limitations accompanied with organ transplant such as graft rejection due to dysfunction in the liver forces us to re-think on the much-needed new diagnostic tools.

Hepatitis C virus (HCV) associated cirrhosis is the most common indication for liver transplantation worldwide. Conventional interferon-based anti-viral therapy for HCV is only partly effective and is often poorly tolerated. Therefore, many patients are not cured and progress to advanced liver disease and malignancy, and potentially require liver transplantation as treatment for their liver failure. However, HCV remains a problem after transplantation because of recurrent hepatic infection<sup>2, 3</sup>, which is also one of the leading causes for graft failure.

Therefore, my thesis focuses on different aspects of HCV infection and liver transplantation, aiming at developing alternative therapeutic options such as novel antivirals, and understanding better the various routes of transmission of HCV. In parallel, research was performed to develop biomarkers that will easily and early detect liver injury.



## Release of hepatocyte-derived miRNAs in response to injury

Currently, protein based biomarkers aspartate transaminase (AST) and alanine transaminase (ALT), are widely used in the clinic for detection of liver injury. But the assessment is never complete unless a tissue biopsy is taken to confirm hepatic injury. This is due to the presence of AST and ALT in muscle tissue, which could give false elevations during muscle injury<sup>4</sup>. Retrieving trough-cut biopsies after liver transplantation is an invasive procedure and is associated with pain, bleeding and increased risk of infections<sup>5-8</sup>. Therefore, there is a strong need for non-invasive biomarkers to provide early and more sensitive diagnosis of rejection and liver graft dysfunction.

MiR122 is the most abundantly expressed miRNA in hepatocytes<sup>9</sup>. MiR122 was found to regulate various metabolic functions<sup>10,11</sup> and is a crucial host factor for HCV infection and replication<sup>12</sup>. A recent study in rodents demonstrated the release of miR122 and other hepatocyte abundant miRNAs from cells during drug-induced liver injury<sup>13, 14</sup>. These miRNAs termed as hepatocyte-derived miRNA (HDmiRs) were detectable in serum or plasma. They correlated with AST and ALT and liver histology. The rise in serum miRNA was earlier than that of transaminases. In humans, it was shown that miR122 was detectable in serum and elevated in patients with hepatocyte injury<sup>15, 16</sup>. Here also, a close co-relation with transaminases was shown. However, this has not been evaluated in the setting of liver transplantation. In **Chapter 2** we explored the potential of HDmiRs to be a sensitive biomarker for various types of liver injury in liver transplant recipients. We demonstrated that serum levels of HDmiRs were elevated in patients with liver injury after liver transplantation (Figure 2) and during acute rejection (Figure 4). During acute rejection, serum HDmiRs showed similar kinetics, however, miRNA levels increased and decreased earlier than transaminases (Figure 4B and C). Of all the circulating HDmiRs, miR-122, miR-148a and miR-194 were shown to be stable and detectable during hepatic injury in patients after liver transplantation. The limitation of this study was low yields of RNA that prevented us from doing any further research on understanding the mechanism and biology of the release of these miRNAs in response to liver injury. Our data support the potential use of miRNAs as biomarkers and provides us with the opportunity to determine liver transplant rejection and injury with minimally invasive

procedures.

The miRNAs were shown to be stable, early and sensitive markers of liver injury<sup>17</sup>. Also in the absence of liver disease or injury, HDmiRs are detectable in serum, indicating release from hepatocytes is constitutive and merely increased in response to cellular stress or injury. This could indicate that this release is selective and not non-specific or passive. For polarized cells like hepatocytes, the question remains whether the release of miRNA can occur bidirectionally into bile and blood. Hence in **Chapter 3** we investigated paired bile and serum samples during liver injury and reduced liver function after transplantation. We observed that during cellular stress and injury, hepatocytes mainly direct the release of HDmiRs into the circulation, whereas cholangiocytes seem to direct CDmiR release into bile (Figure 4). Besides the release of HDmiR-122 to serum during injury, we also observed a strong correlation between bilirubin and HDmiR-122 secretion to bile (Figure 5). This not only suggests that HDmiR-122 could be useful marker for hepatocyte function, but also that this miRNA might be involved in conjugating and exocrine function of hepatocytes.

Several studies confirmed that serum levels of HDmiR-122 are sensitive for the detection of liver injury<sup>7, 17, 23</sup>. The finding that HDmiR-122 is also secreted to bile and the strongly correlates with hepatocyte function in terms of conjugation however is new. The first study reported on the presence of specific miRNAs in bile, identified miR-9 as a potential biomarker for biliary tract cancer. Despite the hostile environment of human bile, in general, biliary miRNAs were found to be highly stable and protected from degradation<sup>20</sup>. Though this study focused on tumor-derived miRNAs, small RNA library sequencing analysis verified the presence of two hepatocyte-abundant miRNAs, miR-122 and miR-192 in bile. Of all miRNA clones analyzed, approximately 19% consisted of these two HDmiRs. Real-time PCR analysis of over 667 specific miRNAs in these same samples suggests this percentage is likely lower<sup>18</sup>. The results of this study suggest that patients suffering from cholangiocarcinoma have higher miRNA contents in bile extracellular vesicles compared to patients with non-malignant biliary obstructions. The authors plea for the analyses of extracellular vesicles rather than whole bile, in order to have a better discrimination between pathologies. Evidence that whole bile analysis could be insufficient was however not provided. Furthermore, the results from previous studies

as well as in the current, the percentage of miRNAs in vesicles like exosome were demonstrated to be very low<sup>18</sup>. By only looking at miRNAs in the vesicle fraction, over 90% of the miRNA signal in bile would be overlooked and ignored for analyses. For chronic disease like cholangiocarcinoma, miRNAs in bile fractions like vesicles might indeed be more suitable for diagnostic purposes, but this mechanism of miRNA release is less frequent in severe acute injury like ischemia-reperfusion<sup>19</sup>. Beside HDmiR-122, the study of Li et al. also reported on CDmiR-222 as one of the enriched miRNAs in cholangiocarcinoma<sup>20</sup>, confirming that this miRNA is a potentially relevant marker for various cholangiopathies. Earlier work from our group found CDmiR-222 release to be lower in preservation solutions that were used to flush grafts which later developed ischemic-type biliary lesions after liver transplantation<sup>19</sup>. Then, we hypothesized that cholangiocytes release their miRNA content to the bile rather than to the blood. The results from the current study further support this hypothesis by the inverse release between HDmiRs and CDmiRs to bile and blood. Furthermore, in esophageal adenocarcinoma cells, bile acid has been shown to increase CDmiR-222 expression in tissue, along with the farnesoid X receptor (FXR) activity<sup>21</sup>. Together with the finding that HDmiR-122 is strongly correlated with bilirubin secretion into bile, this supports a role of bile miRNAs in the enterohepatic circulation, though this hypothesis should be further investigated<sup>22, 23</sup>.

A remarkable finding from our study was the difference in ProtK-treated sensitivity between HDmiRs and CDmiRs. As mentioned before, CDmiR levels were less influenced when protein was broken down, while HDmiR levels drastically decreased. This implicates that cholangiocytes release their miRNAs in a different manner to the bile than hepatocytes. Previous studies suggested that miRNAs can be released and bound to lipoproteins as HDL<sup>24, 25</sup>, which perhaps could explain the protein-independence of CDmiRs, though no evidence for this hypothesis is provided in this study. The results from our study not only confirm that HDmiR-122 is a suitable injury marker in serum, but also that its levels in bile are strongly correlated with hepatocyte function. This provides insight in the mechanism and direction of miRNA, which is relevant for the development of diagnostic assays. In particular for cholestatic diseases, the miRNA composition of the bile in different fragments could be helpful in distinguishing different pathologies. But also in the setting of liver transplantation, analysis of miRNAs in bile which is produced during graft machine

preservation might be informative on the degree of biliary injury which is currently the second cause of graft failure after liver transplantation<sup>26</sup>. In conclusion, this study demonstrates the polarized release of hepatocyte and cholangiocyte abundant miRNAs into bile and blood during liver injury and impaired cellular function. The variety in HDmiR and CDmiR release is further underlined by the difference in protein dependent stability in bile. MicroRNAs in bile are potential markers for assessing cell function and as a marker for biliary injury in liver transplantation and cholestatic diseases.

The genetic delivery of miR-122 would enable us to divulge various biological functions of miR-122 in hepatocytes and also further explore its role in HCV replication and production. Also the role of this liver abundant miR-122 in the differentiation of stem cells and progenitor cells into mature hepatocytes is not clear. To be able to study the effect of overexpressing miR-122, a 3<sup>rd</sup> generation lentiviral vector expression system was designed to deliver miR-122 to human cells. The presence of eGFP in this vector enabled us to identify transduced cells easily. Once the vector was established the expression of miR-122 in HEK293T transduced cells was tested and compared to Huh7 cells that are known to express miR122 endogenously (Figure 1) (manuscript in preparation).

### **RNA interference in the biology and therapy of HCV**

A small part of this thesis aimed to study RNA interference (RNAi) mediated gene silencing against HCV and the related transmission of small interfering RNAs between hepatic cells.

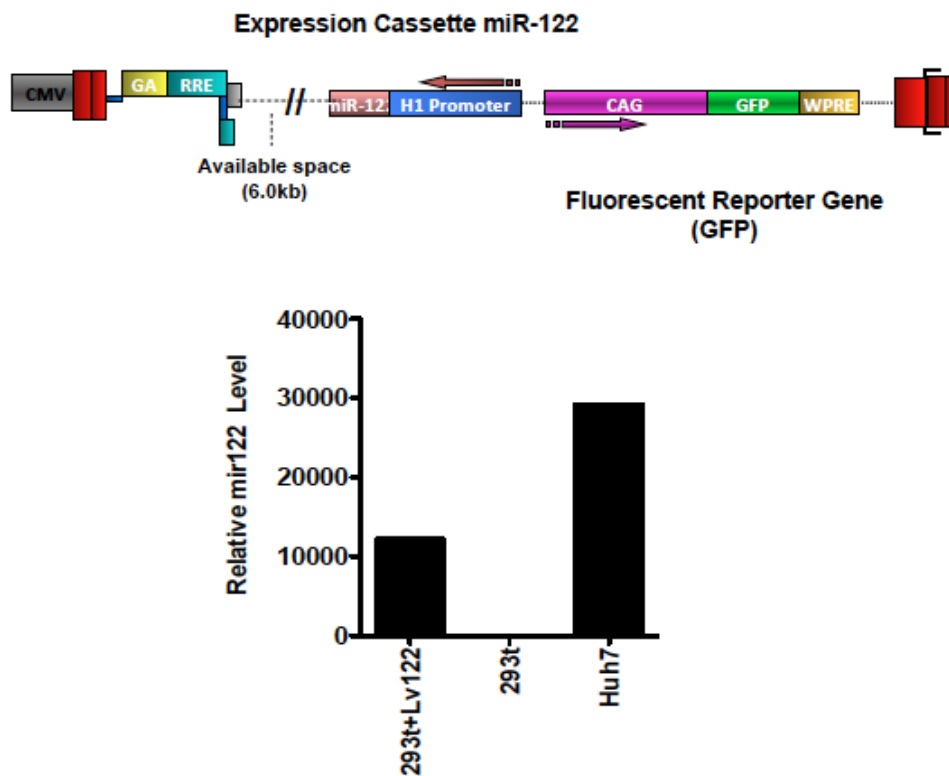
RNAi is a promising new therapeutic tool. RNAi provides an important mechanism of cellular defense against viral pathogens in plants, insects and invertebrates, dependent on the spread of small silencing siRNA to neighbouring cells<sup>27-29</sup>. The conventional antiviral therapy for HCV is interferon-based. Half of the patients can attain sustained virologic response (SVR) with current standard peg-IFN- $\alpha$  in combination with ribavirin therapy, whereas in genotype 1 infection (the most common) the SVR rate is only about 40%<sup>30</sup>. With the launching of directly acting antivirals (DAAs), triple combination of DAAs, peg-IFN- $\alpha$  and ribavirin, it is shown that SVR rates in genotype 1 increase up to 90%<sup>31</sup>. However, given the large percentage of non-responders, poor toleration of interferon or DAAs and the large

infected population, novel antiviral strategies remain a challenge as well as a need. Therefore, research on RNAi based therapy has been very promising to combat this challenge. The basic understanding of HCV has also progressed due to RNAi based gene silencing technologies by revealing numerous host (CD81<sup>32</sup>, glycosaminoglycan<sup>33</sup>, scavenger receptor class B type-1<sup>34</sup>, low-density lipoprotein receptor<sup>35</sup>, DC-SIGN<sup>36</sup>, L-SIGN<sup>37</sup> and Claudin-1,-6,-9<sup>38-40</sup>) and viral targets<sup>41-45</sup> for therapy.

In **Chapter 4**, we explored whether vector delivered RNAi can transfer between hepatic cells in vitro and in vivo and if this exchange can extend the therapeutic reach of RNAi against HCV infection. In Figure 1, we show evidence for intercellular transmission of functional siRNAs. We observed that there was silencing of CD81 expression in non-transduced Huh7 cells when Huh7 cells were transduced by lentivector-GFP containing CD81 targeting shRNA (Fig 1A). Similarly, on co-culturing HCV replicon cells (Huh7-ET) with cells stably expressing shNS5b, a significant, ratio dependent inhibition of HCV replication was observed (Fig 1B). Transfer of miR122 was demonstrated in freshly isolated human peripheral blood mononuclear cells incubated with Huh7-CM, that is known to have high miR122 levels (Figure 2). All these results suggest that transmission of RNAi involves the uptake of released silencing RNA components and that there is functional regulation of target reporter gene expression by transferred miRNA. Finally, also in vivo studies in mice revealed transmission of RNAi, where non-transduced liver tissue showed marked reduction in CD81 expression (Figure 5). From our study, there is evidence that small RNA can be exchanged between hepatic cells and this could be used to target or inhibit HCV infection.

We explored integrating a lentiviral vector expressing shRNA to develop a stable and long-term siRNA expression system that could serve well as an antiviral RNAi based therapy for HCV. This expression system is an alternative for the adeno-associated viral (AAV) vectors based anti-HCV RNAi regimen that has been shown to be able to evoke liver toxicity in mice when used at high doses<sup>46</sup>. The possible cause for this toxicity could be the saturation of the endogenous miRNA processing machinery by overexpressed shRNA<sup>47</sup>, but the exact mechanism remains unclear. Although certain lentiviral RNAi systems, such as some commercial RNAi libraries, express high levels of shRNA and cause disturbance of the cellular miRNA

machinery, no significant cell toxicity was observed<sup>48</sup>. Lentiviral RNAi vectors used in our study express moderate levels of shRNA without clear effects on the miRNA pathway<sup>48</sup>. But the major challenge to produce sufficient vectors to target the entire liver remains. Hence, the transfer of gene silencing to neighbouring non-transduced cells could indeed help overcome this issue.



**Figure 1:** Lentiviral vector design and miR-122 expression in epithelial cell lines.

### **Exosomes transmit small RNAs and HCV from one cell to another in hepatocytes.**

Exosomes are believed to be present in all body fluids, and represent a new way of thinking about cell signaling. These small extracellular vesicles are thought to play a role in a large number of biological functions. Exosomes have been described to act as vehicles of genetic exchange between cells<sup>49</sup>. In my thesis we focused on exosomes as carriers of small RNAs and virus from one cell to another, that could potentially affect the functioning of a recipient cell.

Exosomes isolated from Huh7 cells were first put through a mass spectrometric (MS) analysis to detect the various proteins present. This is shown in

**Chapter 4 and 5.** These exosomes from Huh7 cells contained small RNAs and RNA binding proteins (Chapter 2, Figure 3) along with many common proteins and established exosome markers<sup>50</sup> (Chapter 4, Figure 1). MiR122 as demonstrated by Valadi et al.,<sup>49</sup> was confirmed to be present in exosomes derived from hepatocytes. We further show the involvement of exosomes isolated from Huh7 cells in transmission of small silencing RNAs (Chapter 2, Figure 4). Treatment of Huh7-ET replicon cells with purified exosomes derived from shNS5b-CM significantly reduced viral replication (Fig 4C) and similarly, treatment of normal Huh7 cells with purified exosomes derived from shCD81-CM resulted in a significant down regulation of CD81 cell surface expression (Fig 4D). These results indicate that there is exosome-mediated functional transmission of small silencing RNAs. Hence, transmission of gene silencing is partially mediated by exchange of secreted exosomes. Similar exchange of small RNAs was shown in other mammalian cells, like mouse or human mast cell lines,<sup>49</sup> the African green monkey kidney fibroblast-like cell line,<sup>51</sup> and human glioma, embryonic kidney, Epstein–Barr virus positive nasopharyngeal carcinoma and B lymphocyte cell lines<sup>51-54</sup>.

The role of vesicles in the transfer of pathogen-derived antigens and virulence factors has emerged<sup>55-57</sup>. There has been a link with the possible involvement of exosomes in liver diseases such as hepatocellular carcinoma (HCC)<sup>58</sup>, hepatitis C (HCV)<sup>59</sup> and liver inflammation<sup>60</sup>. Here we investigated the role of exosomes in the cell-to-cell transmission route of HCV between hepatocytes. Studies demonstrate that viral RNA is associated with exosomes in HCV infected patients<sup>61</sup> and HCV envelope proteins are associated with exosomes<sup>59</sup>. There are also studies to show that hepatocyte-derived exosomes can transfer viral RNA to plasmacytoid dendritic cells triggering their activation and interferon- $\alpha$  production<sup>62</sup>. HCV, unlike hepatitis B virus, is known to evade neutralizing antibodies. One of the strategies the virus might be exploiting is by recruiting the biogenesis of exosomes. This route of infection for HCV is investigated in **Chapter 5**. In this chapter we explored the possibility of exosomes being carriers of HCV from one cell to another resulting in a productive infection. In other words, is HCV transmitted to uninfected cells via the exosome-route of transmission? In our study, we first confirmed the presence of exosomes in our isolations through mass spectrometry and electron microscopy (Figure 1). Further, through immunogold labeling we could detect the presence of viral envelope proteins

in exosomes isolated from HCV infected Huh7.5.1 but not in exosomes from naïve Huh7.5.1 cells. We further determined that infected exosomes could transfer HCV to naïve cells and establish a productive infection (Figure 2). However, this transmission is only partially resistant to neutralizing antibodies (nAbs). We show in the presence of primary neutralizing immunoglobulins that the HCV transmission via exosomes was partial and that it varied for serum from patient to patient (Figure 3). The partial neutralization can be attributed to various reasons, such as the presence of free virus in our samples. So, the fact that free virus particles are present in the isolated exosome samples could be a contributing factor. We have however tried to address this question with the use of a subgenomic replicon HCV model, which does not produce free virus in culture. The exosomes derived from Huh7-ET cells were not inhibited by neutralization (Fig. 4B). Our findings support the conclusion that the HCV viral genome can be transmitted to naïve cells in the absence of viral core and envelope proteins, be it at a much lower level of infection. Surprisingly, several of the IgGs enhanced the transmission of Huh7-ET derived exosomes, but the reason for this effect is unknown. The requirement of HCV receptors for the uptake of HCV positive exosomes was tested. To specifically address the effect of entry receptor blocking antibodies on exosome-mediated transmission of HCV, we examined exosomes derived from Huh7-ET. Results for three entry receptors are shown in Figure 4C. For this, naïve Huh7.5.1 cells were blocked with anti-CD81, anti-SR-BI and anti-CLND1 antibody prior to adding the Huh7-ET derived exosomes. Some inhibition of HCV RNA transfer was observed for all three-entry receptors but their role in exosome uptake is unclear. HCV entry receptors CD81 and SR-BI are known to localize in lipid rafts<sup>63, 64</sup> and study on endocytosis of exosomes identified an important role for lipid rafts and caveolins as factors for uptake<sup>65</sup>, hence supporting a hypothetical role of these receptors in exosome uptake by hepatocytes. Further research is required to determine different pathways involved in uptake of exosomes and HCV virions.

We extend our hypothesis in **Chapter 6**, where we discuss the transmission of HCV via exosomes through the feco-oral route. The Nature article by Feng et.al., compellingly demonstrated the importance of the exosome pathway in the biogenesis and transmission of Hepatitis A virus (HAV)<sup>66</sup>. They state that HAV is cloaked in host derived membranes, resembling exosomes, thereby preventing it from antibody mediated neutralization. They term this virus as enveloped HAV (eHAV) which was



shown to be the dominant form released from infected cell cultures and circulates in the blood of infected humans and chimpanzees. They conclude that HAV exploits the exosome-like vesicles for its transmission and show that proteins involved in the exosomal pathway (ESCRT proteins) play an important role in virus transmission. This promotes viral spread in the liver and makes the distinction between enveloped and non-enveloped viruses blurry. HAV is known to be excreted from the liver via blood and bile and exits the host via the stool<sup>67</sup>. There is no direct evidence that the membranes protect the otherwise very stable HAV capsid in the intestinal tract or outside environment. However, exosomes were shown to be resistant to bile toxicity and hence protect their contents from degradation<sup>68</sup>.

We further speculated on this concept in the setting of chronic HCV infection. Studies in the past have shown the presence of HCV RNA in bile<sup>69,70</sup>. Could the virus be set in the context of an exosome and hence protected from degradation, like was demonstrated for HAV? We isolated exosomes from bile obtained from the gallbladders of chronic HCV patients at the time of liver transplantation. HCV genomic RNA could be detected in all exosome fractions of our HCV positive bile samples. HCV was not detectable in the total bile fraction, suggesting that viral RNA is enriched and protected in the biliary exosome fraction. This could open up a discussion on the spread of HCV via fecal transmission, like HAV.

### **Concluding statements (take-home messages)**

1. The ability of small interfering RNAs to transmit from one hepatic cell to another extends the research on RNAi based therapy for the treatment of liver disease.
2. The potential of miRNAs as early biomarkers for liver injury and rejection can in the future make a non-invasive diagnostic tool as a 'state of the art' technique.
3. Never underestimate the competence of viruses; they have more than one way to escape war against them. The transmission of HCV via exosomes could be one of these ways.
4. Evidence of secretion of HCV from hepatocytes to bile could re-ignite the discussion about the feco-oral route of transmission of this virus.

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## Summary

The liver mass constitutes about 75%-85% of hepatocytes. These cells take part in various metabolic functions like protein synthesis, breaking down of triglycerides and lipids. In case of some liver diseases, the hepatocytes are irreparably damaged resulting in cell death and fibrosis. Liver disease is an important cause of morbidity and mortality across the world. It also disproportionately affects minority individuals and the economically disadvantaged. In end-stage liver failure, liver transplantation is still the only treatment option. In order to establish alternative treatment options for liver diseases, it is important to develop early diagnostic markers that would provide a tool for the early detection of liver transplant rejection. Early detection of rejection can help in preventing graft loss due to timely start of immunosuppressive treatments. Hepatitis C virus (HCV) associated cirrhosis, being the most common indication for liver transplantation, remains a problem after transplantation and recurrent hepatic infection is also one of the leading causes for graft failure.

In this thesis, I therefore aimed at investigating HCV infection routes, in the context of liver transplantation, and the routes of RNA secretion by hepatocytes that may serve as novel biomarkers for hepatocyte damage and extend use of RNA interference (RNAi) mediated gene silencing against HCV. Hence, we structured out research in an attempt to obtain early biomarkers for liver injury and augment the understanding of the biology of HCV to enhance the current knowledge on the transmission of HCV infection.

### **Release of hepatocyte-derived miRNAs in response to injury**

Hepatocytes contain micro RNAs (miRNAs) along with small RNAs. These miRNAs termed as hepatocyte-derived miRNAs (HDmiRNAs) hugely influence the biogenesis of a cell. In **Chapter 2**, we demonstrate how useful these miRNAs can be in detecting post transplant liver injury and also aid in indicating acute transplant rejection. Hence asserting them as liver biomarkers apart from their various biological roles in gene expression. Circulating HDmiRs, miR-122, miR-148a and miR-194, are shown to be stable and detectable during hepatic injury in patients after liver transplantation. The levels of two of these HDmiRs showed higher sensitivity than serum transaminases during post-transplant acute rejection. This could help us bring a

better balance in using immunosuppressive drugs. Often liver biopsies are taken to confirm acute rejection, as transaminases are not adequate. This poses a risk for complications in the setting of liver transplantation. The use of miRNAs as potential biomarkers provides us with the opportunity to determine rejection with minimally invasive procedures.

Studies on human miRNAs in bile have shown that despite the hostile environment they were stable and protected from degradation. In this study two HDmiRNAs, miR122 and miR192 were also present in bile. Correlation between their levels in serum was not focused. For polarized cells like hepatocytes, the question remains whether the release of miRNAs can occur bidirectionally into bile and blood. Hence in **Chapter 3** we investigated paired bile and serum samples during liver injury and reduced liver function after transplantation.

We describe how miRNAs are also excreted into bile from hepatocytes. Only a small portion of HDmiRs were found in exosomes. Most of the bile HDmiRs were associated with a protein complex that protected the miRNAs against degradation. The release of HDmiRs into bile was not increased during liver injury but rather increased during good bilirubin secretion from the liver into the bile. These results indicate that in a healthy liver, the release of miRNAs from hepatocytes is regulated, and is directed towards the bile. During liver damage, this seems to turn around and the release of miRNAs from hepatocytes is towards the blood.

### **RNA interference in the biology and therapy of HCV**

The conventional antiviral therapy for HCV is still based on interferon- $\alpha$  (IFN- $\alpha$ ). Given the large infected population and the large number of non-responders to IFN- $\alpha$  based therapy, novel antiviral strategies are emerging but as well remain a challenge. The basic understanding of HCV has progressed heavily due to RNAi based gene silencing library technologies. In **Chapter 4**, we explored integrating lentiviral vector expressing shRNA to develop a stable and long-term siRNA expression system that could serve well as an antiviral RNAi based therapy for HCV. This expression system overcomes the Adeno-associated viral (AAV) vector based anti-HCV RNAi regimen shown to evoke liver toxicity in mice ultimately causing death. We observed in our study that small RNA was transferred to non-transduced cells, which overcame the pitfall of suboptimal vector transduction. However, it is impossible to achieve 100%



transduction efficacy. The transmission of gene silencing was seen to be cell-cell contact independent and appeared to recruit a secretory pathway that partially involved exosomes. This extends the therapeutic reach of RNAi and bears a clinical relevance in the treatment of liver diseases, especially HCV. It would ensure long-lasting beneficial effects and can prevent recurrence in HCV positive transplant patients.

### **Exosomes transmit small RNAs and HCV from one hepatocyte to another**

Exosomes have been described to serve as vehicles of genetic exchange between cells. We focused on exosomes as carriers of small RNAs and viruses from one cell to another, which could potentially affect the functioning of a recipient cell. We show the involvement of exosomes isolated from Huh7 cells in transmission of small silencing RNAs in Chapter 2.

In **Chapter 5** we investigated the possibility of exosomes being carriers of HCV from one cell to another and establishing a productive infection. In other words, is HCV transmitted to uninfected cells via the exosome route of transmission? Though there were previous studies that associated HCV with exosomes, we are the first to demonstrate that exosomes can shuttle virus to hepatocyte-like cells. We further found that this route of transmission of infection is partly resistant to neutralizing antibodies. HCV is known to evade neutralizing antibodies. So, this could be a contributing factor to the known evasive properties of the virus. We have also speculated as to why the transmission was not completely resistant to neutralizing antibodies. It could be due to the presence of free virus particles in the isolated exosome samples. With the current knowledge it is impossible to quantify the relative contributions of cell free and exosome associated virus to HCV spread. We have however tried to answer this question to our best with the use of a subgenomic replicon HCV model (no free virus in culture). The data support the conclusion that the HCV viral genome can be transmitted to naïve cells in the absence of viral core and envelope proteins. However, there has been no strong conclusion reached and this discussion remains speculative. The other interesting observation made in these experiments is that HCV entry receptors may partly contribute to exosome uptake even in the absence of viral envelope or core proteins. Additional experiments would go a long way toward firming up the conclusions and to unravel the pathways

involved in exosome and HCV uptake.

It was interesting to extend this study to the spread of HCV via other routes, especially the feco-oral route of transmission. In **Chapter 6** we discuss the Nature article by Feng *et.al.*, on Hepatitis A virus (HAV) and the importance of the exosome pathway in HAV biogenesis. They conclude that HAV exploits the exosome-like vesicles for its transmission and show that proteins involved in the exosomal pathway (ESCRT proteins) play an important role in virus transmission. Exosomes were shown to be resistant to bile toxicity and hence protect their contents from degradation. An encapsulated HAV is excreted from the liver via the bile and exits the host via the stool. We further speculate on this concept in the setting of HCV. Studies in the past have shown the presence of HCV RNA in bile. Could the virus be set in the context of an exosome and hence be protected from degradation too? We explored this hypothesis by studying exosomes isolated from bile obtained from the gallbladders of chronic HCV patients at the time of liver transplantation. HCV genomic RNA could be detected in all our HCV positive exosome samples and not in total bile. Like HAV, this could open up a discussion on the spread of HCV via fecal transmission.

## **Nederlandse Samenvatting**

Een groot deel van de massa van de lever bestaat uit hepatocyten. Deze cellen zijn verantwoordelijk voor de belangrijkste metabole functies van de lever. In de hepatocyten vindt bijvoorbeeld eiwitsynthese plaats, en worden lipiden en triglyceriden afgebroken. Bij ziekten van de lever zoals hepatitis of kanker, of door bijvoorbeeld alcoholmisbruik, kunnen de hepatocyten onherstelbaar beschadigd worden. Bij eindstadium leverfalen is levertransplantatie dan nog de enige behandelbaarheid.

De meest voorkomende indicatie voor levertransplantatie is Hepatitis C virus (HCV) geassocieerde lever cirrose. Een groot deel van met HCV geïnfecteerde patiënten reageert niet op de standaard antivirale therapie, of verdraagt deze niet, waardoor uiteindelijk een levertransplantatie noodzakelijk is. Helaas blijft de HCV infectie ook na transplantatie een probleem, en is vaak een van de oorzaken van leverfalen na transplantatie. Ook is het detecteren van schade aan hepatocyten door HCV met de huidige serum biomarkers vaak niet goed mogelijk.

In dit proefschrift heb ik mij daarom gericht op het onderzoeken van HCV infectie routes, bijvoorbeeld in de context van levertransplantatie, en de routes van RNA secretie door hepatocyten die mogelijk als nieuwe biomarkers voor hepatocyte schade kunnen dienen.

### **Het vrijkomen van miRNAs uit hepatocyten bij leverschade**

Op dit moment zijn de leverenzymen aspartaat transaminase (AST) en alanine transaminase (ALT) de meest gebruikte biomarkers voor het vaststellen van leverschade. Bij patiënten die een levertransplantatie hebben ondergaan is het belangrijk om een vroege en gevoelige marker te hebben om schade aan de lever of afstotingsverschijnselen te kunnen vaststellen. Voor een juiste diagnose is het echter altijd nodig om een leverbiopt te nemen, omdat AST en ALT in het bloed ook verhoogd kunnen zijn bij spierschade. Het nemen van een leverbiopt is echter belastend voor de patiënt, en is geassocieerd met pijn, bloedingen en infecties.

Het microRNA (miRNA), miR-122 is het meest voorkomende microRNA in hepatocyten. Het is betrokken bij de regulatie van verschillende metabole

processen, en is een essentiële factor voor infectie en replicatie van HCV. In proefdieren is aangetoond dat miR-122 en andere hepatocyt-abundante miRNAs (HDmiRs) in serum of plasma kunnen worden aangetoond bij leverschade. Dit correleerde met AST, ALT en lever histologie. Ook bij patiënten met leverschade is miR-122 in serum verhoogd. Bij patiënten die een levertransplantatie hebben ondergaan was dit echter nog niet onderzocht. In **Hoofdstuk 2** hebben we onderzocht of HDmiRs gebruikt kunnen worden om leverschade bij transplantatiepatiënten aan te tonen. Zowel bij patiënten met leverschade na transplantatie als bij patiënten met acute afstoting bleken HDmiRs in het serum verhoogd te zijn. De niveaus van HDmiRs bleken eerder te stijgen en te dalen dan de transaminases, en HDmiRs zouden daarom kunnen dienen als biomarkers voor het bepalen van leverschade en afstoting zonder dat er uitgebreide invasieve procedures nodig zijn.

### **RNA interferentie in de biologie en therapie voor HCV**

In het planten en dierenrijk is RNA interferentie (RNAi) is een belangrijk onderdeel van het defensiemechanisme van een cel tegen virale pathogenen, en dit is afhankelijk van de overdracht van klein interfererend RNA naar naburige cellen. Met behulp van interfererend RNA kunnen genen in de cel specifiek uitgeschakeld worden. Wij hebben onderzocht of RNAi, dat met behulp van een vector in de cel is ingebracht, kan worden uitgewisseld tussen levercellen in vitro en in vivo, en of deze uitwisseling ingezet kan worden voor therapeutische doeleinden bij HCV infectie. Zoals beschreven in **Hoofdstuk 4**, is gevonden dat klein RNA inderdaad kan worden overgedragen tussen levercellen, en dat dit mechanisme effectief kan zijn bij het remmen van HCV infectie.

Ongeveer de helft van de HCV patiënten die worden behandeld met peg-IFN- $\alpha$  en ribavarine bereiken een volledige virologische respons. Bij patiënten met een genotype 1 infectie gebeurt dit bij ongeveer 40%. Met de introductie van de zogenaamde ‘directly acting antivirals (DAAs)’, in combinatie met peg-IFN- $\alpha$  en ribavarine zal naar verwachting het percentage patiënten met genotype I infectie dat reageert op therapie stijgen naar 90%. De ontwikkeling van nieuwe antivirale middelen blijft echter nog steeds noodzakelijk, en op RNAi gebaseerde therapieën kunnen hier mogelijk een bijdrage aan leveren. Wij hebben onderzocht of met behulp van lentivirale vectoren een stabiel systeem kan worden verkregen voor de expressie

van siRNA, wat als basis kan dienen voor antivirale therapie. Dit expressie systeem is een alternatief voor expressie met adenovirale vectoren, waarvan is gebleken dat dit in muizen bij hogere dosis levertoxiciteit kan geven. De lentivirale vectoren in ons onderzoek brachten gematigde hoeveelheden klein interfererend RNA tot expressie, zonder duidelijke negatieve effecten op de microRNA biosynthese.

### **Exosomen transporteren kleine RNAs en HCV tussen hepatocyten**

Exosomen zijn kleine extracellulaire blaasjes die een rol spelen bij allerlei biologische processen, zoals het uitwisselen van genetische informatie tussen cellen. In dit proefschrift hebben wij onderzocht wat de rol van exosomen zou kunnen zijn bij het overdragen van kleine RNAs en virussen tussen cellen, en welke gevolgen dit kan hebben voor het functioneren van de cel.

Exosomen die geïsoleerd zijn uit Huh7 hepatoma cellen zijn geanalyseerd met massaspectrometrie. Naast een groot aantal eiwitten bleken de exosomen ook RNA moleculen, RNA bindende eiwitten en oa. miR-122 te bevatten. Zoals beschreven in **Hoofdstuk 4**, bleken exosomen in staat te zijn om RNAs tussen hepatocyten over te dragen: exosomen van cellen die getransfecteerd zijn met shNS5b (gericht tegen HCV) en exosomen van cellen die getransfecteerd waren met shCD81 waren in staat om respectievelijk HCV replicatie en expressie van CD81 te verminderen in cellen die met deze exosomen werden behandeld. Hiermee kon worden aangetoond dat exosomen in staat zijn tot functionele transmissie van siRNAs.

Exosomen kunnen ook een rol spelen bij leverziekten zoals hepatocellulaire carcinoma (HCC), HCV en leverontsteking. Wij hebben onderzocht of HCV via exosomen naar ongeïnfecteerde cellen kan worden overgedragen. Zoals beschreven in **Hoofdstuk 5**, konden specifieke virale eiwitten in exosomen afkomstig van geïnfecteerde cellen worden aangetoond. Deze virus-positieve exosomen waren in staat om nieuwe cellen te infecteren, en een productieve infectie te veroorzaken. Deze overdracht van infectie kon slechts gedeeltelijk worden voorkomen met neutraliserende antilichamen. Het feit dat virus partikels in exosomen kunnen worden aangetroffen kan bijdragen aan de verklaring waarom HCV kan ontsnappen aan het immuunsysteem. Om de rol van exosomen bij de overdracht van HCV infectie volledig te verklaren is echter meer onderzoek nodig.

### **Hepatocyten secreteren miRNA en HCV exosomen in gal**

Exosomen kunnen misschien betrokken zijn bij overdracht van HCV door gal en transmissie via de fecale-orale route. Zoals beschreven in **Hoofdstuk 6**, is recent voor Hepatitis A virus (HAV) aangetoond dat het virus zich in exosoom-achtige partikels kan bevinden. HAV wordt via de lever uitgescheiden in bloed en gal, en verlaat het lichaam via de ontlasting. De exosoom-achtige partikels beschermen HAV tegen afbraak in de toxische omgeving van de gal. Via eerdere onderzoeken is aangetoond dat ook HCV RNA in gal aanwezig kan zijn. Wij hebben daarom onderzocht of HCV in gal zich in exosomen bevindt. Wij konden exosomen isoleren uit gal van verschillende met HCV geïnfecteerde patiënten en waren in staat om in de exosoom fractie HCV RNA aan te tonen, in tegenstelling tot in ongefractioneerde gal. Dit zou er op kunnen duiden dat ook HCV via de orale-fecale route kan worden overgedragen.

Ten slotte, in **Hoofdstuk 3** beschrijven we hoe ook miRNAs uit hepatocyten worden uitgescheiden naar gal. Slechts een kleine fractie van de HDmiRs waren terug te vinden in exosomen. Het merendeel van de gal HDmiRs waren geassocieerd met een eiwit complex dat de miRNAs beschermd tegen afbraak. Het vrijkomen van HDmiRs in gal was niet verhoogd tijdens leverschade maar juist verhoogd tijdens goede bilirubine secretie van de lever naar het gal. Deze resultaten geven aan dat het vrijkomen van miRNAs uit hepatocyten is gereguleerd en bij een goede leverfunctie met name is gericht richting het gal. Bij leverschade lijkt dit zich om te draaien en blijkt het vrijkomen van miRNAs uit hepatocyten met name richting het bloed plaats te vinden.



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## **List of Publications**

**V.Ramakrishnaiah, C.Thumann, I.Fofana *et.al.***, Exosome-mediated transmission of hepatitis C virus between human hepatoma Huh7.5 cells.

*Proc Natl Acad Sci U S A. 2013 Aug 6;110(32):13109-13*

**V.Ramakrishnaiah and LJ van der laan.** Hepatitis Virus Hijacks Shuttle: Exosome-Like Vesicles Provide Protection Against Neutralizing Antibodies.

*Hepatology. 2013 Nov 22*

**Q.Pan, V.Ramakrishnaiah, S.Henry *et al.***, Hepatic cell-to-cell transmission of small silencing RNA can extend the therapeutic reach of RNA interference (RNAi).

*Gut. 2012 Sep;61(9):1330-9*

**WR Farid, Q Pan , AJ van der Meer , PE de Ruiter , V Ramakrishnaiah *et al.***, Hepatocyte-derived microRNAs as serum biomarkers of hepatic injury and rejection after liver transplantation.

*Liver Transpl. 2012 Mar;18(3):290-7*

**WR Farid , CJ Verhoeven, V Ramakrishnaiah *et al.***, Bi-directional release of hepatocytes-derived microRNAs to bile and blood: Relation with liver injury and bilirubin secretion.

*In preparation*

## **Curriculum vitae**

Vedashree Ramakrishnaiah was born in Bangalore on the 11<sup>th</sup> of August 1979. In 1997 she started her Bachelor studies in Dentistry at the Yenepoya Dental College, Mangalore, Karnataka, India from which she graduated in 2002. At the university of Wageningen in The Netherlands, she started her Master education in Medical Biotechnology. As a student she performed research on 'Role of DMI1 and DMI2 genes in the evolution of *Medicago truncatula*' at the department of molecular biology under the supervision of Dr. Rene Gerts. For her intership she researched on 'Differentiation of mesenchymal stem cells to endothelial cells' at the University of Twente, Netherlands, under the supervision of Dr. Nicolas Rivron. She started her PhD in January 2009 at the Department of Surgery under the supervision of her promoter Prof.dr. H.W.Tilanus and co-promoter Dr. L.J.W. van der Laan and the results of her work are presented in this thesis.

## **PhD Portfolio**

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PhD Period	January 2009 – August 2012
Promotor	Prof.dr. H.W. Tilanus
Copromotor	Dr. L.J.W. van der Laan

### **General Courses**

The international course on laboratory animal science (Art.9)  
Animal imaging workshop  
English Biomedical Writing and Communication  
Photoshop CS3 workshop

### **Oral Presentations at National and International Conferences**

- 2010, The 61<sup>st</sup> annual meeting of the American Association for the Study of Liver Disease, San Francisco, CA, USA
- 2011, The annual American Transplant Congress, Philadelphia, USA
- 2011, The annual conference of the Dutch Association of Hepatology, Netherlands
- 2011, The joint International Congress of ILTS, ELITA and LICAGE, Valencia, Spain.

### **Academic Awards**

- 'Best Student Research Award' at international conference AASLD conference 2010, held at Boston, USA. (1000 US\$ prize money)
- Awarded 'Best Abstract Prize' DEGH Dutch conference 2011, Veldhoven (€500 prize money)
- Awarded 'Best Abstract Prize' Surgery day, Erasmus Medical Center, 2011
- Novartis Transplantation Award 2014 for the best basic science article published (€1500 prize money)



