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Characterization of Structural and Functional Liver Changes in a Transgenic Mouse Model Expressing Genotype 1a Hepatitis C Virus Core and Envelope Proteins 1 and 2

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Characterization of Structural and Functional Liver Changes in a Transgenic Mouse Model Expressing Genotype 1a Hepatitis C Virus Core and Envelope Proteins 1 and 2

By

Turaya Naas

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ABSTRACT

Hepatitis C virus (HCV) is a major cause of chronic hepatitis affecting over 170 million people worldwide. The mechanisms behind the pathogenesis of chronic HCV infection are not well understood. The aim of this thesis is to elucidate how the HCV structural proteins affect the dynamic structural and functional properties of hepatocytes and measure the extra-hepatic manifestations induced by these viral proteins. To pursue this goal, a transgenic mouse model was established by expressing Core, E1 and E2 proteins downstream of a CMV promoter. HCV RNA was detected in transgenic mouse model tissues, such as liver, kidney, spleen, and heart. Immunofluorescence analysis revealed the expression of core, E1 and E2 proteins predominantly in hepatocytes. Histological analysis of liver cells demonstrated steatosis in transgenic mice older than 3 months, which progressed with mouse age. Electron microscopy analysis revealed alterations in mitochondria, and in the endoplasmic reticulum. The animals became more prone to liver and lymphoid tumor development and hepatocellular carcinoma. It is likely that the HCV structural proteins mediate some of the histological alterations in hepatocytes by interfering with liver biological processes. To study that, we compare differential gene expression patterns in the liver of HCV transgenic and non-transgenic mice using complementary DNA (cDNA) microarrays and confirmed the expression of altered genes by real-time RT-PCR. 15 600 genes were analyzed and 394 genes were identified to be differentially expressed at a statistically significant level, while 196 genes were identified as up-regulated and 198 genes were identified as down-regulated. The expression of HCV structural proteins in transgenic mouse livers induces alterations in the expression of genes involved in many important biological processes such as lipid metabolism and transport, antigen processing and presentation, protein biosynthesis,

carcinogenesis, etc. Also, some of the differentially expressed genes were associated with important cell signaling pathways such as Mitogen activated protein kinase (MAPK) pathway and Wnt oncogenic pathway. The third study aims to elucidate the role of immune-mediated cell damage in hepatitis C infection using HCV Transgenic mice. Adoptive transfer of carboxyfluorescein diacetate suuccinimidyl ester (CFSE) labelled splenocytes from HCV immunized mice into HCV transgenic mice was performed. After the adoptive transfer, we observed that there was a significant decrease in the percentage of CFSE-labeled CD4+ and CD8+ T cells in the blood of transgenic mice receiving transfers from immunized donors. Moreover, the percentage of CFSE-labeled CD4+ and CD8+ T cells were significantly higher in the spleen of transgenic and non-transgenic mice when they received transfer from non-immunized mice. Interestingly, transgenic livers of mice received transfers from immunized mice had significantly high percentage of CFSE-labeled T cells than non-transgenic livers receiving non-immunized transfers. This suggests that the T cells from HCV immunized mice recognized HCV antigens in the liver. Taken together, the research results indicate that our transgenic mouse model is a suitable model to study hepatitis C pathogenesis.

DEDICATION

I dedicate this thesis to my husband, for his endless support and encouragement during the course of my studies, to my daughter to inspire her, to my family for their never-ending encouragement, and to my father's immortal soul.

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Acaa1	Acetyl-Coenzyme A acyltransferase 1
Acox1	Acyl-Coenzyme A oxidase 1
Acsl-3	Acyl-CoA synthetase long-chain family member 3
ALT	Alanine aminotransferase
AOX	Acyl CoA oxidase
AP-1	Activator protein 1
Apoa1	Apolipoprotein A-I
Apoa4	Apolipoprotein A-IV
Apoc2	Apolipoprotein C-II
Arl6ip2	ADP-ribosylation factor–like 6 interacting protein 2
Bag4	Bcl2-associated athanogene 4
Bcap31	B-cell receptor associated protein 31
BSA	Bovine serum albumin
C4bp	Complement component 4 binding protein
CCR7+	Chemokine receptor 7 positive
CDK	Cyclin-dependent kinase
CFSE	Carboxyfluorescein diacetate succinimidyl ester
CHO	Chinese hamster ovary
c-Jun	Jun oncogene
CLIP	Class II-associated Ii peptide
CMV	Cytomegalo virus
CPT-1	Carnitine palmitoyl transferase -1
CTL	Cytotoxic T lymphocyte
Ctsl	Cathepsin L
DA	Dominancy Analysis
DAG	Diacylglycerol
DC	Dendritic cell
DC-SIGN	DC-specific- intercellular adhesion molecule-3-grabbing nonintegrin.
DMSO	Dimethyl Sulfoxide
ds-RNA	Double stranded RNA
E1	Envelope 1
E2	Envelope 2
Eef1α	Eukaryotic translation elongation factors 1 alpha
ELISA	Enzyme- linked immunosorbant assay
ER	Endoplasmic reticulum
ERKs	Extracellular signal-regulated kinases
Fas-L	Fas ligand
FCS	Fecal calf serum
FITC	Fluorescein isothiocyanate
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GAPs	GTPase-activating proteins
GO	Gene Ontology

Gp	Glycoprotien
GTP	Guanosine triphosphate
H2-Aa	Histocompatibility 2, class II antigen A, alpha
H2-DM b1	MHC class II, locus Mb1
H_2O_2	Hydrogen peroxide
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HDL	High-density lipoproteins
HIV	Human immunodeficiency virus
HMG	High mobility group
Hsp70	Heat shock proteins 70
HVR1	Hypervariable region 1
HVR2	Hypervariable region 2
HVR3	Hypervariable region 3
ICAM	Intercellular adhesion molecule
IFN	Interferon
IFN-α	Interferon alpha
IFN-γ	Interferon gamma
IgG	Immunoglobulin G
IgG1	Immunoglobulin G 1
IgG2a	Immunoglobulin G 2a
Ii	Invariant chain
IL	Interleukin
IL-10	Interleukin-10
IMDM	Iscove's Modified Dulbecco's Medium
IRES	Internal ribosomal entry site
ISGs	IFN-stimulating genes
JNKs	c-Jun amino-terminal kinases
KEGG	Kyoto Encyclopedia of Genes and Genomes
LDL	Low density lipoprotein
LDLR	Low density lipoprotein receptor
LIT	Low-intensity threshold
LKM1	Liver and kidney microsome
LPL	Lipoprotein lipase
L-SIGN	Liver/lymph node–specific intercellular adhesion molecule-3-grabbing nonintegrin.
MAPK	Mitogen activated protein kinase
MAPKK	Mitogen activated protein kinase kinase
MAPKKK	Mitogen activated protein kinase kinase kinase
MAPKKKK	Mitogen activated protein kinase kinase kinase kinase
MHC II	Major Histocompatibility Complex class II
MKK3	Mitogen activated protein kinase kinase 3
Mod1	Malic enzyme
MTP	Microsomal triglyceride transfer
NANBH	Non-A, non-B hepatitis
NF-κB	Nuclear Factor kappa B

NK	Natural killer
NS	Non-structural
OD	Optical density
Osbpl3	Oxysterol binding protein-like 3
Pak2	p21-activated kinase 2
PAKs	p21-activated kinases
PBMCs	Peripheral blood mononuclear cells
PKC-βI	Protein kinase C, beta 1
PKR	Protein kinase R
PMA	Photbol myristate acetate
PPAR	Peroxisome proliferators- activated receptor
PPARα	Peroxisome proliferators- activated receptor α
Rasa2	RAS p21 protein activator 2
pRb	retinoblastoma protein
RBCs	Red blood cells
RER	Rough ER
rRNA	Ribosomal RNA
ROS	Reactive oxygen species
RT-PCR	Reverse transcriptase polymerase chain reaction
RxRα	Retinoid receptor α
Scp2	Sterol carrier protein 2
SFC	Spot forming cell
SFCs	Spot-forming cells
SREBP-1c	Sterol regulatory element binding protein-1c
STAT3	Signal transducer and activator of transcription
Tfdp1	Transcription factor Dp1
TNF-α	Tumor necrosis factor alpha
TNFR1	Tumor necrosis factor receptor 1
UTR	Untranslated region
VLDL	Very low density lipoprotein
WAF	Wild-type p53-activated fragment

CHAPTER 1:

INTRODUCTION

1 Background:

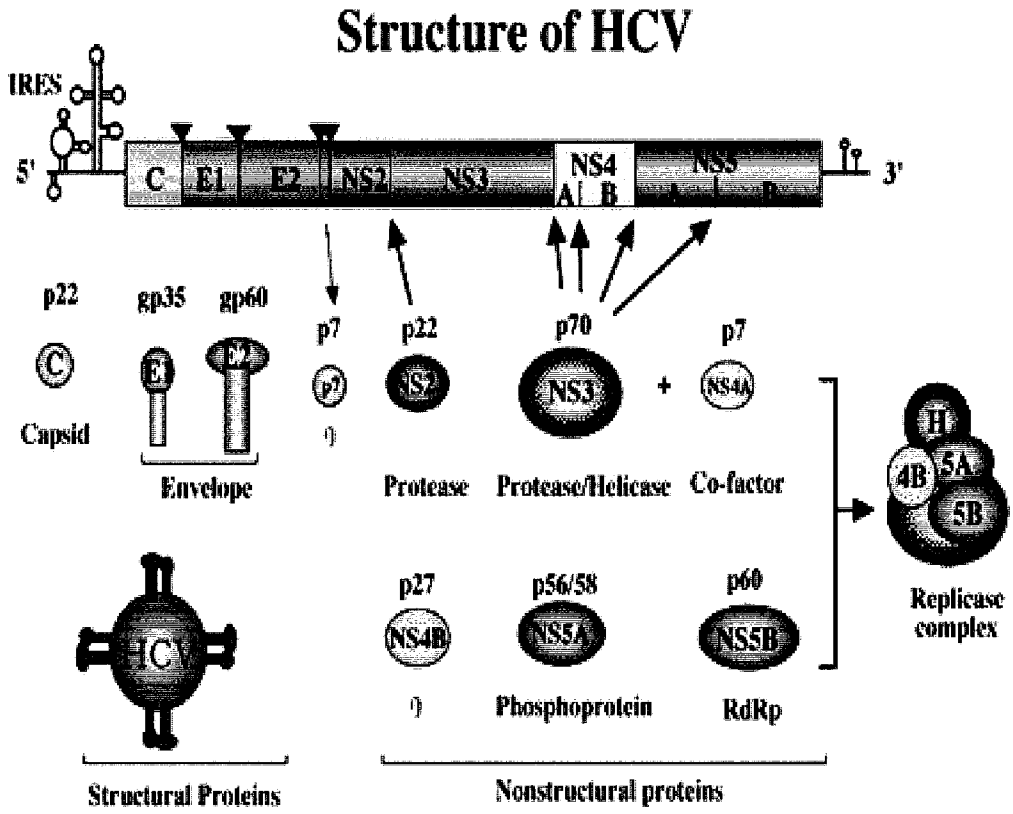
Hepatitis C virus (HCV) is a major cause of chronic hepatitis [1-3]. More than 170 million people are infected with HCV worldwide including 4 million within the United States and 250,000 individuals within Canada [4,5]. About 85% of individuals acutely infected with HCV, develop a chronic infection and they are at risk of developing liver cirrhosis and hepatocellular carcinoma. HCV infection causes clinically acute disease in some cases, however, most cases of acute infection are clinically undetectable. Hepatitis C infection accounts for the death of at least 10,000 people in North America each year and it has a major economic consequences. This infection is now the leading cause of liver transplantation in developed countries [6], which accounts for 30% of all liver transplants [7,8]. Currently the only therapy available for hepatitis C infection is a combination of interferon-α and ribavirin, however, this treatment is effective in less than 50% of infected individuals [9]. The response to this treatment is affected by several factors; including intrinsic viral resistance, defective viral pathways induced by HCV proteins, or other host factors, such as obesity [10-12]. No vaccine is available yet for HCV because of the viral genomic variability and the lack of an efficient cell culture system that supports virus replication. Most of the knowledge about HCV replication is based on *in vitro* HCV models using HCV subgenomic and genomic replicons [13]. In addition, progress in research is hampered by the lack of a small susceptible animal model to study the pathogenesis. The chimpanzee is the only animal naturally susceptible to HCV infection, however this animal is very expensive and its immune response is different from humans. Thus hepatitis C infection is a major public health problem impacting the worldwide health and economy.

1.1 Hepatitis C virus:

1.1.1 Virus structure and genome organization:

Hepatitis C virus is a small (55-65 nm), enveloped positive stranded RNA virus belonging to the family *Flaviviridae*, genus *Hepacivirus* [2,14]. The viral particle consists of an envelope derived from host cell membrane, neucleocapside, and RNA genome. Two virally encoded glycoproteins (E1 and E2) are inserted into the envelope. The viral envelope surrounds a nucleocapsid and the HCV genome. The virus has a single-stranded RNA genome of positive sense, about 9,600 nucleotides (9.6 kb) in length (Fig. 1.1). The genome contains a single large open-reading frame that encodes a precursor polyprotein of approximately 3000 amino acids length, flanked by un-translated regions (UTR) at both the 5` and 3` ends, which have been shown to be essential in both initiation of translation and viral replication [15]. The 5` UTR contains an internal ribosomal entry site (IRES), the site at which the protein translation starts [16]. In addition to the polyprotein, another HCV protein with yet unknown function has recently been described. This protein is called F-protein and it is generated by ribosomal frame shifting [17]. The amino-terminal portion of the viral RNA encodes for the structural (S) proteins, Core, E1 and E2, followed by the nonstructural (NS) proteins (NS2, NS3, NS4A, NS4B, NS5A and NS5B) in the carboxy-terminal portion of the polyprotein [18,19]. Both host signal peptidase and viral-encoded proteases cleave the polyprotein into at least 10 mature structural and non-structural proteins. The structural proteins are cleaved by cellular peptidases associated with the lumen of the endoplasmic reticulum, while the non structural proteins are cleaved by viral-encoded proteases [18,19].

Fig. 1.1: Schematic representation of the HCV genome and encoded viral proteins. The boxed area corresponds to the single open reading frame of the hepatitis C virus (HCV) genome. The stem–loop structures represent the 5' and 3' Un-translated (UTR) regions, including the internal ribosome-entry site (IRES) at 5' and poly-A region at 3'. Core (C)–E1, E1–E2, E2–p7 and p7–non-structural protein 2 (NS2) junctions are cleaved by a cellular signal peptidase(s) (Red triangles) to yield structural proteins. The NS2–NS3 metalloproteinase undergoes autocatalytic cleavage, which releases the mature NS3 serine protease. NS3 cleaves the remainder of the NS polypeptide (Black arrows). The function and molecular mass (in kDa) of the gene products after polyprotein processing are shown. (Modified from www.biken.osaka-u.ac.jp/kenkyu/fuzoku/emajing_e.html)



1.1.2 HCV structural proteins:

The hepatitis C genome encodes three structural proteins, namely core, E1 and E2. The core protein is non-glycosylated and associates with genomic RNA to form the virion nucleocapsid [20]. The core protein has RNA binding activity in the conserved N-terminus which plays a role in the encapsidation of the RNA genome and formation of the viral particles [21]. Additionally, the core protein is highly conserved and immunogenic and it contains several B-cell epitopes near the N-terminus [22]. It is highly basic and has hydrophobic region at the C- terminus, which acts as a signal for the translocation of E1 to the endoplasmic reticulum and it is important for membrane-dependent processing of the core [22].

Immediately downstream from the core are two proteins, designated E1 and E2, which correspond to the envelope glycoproteins. They are produced after the cleavage of the polyprotein precursor by host signal peptidase [23]. These proteins are heavily N-glycosylated with a large N-terminal ectodomain and a C-terminal hydrophobic anchor, and they function as transmembrane proteins which are essential for virus assembly [24]. E1 and E2 glycoproteins have been shown to assemble as a non-covalent heterodimer in the endoplasmic reticulum [25], and this assembly requires the transmembrane domains of E1 and E2. The formation of E1 and E2 complex is slow and inefficient process and it may lead to the formation of misfolded and aggregated molecules [24]. The two glycoproteins likely play different functions in HCV entry. While E2 glycoprotein is involved in receptor binding [26,27], E1 has been proposed to be the fusion protein [28].

The N-terminal portion of the E2 glycoprotein contains a high degree of amino acid variation, which referred to as the hypervariable region 1 (HVR1). This region is located

between amino acids 384-410 of the polyprotein (aa 1-27 of E2) [29]. It exhibits a significant variation not only among HCV isolates but also within infected individuals over the course of time. HVR1 is located on the surface of the HCV virion and carries a neutralizing epitopes to which a humoral immune response is produced. However, sequence variation in HVR1 during infection results in the generation of variants or quasispecies which are not recognized by pre-existing antibodies[30]. The formation of these variants is considered as an essential mechanism by which HCV evades host immune response and establishes persistent infection. In addition to HVR1, there is a second hypervariable region in the E2 glycoprotein, termed HVR2, located downstream of HVR1 and consists of 9 amino acids [31]. It has a potential involvement in the cell surface receptor binding [32]. The existence of a third hypervariable region, termed HVR3, located between HVR1 and HVR2 has also been reported [33].

The viral structural proteins are flanked by p7, a small membrane polypeptide that precedes the non-structural protein region of the HCV polyprotein. It was recently shown that p7 protein is a member of the viroporin family of proteins involved in membrane permeabilization. p7 protein forms an ion channel, thus it plays an important role in viral particle release and maturation [34-36].

1.1.3 HCV non-structural proteins:

The non-structural (NS) proteins include NS2, NS3, NS4A, NS4B, NS5A, and NS5B. These proteins are likely to have a role in HCV replication and encode for proteases that are important in the cleavage of non-structural ploy protein [15]. The NS2 protein is released from its polyprotein precursor by two proteolytic cleavages. The N terminus of NS2 is cleaved from the E2/p7 polypeptide by a cellular peptidase, while the NS2-3 junction located at the C terminus is processed by a viral protease [37]. NS2 binds with the N-terminus of

NS3 and forms NS2/3 autoprotease which is responsible for the cleavage at the NS2/3 junction [38].

The NS3 protein is a bi-functional molecule having three different enzymatic activities; including serine protease activity at its N-terminus, nucleotide triphosphatase, and RNA helicase at its C terminus. The serine protease activity has been extensively characterized [39,40] and is required, in conjunction with NS4A, for optimal viral polyprotein cleavage and processing [41]. The nucleotide triphosphatase and helicase activities of NS3 are less well characterized. The RNA helicase activity is involved in strand separation during viral replication and transcription [42].

The NS4 region of the HCV genome encodes two viral proteins; NS4A and NS4B. The NS4A protein functions as a cofactor in NS3 activity. It is essential for the cleavage at NS3/4A and NS4B/5A junctions and it also enhances cleavage at the NS4A/4B and NS5A/5B sites [41,43,44]. Since the interaction of NS4A and NS3 is vital for the viral polyprotein processing, it is considered as an important target in the development of antiviral agents. Although the function of NS4A is well characterized, the function of NS4B remains unknown.

The NS5 region is processed into NS5A and NS5B. The function of NS5A has not been clearly elucidated, but it has been shown to be a part of the HCV replication complex and important for viral replication [45,46]. It also plays a role in interferon sensitivity [47]. It has been shown that there is a variable sequence in the C terminus of NS5A and this is associated with the viral resistance to interferon therapy. NS5A binds with interferon-induced double-stranded RNA activated protein kinase receptor (PKR) and causes viral resistance [48,49]. The NS5B region of the genome encodes RNA-dependent RNA polymerase, which

is critical for viral replication [15]. Thus, it has been recognized as a primary target for therapeutic agent development. Non-structural proteins are localized to the membrane of the endoplasmic reticulum, suggesting that it is where the polyprotein maturation and viral particle assembly take place.

1.1.4 HCV life cycle:

Very little is known about the replication cycle of HCV, because of the limited *in vitro* cell culture systems or a convenient animal model that can support the viral replication. The current understanding of HCV replication is based primarily on analogies to the closely related flavi- and pestiviruses and on the characterization of recombinant HCV proteins. The HCV replication cycle can be summarized as follows: (1) the virus enters the cell and is uncoated in the cytoplasm to liberate the positive stranded RNA from the virus particle into the cytoplasm; (2) there is translation of viral RNA to form the polyprotein, processing of the polyprotein, and formation of the replicase complex; (3) viral RNA is transcribed to form a complementary negative-sense RNA molecule which serves as a template for the synthesis of positive-strand RNA molecules associated with intracellular membranes; (4) packaging into progeny virions; (5) release of virus from the infected cell.

The first step in HCV life cycle is the attachment of the viral particle to the host cell. There is a specific interaction between a cell surface receptor and a viral attachment protein on the surface of the virus. Several cellular molecules have been identified as a receptor for HCV, such as CD81 tetraspanin [27], scavenger receptor class B type I [50], mannose-binding lectins DC-SIGN and L-SIGN [51-53], low-density lipoprotein receptor [54], heparin sulphate proteoglycans [55] and the asialoglicoprotein receptor [56]. Because of the lack of cell culture system supporting HCV replication, the discovery of these receptors was based

on their interaction with soluble, truncated forms of HCV glycoprotein E2. The envelope glycoprotein E2 is thought to be responsible for initiating virus attachment to the host cell because E2-specific antisera can block HCV binding to the cells [57,58]. It is not known if the interaction between HCV E2 glycoprotein and HCV receptors leads to internalization of the virus and results in a productive infection. However, recent studies have indicated that HCV enters target cells by clathrin-mediated endocytosis, followed by a fusion step from within an acidic endosomal compartment [59,60].

Once the HCV RNA enters the cell, it is directly translated to a polyprotein precursor. The translation of the viral RNA is mediated by IRES dependent mechanism [16,61]. The polyprotein is translated at the rough endoplasmic reticulum (RER) and cleaved co- and post-translationally by cellular peptidase and via NS2-3 and NS3 proteases, as mentioned above. After the translation of the polyprotein, RNA replication occurs and the NS5B (RNA-dependent RNA polymerase) catalyses the synthesis of plus-strand RNA using minus-strand RNA as a template [62]. Although most of the information about RNA replication was obtained using replicon systems, neither HCV particles nor even nucleocapsids are produced in these systems [63,64]. There is limited information on the critical steps in virion formation, including capsid assembly, genome encapsidation, budding, and release. When the structural proteins are cleaved from the polyprotein, the mature core protein assembles into HCV capsids at the cytoplasmic face of the ER [65-67]. Core protein is known to interact with the HCV envelope glycoprotein E1 at the ER, and assembled capsids are thought to acquire their envelopes by budding into the ER [65,66,68]. However, the specific details of these late events in the viral life cycle have not been elucidated.

1.1.5 HCV genotypes:

Based upon phylogenetic analysis of HCV strains isolated in different parts of the world, hepatitis C virus species is classified into six genotypes (1-6) with each genotype containing several subtypes. These subtypes are classified by sequence variation and identified by lower-case letters (i.e. 1a, 1b, 1c... 2a, 2b, 2c,..., and so on). These subtypes are further classified into quasispecies based on their genetic diversity [69]. The existence of HCV as a heterogeneous mixture of quasispecies is a consequence of the immune pressure, low fidelity of the RNA replicate machinery, and interaction with host cellular proteins. This leads to the development of escape mutants to humoral and cellular immunity, the generation of defective viral particles, variable cell tropism, and the development of drug resistance [70]. The distribution of HCV genotypes varies globally. For example in North America, genotype 1a is the predominant genotype, followed by 1b, 2a, and 3a. In Europe, genotype 1b is the predominant genotype followed by 2a, 2b, 2c, and 3a. Genotypes 4 and 5 are mostly found in Africa. The genotype of the virus is clinically important in determining potential response to interferon-based therapy. For example, genotypes 1 and 4 are less responsive to interferon treatment than are the other genotypes (2, 3, 5 and 6) [71].

1.2 Hepatitis C infection:

1.2.1 History:

In the mid 1970's, scientists from National Institutes of Health demonstrated that most hepatitis cases after blood transfusion were not due to hepatitis A and B viruses. The virus identified initially as non-A, non-B hepatitis (NANBH). In 1987, Choo *et al* started to use molecular cloning to identify the unknown virus. In 1988, the virus was confirmed by Alter *et al* by verifying its presence in a panel of NANBH specimens. In 1989, the discovery

of the virus, which was re-named Hepatitis C virus, was a major breakthrough and published in two articles in the journal *Science* [1,3].

1.2.2 Epidemiology and transmission of HCV:

Hepatitis C virus is a common pathogen, which infects more than 3% of the world population as estimated by World Health Organization in 1999 [72]. In the United States 4 million people are infected with HCV as well as 240,000 individuals in Canada [4,5,73]. Hepatitis C infection accounts for the deaths of at least 10,000 to 20,000 Americans each year and is now the most common indication for adult liver transplantation in developed countries [6]. The prevalence of HCV infection varies widely in different parts of the world. The highest prevalence of HCV infection has been reported in Northern Africa (particularly Egypt), moderate prevalence in Eastern Europe and most of Asia, low prevalence in Western Europe, North and South America, and Australia, and very low prevalence in Northern Europe and the UK [74]. For example, Central Africa is reported to have a prevalence rate of 6% while Sub-Saharan Africa has an estimated prevalence of 3% [75,76]. Other countries such as Egypt, Japan and parts of Southeast Asia have a moderate to high prevalence (4-10%) [77-79]. In the UK, Germany and France, the prevalence of HCV among the general population is around 1% [80-82].

The means of transmission associated with HCV infection included transfusion of blood and blood products from unsecreened infected donors, organ transplantation from unsecreened infected donors, illegal injection drug use (in developed world), unsafe therapeutic injections (in developing world),, occupational exposure to infected blood, birth from an infected mother, high-risk sexual practices with one or multiple infected partner [83,84]. Blood or blood

products transfusions were accounted for 10-20-% of cases previous to the introduction of testing blood donors for antibodies to HCV. Today, unsafe drug injection is the major route of transmission and accounts for the most new infections especially in high or moderate prevalence countries [74]. In most low prevalence areas, illegal injection drug use is the predominant mode of transmission. Although transfusion- and transplant-associated HCV infections have been almost eliminated through routine testing of donors [74,84], outbreaks of HCV infection are associated with contamination of multiple dose medication vials and intravenous solutions by reuse of disposable needles and syringes.

1.2.3 Hepatitis C infection and its clinical manifestations:

The incubation period for HCV ranges from 2 – 26 weeks, with an average of 6 to 7 weeks. HCV infection may result in one of three general outcomes: 1) acute infection with recovery, 2) development of persistent infection without apparent disease, or 3) persistent infection with chronic hepatitis [85-87]. In acute hepatitis C infection, the majority (60-70 %) of HCV infected individuals are asymptomatic [88], and 10-20% may show non-specific symptoms such as anorexia, abdominal pain, fatigue and jaundice [85,86]. The first biochemical evidence of the acute infection is the presence of HCV RNA in the serum or liver of infected individuals. The HCV RNA can be detected in the serum by PCR within one to eight weeks after exposure [87]. Virtually all patients with acute infection develop liver cell injury as shown by the elevation of serum alanine aminotransferase (ALT) which becomes elevated approximately 6 to 12 weeks after exposure to the virus. Liver biopsy is not usually performed in acute hepatitis. There is only one histological study which indicated a spotty necrosis of hepatocytes and inflammatory infiltrates in lobular parenchyma and portal tract in acute hepatitis [89]. During the acute phase approximately 15-20% of HCV

infected patients clear the virus from their blood as is indicated by the disappearance of HCV RNA from the blood [87].

The most striking feature of HCV infection is its ability to cause chronic hepatitis. Eight five percent of patients with acute hepatitis C infection fail to clear the virus by six months post infection and develop chronic infection with persistent viremia [87]. The majority of patients with chronic hepatitis are asymptomatic and 20% will have non-specific symptoms. The majority of chronically infected patients will have persistently normal serum ALT levels and high level of antibodies against HCV in their blood [85,87]. Patients with chronic hepatitis have evidence of necroinflammation on liver biopsy as indicated by the infiltration of inflammatory cells to the hepatic portal tracts and liver parenchyma. This necroinflammation may progress slowly to develop severe hepatic fibrosis which may lead to bridging between portal tracts, which is known as "bridging fibrosis''. Ten to fifteen percent of individuals with chronic HCV infection will end up with liver cirrhosis in less than 20 years [90,91]. Once cirrhosis is established, adverse consequences of chronic hepatitis C start to appear, leaving large numbers of patients at particularly high risk for the life threatening sequelae to this disease. Liver cirrhosis is associated with an increased risk of hepatocellular carcinoma (HCC) and 1-4 % of patients with chronic hepatitis C will develop HCC after 20 - 30 years [92,93].

There are many factors that have been reported to affect the rate of chronic HCV infection. These include: age (increased age associated with a more rapid progression) [73], gender (males have more rapid disease progression than females) [94,95], alcohol consumption (associated with an increased rate of disease progression) [96,97], HIV coinfection (associated with a markedly increased rate of disease progression) [98,99], and

fatty liver (the presence of fat in liver cells has been associated with an increased rate of disease progression) [100].

Although the liver is the major site of HCV replication, a broad spectrum of extrahepatic complications is linked with chronic hepatitis C infection. It has been reported that chronic hepatitis C infection is closely related to a certain type of autoimmune disorder, especially mixed cryoglobulinemia [101,102] and B-cell non-Hodgkin's lymphoma [103-105]. HCV infection is also associated with membranoproliferative glomerulonephritis, which represents 70 to 80% of HCV-associated glomerularnephritis [106,107]. In addition, HCV infection may result in neuropathy [108]. Furthermore, HCV is linked with porphyria cutanea tarda, lichen planus and Sjogren's syndrome [109]. The association between HCV infection and the Sjogren syndrome and lichen planus remains weak. All these extrahepatic manifestations indicate that HCV can replicate in extrahepatic tissues. There is evidence suggest that HCV replication may occur within peripheral dendritic cells, granulocytes, B-lymphocytes and monocytes/macrophages [110,111].

1.2.4 Therapy of HCV infection:

Treatment of hepatitis C infection may prevent a progression to cirrhosis, hepatocellular carcinoma and end-stage liver disease. The initial treatment of HCV infection was based on interferon-α (IFN-α), which is able to induce a non-virus-specific antiviral state in infected and non-infected cells, resulting in direct inhibition of viral replication [112-114]. However, using IFN-α was effective only in 15 to 25% of the treated patients and majority of treated patients relapse after the treatment is discontinued [113]. Recently, a combination of IFN-α and the guanosine analogue, ribavirin, significantly improved the rate of viral eradication and resulted in a ~47% sustained viral clearance rate [9,115,116]. Ribavirin may exert its antiviral activity

indirectly by stimulating IFN-γ production [117]. However, the exact mechanism behind the improvement in the response to IFN-α during the combination therapy is not well known. It has been hypothesized that ribavirin could decrease the viral infectivity in the presence of IFN-α [118].

The response to the combination therapy is affected by many host and viral-related factors [115,116]. Young age, female sex, and low degree of hepatic fibrosis are associated with a greater sustained response to the treatment. HCV genotype and RNA level are also significant factors. Patients infected with HCV genotype 2 and 3 show higher sustained response rate than patients infected with genotype 1. However, interferon-based therapies (either alone or in combination with ribavirin) are associated with several potentially serious adverse reactions [119], do not lead to fully satisfactory results in all patients, and are too costly for most individuals in developing countries to afford. Therefore, there is a greater need for better, safer and more potent antiviral therapy.

No vaccine is currently available to prevent hepatitis C infection. The development of an HCV vaccine is hampered by the virus genome variability, which leads to emergence of mutants that are capable of escaping the host immune response [120]. Furthermore, protection from re-infection may not occur even if a past acute HCV infection has been cleared by the immune system [121]. Also, the lack of a reproducible and highly productive *in vitro* replication assays dramatically hampers the identification of putative neutralizing antibodies or passage of attenuated viral strains. Additionally, the absence of a small animal model that can naturally support HCV replication complicates the identification of a protective or curative vaccine. Chimpanzees are the only animal model that is naturally susceptible to HCV infection. However, the course of HCV infection in the chimpanzee is not necessarily representative of

the one which occurs in humans [122]. The finding of an effective therapy and preventive vaccine for HCV infection requires understanding of the mechanisms responsible for HCV-related liver damage.

1.3 Immune responses in hepatitis C:

The immune response to HCV plays an important role at different stages of HCV infection. Many studies demonstrated that cellular immune responses are important during the acute stage of the infection, and they may control the viral replication in patients who are able to clear the virus. In chronic hepatitis, the immune responses significantly determine the rate of the disease progression by limiting the viral replication or by causing immunopathology. Therefore for the immune response to be effective against HCV infection it is essential that it contains an effective innate immune response; such as type I interferon [123]), intact lymphoid organs in which generation of efficient B and T cells responses take place [124], antibodies able to induce neutralizing response [125], and strong and sustained CD4+ and CD8+ T cell responses[126].

1.3.1 Role of Innate immune response in hepatitis C infection:

The first immune response in HCV infection is the innate immune response. The type I interferon (IFN) system is a powerful and universal defense system against viruses and it is rapidly produced as a response to the double-stranded RNA viral genome intermediate produced during viral replication. Double-stranded RNA activates protein kinase R (PKR) which will initiate signal transduction in part through the phosphorylation of interferon regulatory factors (IRFs), which can then serve as transcription factors that upregulate the expression of a number of antiviral gene products [127], including type I interferons. This pathway is activated in the liver as early as 2 days after HCV infection and results in the

upregulation of several type I interferon-inducible genes and immune transcription factors associated with the innate immune response [128]. However, some studies in HCV-infected chimpanzees suggested that intra-hepatic type I IFN responses do not correlate with the outcome of the infection [86,128]. These findings suggest that HCV might not be sensitive to the antiviral effects of type I IFN *in vivo* regardless of the presence of IFN response.

In addition to the type I interferon system, natural killer (NK) cells play an important role in the host defense against viruses including HCV. NK cells recognize infected cells and destroy them by producing large amounts of IFN-γ, which then induces cytotoxic activity [129]. The role of NK cells in early HCV infection was recently studied, indicating their association with HCV clearance and clinical recovery [130]. Despite these results, some *in vitro* studies showed that NK cells from healthy individuals, as well as patients with chronic HCV infection, can be inhibited at high concentrations of HCV E2 protein [131,132] and that NK cells of HCV infected patients are not able to produce certain cytokines which are important to activate dendritic cells *in vitro* [133].

1.3.2 Role of adaptive immune response in hepatitis C infection:

1.3.2.a Humoral immune response:

HCV infection results in antibody production to various HCV proteins and these HCV-specific antibodies are usually detectable approximately 7–8 weeks after the primary infection. There is strong evidence supporting the ability of these HCV-specific antibodies to neutralize the virus. It has been shown that antibodies specific for HCV E1 and E2 glycoproteins are able to neutralize the HCV infectivity in chimpanzees [57] and modify HCV RNA levels in vaccinated chimpanzees [134]. However, these neutralizing antibodies

are isolate-specific and are not able to protect against the emergence of the escape mutants which are present in the viral quasispecies [121]. Furthermore, data from chimpanzees and humans showed that generation of antibodies against E2 is not associated with viral clearance [122,135]. Chimpanzees can be repeatedly infected with HCV after recovery from previous HCV infection, despite the development of a specific immune response [136,137]. Also, most patients with anti-HCV antibodies are carriers of infectious virus in their blood [138]. These indicate that antibodies formed during HCV infection may not be protective and unable to clear the virus.

1.3.2.b Cellular immune response:

There is strong evidence for the role of cellular immune response, mediated by CD4+ T helper cells and CD8+ cytotoxic T cells (CTL), in both HCV clearance and liver injury. CD4+ helper T cells are an essential component of the immune response to hepatitis C infection. Many studies of acutely infected patients showed that a strong, sustained and multi-specific HCV-specific CD4+ T cell response is associated with HCV clearance [139,140]. This response remains for several years after viral clearance and these HCV specific CD4+T cells are able to proliferate when they stimulated *in vitro* with HCV antigens and can mediate appropriate effector functions [141-143]. The response is generally directed against highly conserved immunodominant epitopes within the NS3 and core proteins [144]. On the other hand, patients which develop chronic HCV infection have weak, delayed or transient CD4+ T cell response [145]. However, studies in HCV recovered chimpanzees have demonstrated that CD4+ T cell response is critical for the development of effective CTL response and protection from HCV persistence [146]. Thus, it is likely that the early CD4+ T

cell response is critical to determine the initial outcome of the infection either as direct effector cells or by maintenance of the $CD8^+$ CTL response.

The CTL response destroys the virus-infected cells either by a cytolytic mechanisms inducing apoptosis or by producing INF-γ which suppresses the viral replication (non-cytolytic mechanism) [147]. In acutely HCV infected patients, strong HCV multi-specific CTL responses have been observed during the first 6 months after infection and have been associated with the control of viremia [86,147-149]. This response is strong enough in 20% of acutely infected individuals such that they spontaneously resolve the infection [150]. In contrast, in chronic infection there are extensive CTL responses which fail to eliminate the virus and this leads to viral persistence [149]. The presence of CD8+ T cells in the peripheral blood and liver of the chronically infected patients was linked to a lower level of viremia [151], indicating that the presence of HCV specific CTL is adequate to control the virus but not to eliminate it. While a persistent infection is generally associated with a weak CD4+ and CD8+ T cell responses during the acute phase, there is no reasonable explanation as to why this response is strong enough in 15-20% of acutely infected patients such that they spontaneously resolve the infection [150]. It may be that the immune response is inefficient and only partially controls the viral infection. This weak immune response may result in inflammation leading to chronic hepatic damage.

1.3.3 Mechanisms by HCV subverts the immune response:

Once HCV survives after the primary contact with the innate immune response, it uses several mechanisms to escape the selective immunological pressure during chronic infection. One of these mechanisms is the formation of the escape mutants. The virus is able to change its' antigenic epitopes recognized by neutralizing antibodies and T cells, leading to

the formation of quasispecies which are able to escape the immune response [152,153]. It has been demonstrated that HCV genomic mutations occur in the CD8+ T cell epitope region within one year of acute infection, and these mutations are more frequent in patients who develop persistent HCV infection than in patients with resolved infection [154]. Another important mechanism for escaping the immune response is the failure and exhaustion of T cells. Some studies demonstrated the involvement of primary T cell failure in HCV persistence and that this T cell response was exhausted as indicated by the reappearance of HCV [86,139]. This failure in T cell response could be attributed to the impairment of antigen presentation by dendritic cells (DCs). Some *in vitro* studies showed the impaired function of DCs generated from HCV infected patients, which were unable to stimulate allogenic CD4+ T cells [155,156]. Moreover, it has been reported *in vivo* that the number and function of circulating blood DCs in HCV infected patients are altered [157,158].

Other potential mechanisms of immune evasion are that some HCV proteins are able to suppress T cell function through different means. For example, the HCV core protein downregulates T cell proliferation and IL2 production [159]. Also, the E2 protein can bind with NK cells and inhibit their activation [131]. In addition, HCV core and E1 proteins decrease the capacity of DCs to stimulate allogenic T cell responses by unknown mechanisms [160]. Another important mechanism of immune evasion is a defect in the maturation of HCV-specific CD8+ T cells. It has been demonstrated that the differentiation of HCV-specific CD8+ T cells from central memory cells (CCR7+) to effector cells (CCR7-) is hampered in acute infection due to suppression of IL2 production by core protein [161,162]. In addition to this mechanism, the tolerogenic environment in the liver is a potential mechanism used by the virus to escape the immune response. It is well known that

the liver, for which the virus has tropism and where it replicates, is suitable for promoting tolerance to infiltrating T cells [163]. The analysis of liver-infiltrated lymphocytes in chronic HCV infection showed different characteristics from that obtained from peripheral blood [164]. All these mechanisms are used by the virus to abolish the selective immunological pressure and to establish persistent infection.

1.4 Pathogenesis of hepatitis C infection:

The mechanism of hepatitis C pathogenesis is not well understood. The clear understanding of the mechanisms by which HCV infection causes liver damage is hampered by the lack of an effecient *in vitro* cell culture system that can support viral propagation and help in defining the role of different viral proteins in the replication, life cycle and the virus-host cell interactions. Although attempts have been made to develop HCV animal models to help understand the pathophysiological mechanisms of HCV infection, most of these models are difficult to work with. Both viral cytopathicity and host immune response have been suggested to play a role in HCV pathogenesis. However, there is strong evidence that host immunologic response to HCV may significantly contribute to liver damage. The inflammatory changes seen in the liver of chronic HCV infected patients over time results in fibrosis, cirrhosis, and eventually HCC. In the following sections, I will explain the possible mechanisms that have been suggested to play a role in chronic hepatitis infection, liver fibrosis, cirrhosis, and HCC. Also, I will elucidate the potential roles of the immune response and HCV structural proteins in the pathogenesis of hepatitis C infection.

1.4.1 Mechanisms of chronic inflammation of the liver:

The precise mechanisms by which HCV causes acute and chronic hepatitis are still unclear. Both immune-mediated mechanisms and direct viral cytotoxicity have been

suggested as factors in the pathology associated with hepatitis C. It is still controversial whether HCV is directly cytopathic. Many studies in chronically infected patients have suggested a correlation between more severe histological changes and higher HCV RNA level in the serum [165,166] and liver [167,168], and an increasing number of the hepatocytes carrying negative-strand replicative intermediates [169], which may indicate the possibility of a direct cytopathic effect of HCV. On the other hand, several arguments have suggested that HCV is not significantly cytopathic for the many reasons: 1) the presence of normal HCV carriers with normal liver histology, 2) the observation that isolated hepatocytes have no cytopathic changes despite viraemia, 3) the lack of a relationship between that hepatic viral load and disease severity, and 4) low levels of HCV RNA were detected in patients with fulminate hepatitis [170-172]. However, the direct cytopathic effect of HCV can not properly be studied in the absence of a cell culture system. The only lesion that seems to be dueto a direct pathogenic effect of HCV is steatosis, a lipid accumulation in hepatocytes. The prevalence of steatosis in chronic hepatitis C cases is around 40% [173,174]. This evidence suggests that HCV may directly cause steatosis, at least in some patients. An earlier study demonstrated that steatosis was more frequent and more severe in patients infected with the HCV genotype 3 [175,176]. This indicates the presence of steatogenic sequences across the genome of genotype 3. The search for the viral sequence responsible for the genotype-specific effects on steatosis has been so far elusive. An *in vitro* study, has been reported that the HCV core protein affects lipid accumulation in the cells [177,178], but its role in steatosis during natural HCV infection remain to be explained.

The acute phase of hepatitis C infection is usually subclinical (asymptomatic), which makes the diagnosis of the early stages of infection very difficult and it is harder to define the

natural history of the acute infection. Therefore, data on the pathological mechanisms involved in hepatic damage in acute hepatitis are limited. Chronic hepatitis C infection is mainly associated with significant liver disease especially in chronic active hepatitis, fibrosis, cirrhosis and hepatocellular carcinoma [85]. Liver damage in chronic hepatitis is characterized by portal lymphoid infiltration, focal and bridging necrosis, and degenerative lobular lesions. These lesions are due to the local immune responses which lead to infiltration and accumulation of specific T cells at the site of the infection. The lymphoid infiltrate is composed of Th1 CD4+ T cells located within the peripheral space and CD8+ T cells located in the periportal and lobular regions [172,179], suggesting that the host immune system is involved in HCV pathogenesis.

1.4.2 Immunopathogenesis of HCV infection:

Hepatocellular injury during chronic HCV infection is associated with vigorous immune response [180]. However, this immune response is inadequate to eradicate the virus. There is increasing recognition of the role played by the immune response, in particular by the cell-mediated immune response, in the immunopathogenesis of chronic hepatitis C infection. In the following sections I will discuss the roles played by cellular and humoral immune responses in liver damage associated with chronic hepatitis C infection.

1.4.2.a Role of HCV-specific CTL in hepatocellular damage

Cellular immune responses play a critical role in liver damage during the clinical course of hepatitis C infection. HCV-specific $CD4^+$ T cells are involved in eradication of the virus in acute infection but their responses are weak and inadequate in chronic hepatitis [141]. However, there is no clear evidence demonstrating that $CD4^+$ T cells play a direct role

in the liver injury observed in chronic hepatitis infection. $CD4^+$ T cells activate the CTL response and eradicate the virus-infected cells either by inducing apoptosis (cytolytic mechanism) or by producing INF-γ which suppresses viral replication (non-cytolytic mechanism) [181]. Enhanced hepatocyte apoptosis leads to liver damage in chronic hepatitis [182]. HCV-specific $CD8^+$ CTL response are compromised in most patients who fail to clear the infection. In addition, these cells have a diminished capacity to proliferate and produce less INF-γ in response to HCV antigens [183]. These ineffective $CD8^+$ T cell responses may mediate HCV-related liver damage and are inadequate at clearing the chronic infection.

The mechanisms responsible for immune-mediated liver damages associated with HCV are currently poorly understood. One of the important mechanisms for liver damage is that the HCV-activated T cells express the Fas ligand (Fas-L) at the cell surface which will bind with the Fas receptor on the surface of hepatocytes and initiate Fas-mediated signaling which may lead to cell death [184]. It has been shown that the HCV core protein increases the expression of Fas-L on the surface of liver infiltrating T cells leading to the induction of hepatic inflammation and liver damage [185,186]. Another important mechanism of liver-mediated damage is through CD8+ T cell-mediated cytolysis. Previous studies have demonstrated that CD8+ T cells can kill the target cells *in vivo* by cytolytic mechanisms mediated by perforin [187] or requiring IFN-γ [188]. This may also involve additional molecules such as TNF-α [189]. Therefore the level of cytolytic activity or expression of cytolytic mediators from the infiltrating lymphocytes could be involved in the mediation of liver damage.

1.4.2.b Role of humoral immunity in HCV immunopathogenesis:

The role of humoral immunity in the immunopathology of HCV has not completely been studied. Antibodies developing during HCV infection may be involved in immunopathological consequences of chronic hepatitis. For example, immune complexes consisting of HCV and anti-HCV antibodies are associated with type II mixed cryoglobulinemia, which is associated with glomerulonephritis, cutaneous vasculitis and arthritis [190]. In addition, there is evidence that links HCV infection with the presence of autoantibodies against microsomes derived from liver and kidney (LKM1), which are associated with autoimmune hepatitis type 2. HCV infection may lead to altered expression of the hepatocellullar LKM1 target antigen [191], but the association between these antibodies and liver damage is still controversial [192].

1.4.3 Mechanisms of fibrosis progression in HCV infection:

An important manifestation of chronic hepatitis C is fibrosis, which is defined as deposition of collagen and other extracellular matrix components within the liver parenchyma [193]. Chronic inflammation of the liver results from chronic destruction of the liver cells and local production of cytokines and growth factors, eventually leading to the progression of fibrosis. Hepatic stellate cells, which are located in the perisinusoidal space, play an important role in liver fibrogenesis [194]. It has been shown that these cells are activated during HCV infection which leads to the generation of myofibroplasts that are capable of producing large amount of extracellular matrix and collagens [195-197]. The viral factors involved in the progression of fibrosis are still not well characterized. Exogenous factors such as alcohol consumption and co-infection with human immunodeficiency virus are the most important factors involved in the progression of fibrosis to cirrhosis [198].

1.4.4 Mechanisms of hepatocellular carcinoma in hepatitis C infection:

Chronic HCV infection and cirrhosis are major risk factors leading to the development of hepatocellular carcinoma (HCC). 1-4% of patients with HCV-related cirrhosis will develop HCC [93]. In addition to cirrhosis, there are other risk factors for leading to the development of HCC, such as chronic alcohol consumption, viral co-infection, obesity, and diabetes [199]. The specific role of HCV in causing HCC remains controversial since it is not well known whether HCV plays a direct role in the pathogenesis of HCV-associated HCC or whether it simply plays indirect role. Several *in vitro* studies have been shown that some HCV proteins, such as core, NS3, NS4B and NS5A, are able to transform cells and lead to cancer [200-203]. The mechanisms behind the transforming ability of these HCV proteins are still debated. It has been reported that the HCV core protein is involved in cell transformation by interacting with some cellular proteins such as tumor suppressor proteins, p53 [204,205], p73 [206], and retinoblastoma protein (pRb) [207]. However, the results of these interactions have undergone limited study. Also, the core protein has been shown to affect the expression of cyclin-dependent kinase (CDK) [208], which is an inhibitor of Wild-type p53-activated fragment (p21/Waf), a transcriptional target of p53. Furthermore, i*n vivo* studies using transgenic mice expressing HCV core protein or the full-length HCV polyprotein, showed the development of HCC in these mice [209,210]. These findings may be relevant to human HCV infection, but they are still not well elucidated.

1.4.5 Role of HCV structural proteins in hepatitis C pathogenesis:

One of the major issues regarding hepatitis C pathogenesis is whether the HCV proteins have a direct role on the pathogenesis of HCV-associated diseases. Besides their roles in the viral life cycle, HCV proteins also interact with many cellular factors and may lead to

different biological consequences. There have been numerous studies of various HCV proteins expressed in cultured cells or transgenic mice showing the multiple effects of these proteins on cellular signal transduction, growth regulation and apoptosis, transformation, membrane rearrangement, vesicular trafficking and responses to cytokines. The majority of these studies focused on the effect of HCV structural proteins, particularly core, on cellular functions. A study using transgenic mice carrying part of HCV cDNA encoding HCV-core and envelope proteins demonstrated the ability of structural proteins to induce spontaneous focal infiltration of lymphocytes, hepatocyte necrosis, and degeneration and hepatocellular-altered foci with mitotic hepatocytes in mice older than 6 months of age. [211], suggesting that HCV proteins themselves may have a role in chronic viral hepatitis.

1.4.5.a Effect of HCV proteins on apoptosis:

HCV core protein plays an important role in the modulation of several cellular events in the host cells. Many studies showed that core protein effects cellular apoptosis, a key element in host defense against viral infection [212-215]. Since the core protein has a regulatory gene role, its effect on the modulation of the onset of apoptotic cell death has been investigated. It has been demonstrated that core protein inhibits apoptosis in human cervical carcinoma (Hela) cells induced by cisplatin, an anticancer agent [212]. Also, core inhibits c-myc mediated apoptosis in Chinese hamster ovarian (CHO) cells and impairs the regulation of the cell cycle [213]. The core protein has different functions in the case of TNF-α mediated apoptosis. Core expression sensitized HepG2 and Hela cells for apoptosis induced by TNF-α and actinomycin D [215], whereas, stable expression of core in the same cells did not affect the death induced by TNF-α in the presence of cyclohexamide [214]. These results suggest that core is indirectly involved in the control of apoptosis.

Furthermore, the expression of core has been shown to suppress Fas-mediated apoptosis in HepG2 cells and other epithelial cells via activation of NF-κB [216]. In another study, core expression in HepG2 cells sensitized cells for apoptosis upon stimulation with anti-Fas antibody [217]. Also, core protein may promote Fas-mediated apoptosis of human T cell line [218]. In addition, PBMCs of patients with chronic hepatitis express large amounts of Fas ligand on their surface therefore becoming susceptible to Fas stimulated cell death [219]. In more recent studies using transgenic mice expressing core, E1, E2 and NS2, it has been demonstrated that these mice were resistant to Fas antibody stimulated cell death with survival rate correlating with the HCV protein expression levels [220]. However, this study did not investigate the contribution of the individual viral proteins to the inhibition of Fas-mediated apoptosis. The first report that investigated the role of HCV E2 in the induction of apoptosis, showed that HCV E2 can induce apoptosis in cultured mammalian cells by an unkown mechanism [221].

1.4.5.b Dysregulation of lipid metabolism by HCV proteins:

Many studies provide strong evidence that HCV infection is associated with the formation of liver steatosis, a frequently occurring feature of chronic HCV infection [174]. These studies showed that HCV may interfere with lipid metabolism at three levels: impaired secretion of lipids, increased neosynthesis of lipid, or impaired fat degradation. Studies using transgenic mice expressing HCV core showed that these mice frequently developed steatosis within the liver [222]. Also, the core protein localized within the lipid droplets, which indicate a relationship between the expression of HCV core protein and cellular lipid metabolism [177]. Additional supportive studies showed that hepatic steatosis developed in

transgenic mice that express structural protein or the complete HCV polyprotein in the liver [209].

The mechanisms of hepatic steatosis are not completely elucidated. However, based on some experimental transgenic mouse models, the HCV core protein seems to inhibit microsomal triglyceride transfer (MTP) protein activity, which inhibits hepatic very low density lipoprotein (VLDL) assembly and secretion by binding to apolipoproteins A1 and A2 [178]. Recent data in human liver suggest that the intra-hepatic levels of MTP mRNA are reduced in patients with chronic hepatitis C, especially those with steatosis and/or genotype 3 [223]. HCV core protein may also accumulate in the mitochondria and induce liver damage through the production of reactive oxygen species (ROS) [224]. The subsequent production of ROS may lead to the peroxidation of membrane lipids and structural proteins that are involved in the trafficking and secretion apparatus. This would then block VLDL secretion leading to steatosis. In addition, a recent study showed that the core protein interacts with the mitochondrial electron transport chain and inhibits complex I, which results in the production of ROS [225]. This may lead to oxidative stress, which contributes to fibrosis and carcinogenesis in the liver. Another study looking at the lipid composition in the liver of the HCV core transgenic mice as well as in chronic hepatitis C patients, showed that the concentration of carbon 18 monounsaturated (C18:1) fatty acids, such as oleic and vaccenic acids, which are known to increase membrane fluidity leading to higher cell division rates, is significantly increased in the liver [226]. Thus there are many potential roles for HCV core protein in lipid metabolism and liver steatosis in chronic HCV patients.

HCV may also induce hepatic steatosis by neo-synthesis of fatty acids. One study demonstrated that HCV upregulated the sterol regulatory element binding protein-1c

(SREBP-1c) signaling pathway [227]. SREBP-1c is a transcription factor leading to the up-regulation of enzymes involved in lipogenesis, which leads to the accumulation of triglycerides in the cell. Also, it has been reported that HCV core binds and activates the DNA-binding domain of the retinoid receptor α (RxRα), a transcriptional regulator that controls cellular lipid synthesis [228-230]. In addition, HCV core may cause steatosis by impairing fatty acid oxidation. Transfection of a hepatoma cell line with core protein reduced the expression of peroxisome proliferators-activated receptor α (PPARα), a nuclear receptor regulating several genes responsible for fatty acid degradation. The same study reported that core down regulated mitochondrial carnitine palmitoyl transferase -1 (CPT-1), an enzyme involved in mitochondrial β-oxidation which is the main catabolic pathway of fatty acids, and of the acyle CoA oxidase (AOX) [228]. However, the role of steatosis in the progression of the disease remains controversial.

1.4.5.c Cell transformation and liver carcinogenesis:

Accumulating evidence suggests the direct or indirect involvement of HCV proteins, especially the core protein, in liver carcinogensis. It has been reported that core from HCV-1a can transform rat embryo fibroplasts and rodent cell lines when co-produced with cellular oncogenes, such as c-myc or activated h-Ras [231]. Other researchers were not able to transform rat embryo fibroblasts using core from HCV-1b genotype [232]. This controversy may be explained by the different source of the core gene. Also, it has been demonstrated that core protein is able to interact with and activate STAT3 in mouse fibroblast cell lines (NIH-3T3) [233]. This activation results in rapid cell proliferation and the up-regulation of Bcl-XL and cyclin-D1 levels. Over-expression of STAT3 in HCV core expressing cells leads to cell transformation and tumorgenesis. Also, HCV core could affect transcriptional factors,

such as LZIP and Elk-1. However, their role in cellular transformation induced by core and HCC development is unclear [234,235].

In vivo studies using HCV transgenic mice also illustrate the transformation ability of these proteins. Many studies suggested the potential role of HCV core as a central co-factor in regulating cellular proliferation and development of HCC during HCV infection. Moriya *et al* reported the development of hepatic steatosis and HCC in HCV core transgenic mice at an age more than 16 months. In this mouse model, age-dependent hepatic steatosis was observed early in life and the hepatic tumors first appeared as adenomas containing cytoplasmic fat droplets. Next, HCC developed in a "nodule-in-nodule" manner without cytoplasmic fat droplets [210]. These histopathological features resemble that observed in chronic hepatitis C patients [236]. The clear molecular mechanism controlling the ability of core to cause liver cancer remains to be clarified. The appearance of steatosis before tumor formation indicates that impairment of lipid metabolism, with overproduction of lipid peroxidation products due to oxidative stress [225,237], which is one of the possible mechanisms by which core contributes to tumorigenesis. In a more recent study using transgenic mice expressing core protein, it has been reported that this protein caused malignant lymphoma and then HCC in 80% of transgenic mice older than 20 months [238]. These results indicate a potential role of HCV core in the development of cancer. Another study demonstrated that mice expressing envelope proteins at high level do not develop neoplastic lesions [209]. Thus, these studies strongly suggest that expression of HCV core protein in the liver, and possibly in combination with other HCV proteins, significantly enhance the risk of cancer, even in the absence of an immune-mediated response to the infection.

1.4.5.d Role of structural proteins in extra-hepatic manifestations of HCV infection:

Similar to the HCV core protein, the membrane glycoproteins E1 and E2 interact with cellular factors where they may affect cellular processes. E1 and E2 bind to chaperone proteins within the endoplasmic reticulum (ER) [239]. E2 expressed in mammalian cell lines interacts with and inhibits the activity of the double-stranded RNA-dependent protein kinase (PKR) and blocked its inhibitory effect on protein synthesis and cell growth [240,241]. This interaction of E2 and PKR may be one mechanism by which HCV circumvents the antiviral effect of interferon. Confirmation of these interactions in the context of HCV replication in liver or cell culture has not yet been reported.

HCV structural protein expression has been demonstrated in tissues other than the liver, including lung, intestine, kidney, testes, heart, thymus, spleen, and salivary glands [211,242]. One of the studies showed that transgenic mice that carry the HCV envelope genes, E1 and E2, had no hepatitis or hepatic neoplasia, but these mice developed exocrinopathy in the salivary and lachrymal glands resembling Sjogren's syndrome [243,244], which has been associated with HCV infection. Additionally, it has been reported that core protein is expressed in the enlarged lymph nodes of HCV core transgenic mice which develop malignant lymphomas, indicating that these transgenic mice are suitable for understanding HCV-related lymphoproliferative diseases [238]. Taken together, these studies suggest that HCV structural proteins are involved in some extrahepatic manifestations associated with chronic HCV infection.

1.4.6 Transgenic mouse models of HCV pathogenesis:

The lack of efficient tissue culture systems and small animal models to infect and propagate the virus has hampered the progress in understanding the HCV pathogenesis. Also, the host range of HCV is limited to humans and chimpanzees. The chimpanzee model has helped some of the current understanding of HCV infection, including the natural history of infection, genetic drift, the mode of transmission, and the role of the immune response [245,246]. However, chimpanzees are rare, expensive, and difficult to handle, and they need too much care in appropriate non-human primate research facilities. Furthermore, HCV-infected chimpanzees rarely develop chronic liver disease to the extent seen in HCV-infected humans [247], which makes the chimpanzee an inadequate model for studying the mechanisms of HCV pathogenesis. Thus, it is necessary to have a more appropriate experimental animal model to analyze HCV disease mechanisms and carcinogenesis *in vivo.*

The development of mice with chimeric human livers provided a breakthrough in the HCV field [248-250]. Mercer *et al* demonstrated that HCV infection is possible by transplanting normal human hepatocytes into severe combined immunodeficiency disorder (SCID)-beige mice carrying plasminogen-activator gene controlled by the albumin promoter. These mice showed prolonged HCV infection within the human portion of their livers, when they were inoculated with HCV infected human serum. They also showed the ability to transmit this infection to other chimeric mice [248]. However, the development of this animal model requires access to human hepatocytes and transplantation procedures.

Much research is being done to develop a transgenic mouse model in order to understand the HCV pathogenesis. Mice are relatively easy to handle and require low cost

maintenance. However, HCV does not propagate naturally in mouse tissues, which limits this type of research. Despite this, expression of genomic and sub-genomic fragments of HCV in transgenic mice helps researchers to understand the mechanisms of HCV pathogenesis. Conventional transgenic systems, in which a native viral promoter or other cellular promoters control expression of the viral genome, provide an opportunity to study the effects of expression of these HCV transgenes in HCV pathogenesis.

The majority of the existing HCV transgenic mouse models have investigated the role of the expression of HCV structural proteins on the liver pathology. These proteins have been expressed either collectively [242,243,251-253] or individually [210,222] under the transcriptional control of either liver specific or generic promoter elements. Most of these studies showed high expression of HCV structural proteins located within the cytoplasm of the hepatocytes. From some studies, it is clear that expression of HCV proteins results in hepatic steatosis and HCC [209,210,222]. Also, it is clear that these studies did not elucidate the role of host immune response in HCV pathogenesis, since these transgenic mice are immunotolerant to these proteins. To overcome this problem, Wakita *et al* have produced transgenic mice that conditionally express HCV structural proteins using Cre/*iox*P system to produce immunocompetent mice [253]. However, these transgenic mice did not show lymphocyte infiltration or liver pathology. The development of HCV transgenic mice should therefore play a pivotal role in elucidating the molecular mechanisms of HCV-related liver pathology.

1.5 HYPOTHESIS, AIMS AND OBJECTIVES

Hypothesis: The structural proteins (Core, E1 and E2) of HCV induce histopathological changes in the liver due to alteration of hepatocyte metabolism. These structural proteins also induce extra-hepatic pathology in HCV transgenic mice. The adoptive transfer of splenocytes from HCV immunized mice to HCV transgenic mice may provide a model to study mechanisms of immune cell-mediated hepatic injury in chronic hepatitis C infection. Both a direct virus-mediated cytopathic effect and the immune-mediated cell damage result in progression of hepatic injury in chronic hepatitis C infection.

The overall aim of my thesis research proposal is to elucidate the molecular mechanisms of hepatitis C pathogenesis by: 1) establishment and characterization of a transgenic mouse model that encodes the HCV structural proteins (core, E1 and E2) and studying the histological and metabolic alterations in the transgenic mice over a period of time, 2) investigating the effect of HCV structural protein expression on the genes involved in hepatocyte metabolism and cytotoxicity using cDNA microarray analysis, and 3) adoptive transfer of splenocytes from HCV immunized mice into HCV transgenic mice to study the role of cell-mediated immune response in HCV pathogenesis.

Rationale for objective 1: The exact mechanisms of hepatitis C pathogenesis are not completely understood. The progress on the study of HCV pathogenesis has been hampered by the lack of appropriate cell culture systems. In order to study the pathogenesis of HCV, I established an HCV transgenic mouse model expressing HCV structural proteins under the control of a universal transcriptional regulator, a CMV promoter. This transgenic mouse model will be useful to study the mechanisms of hepatitis C pathogenesis.

The specific research objectives are as follows:

1) To establish a transgenic mouse model expressing HCV core, E1 and E2 proteins in the liver and other tissues.

2) To detect the expression of HCV structural proteins in the liver and other tissues of the transgenic mice.

3) To identify the histopathological changes that might occur due to the expression of HCV structural protein.

Rationale for objective 2: DNA microarrays provide a powerful technique for exploring the changes in the gene expression characteristics of different pathological conditions. I applied cDNA microarrays to analyze the pathological changes in liver gene expression due to interaction between HCV structural proteins and hepatocytes in HCV transgenic mice. Finding genes associated with HCV pathogenesis will help us to clarify the mechanisms by which HCV can cause a persistent infection and associated cell injury.

The specific research objective is the identification of liver genes affected by expression of HCV core, E1 and E2 in the HCV transgenic mouse model.

Rationale for objective 3: It is still controversial whether liver damage associated with hepatitis C infection is due the immune response or the direct effect of the virus. Although the vigorous immune response during chronic HCV infection is unable to eradicate HCV, it may play an important role in the liver damage associated with HCV infection. Here I described a model to define the mechanisms of the immune-mediated cell damage during hepatitis C infection using adoptive transfer experiments. This experimental model may be useful to study the immune-mediated antiviral effect and the effects resulting in liver inflammation and injury to the liver. Also, it may be used to study the kinetics of immune

cells that have antiviral specificity. Furthermore, this model could allow the assessment of different vaccination regimens or immunotherapies.

The specific research objective is:

1) To define the relevance and mechanisms of immune-mediated liver cell damage in HCV infection using an adoptive transfer model.

CHAPTER 2:

MATERIALS AND METHODS

2.1 Construction of DNA plasmids:

pVAX-1 core, E1 and E2 vector: The pVAX-1 core, E1 and E2 plasmid was constructed by a post-doctoral fellow in Dr. Diaz-Mitoma's lab as follow: total RNA was extracted from the plasma of a patient infected with HCV genotype 1a using the RNeasy Extraction Kit (Qiagen, Mississauga, ON). The RNA was used as a template to amplify HCV Core, E1, and E2 genes. The HCV fragment containing Core, E1, and truncated E2 genes encoded for amino acid residues 1- 683 (2049 nucleotides) was amplified by RT-PCR using forward primer 5` ACC ATG AGC ACG AAT CCT AAA CCTC 3' and reverse primer 5` TGG TAG GGT TGT GAA GGA ACA CG 3'. The amplified fragment was purified using the QIA quick gel extraction kit (Qiagen, Mississauga, ON) and cloned into the EcoR1 sites of PCR 2.1 vector using the TOPO-TA cloning kit (Invitrogen, Burlington, ON). White colonies (Kanamycin resistant) containing the insert were selected and grown in LB medium (30g Trypton, 15g yeast extract, 15g NaCl, pH 7.4) for 18 hrs, and subjected to plasmid purification using plasmid purification kit (Qiagen, Mississauga, ON). The plasmid was digested with EcoR1 restriction enzyme, and electrophoresed using 1% agarose gel. A 2.1 Kb band corresponding to the core, E1 and E2 HCV genes was excised from the gel and extracted using QIA quick gel extraction kit (Qiagen, Mississauga, ON). The Core, E1, E2 DNA fragment was subsequently sub-cloned into pVAX-1 (Invitrogen, Burlington, ON) downstream of a cytomegalovirus (CMV) promoter (Figure 2.1). The pVAX-1-HCV (Core, E1, and E2) was linearized by Mlu1 restriction enzyme to be used for the development of the transgenic mouse model.

pEF6-Myc/His core, E1 and E2 vector: The pEF6- core, E1 and E2 plasmid was constructed by a post-doctoral fellow in Dr. Diaz-Mitoma's lab as follow: the TOPO-TA

HCV core, E1, and E2 construct was sub-cloned into the pEF6/Myc-His expression vector (Invitrogen, Burlington, ON), which is designed for the over-production of recombinant proteins in mammalian cell lines. All plasmids were amplified using DH5α cells, and purified using the Endofree purification kits (Qiagen).

2.2 Cell culture:

Chinese Hamster Ovary (CHO) cells were grown at 37°C, 5% CO_2 in Iscove's Modified Dulbecco's Medium (IMDM) (Sigma, St. Louis, MO) supplemented with 10% fecal calf serum (FCS) (Life Technologies, Grand Island, NY), 100 U/ml of penicillin and 100 μg /ml of gentamicin.

2.3 Transfection and purification of proteins:

To establish a cell line expressing core, E1 and E2 polyprotein, CHO cells were transfected by electroporation with pEF6/Myc/His vector containing core, E1 and E2 or without any inserted gene. pEF6- core, E1 and E2 DNA (50 ul of 1 ug/ul) was mixed with CHO cells (1 million/1 ml) suspension.. Briefly, transfection was performed by 2 electroporation shocks at 1.4–1.6 KV using an electroporation apparatus (BTX Inc., San Diego, CA). The transfected cells were cultured in IMDM (Sigma-Aldrich, St. Louis, MO) containing 10% FCS (Life Technologies Laboratories, Grand Island, NY) and 50 μg/mL of penicillin-gentamicin for 3 days. This vector encodes blasticidin resistance, thus blastidin-supplemented medium was used to culture these cells. The cells were harvested and lysed in lysis buffer (25 mmol/L Tris base, 2.5 mmol/L mercaptoethanol, and 1% Triton-X100 and a cocktail of protease inhibitors), sonicated, and subjected to protein purification using the TALON affinity resin kit (Clontech, Palo Alto, CA). pEF6/Myc/His vector contains six histidine residues and ultimately allows the purification of the HCV polyprotein by

immobilized metal affinity chromatography (Clontech Talon Metal Affinity Resin Kit, Palo Alto, CA). The purity of the protein was measured by mass spectrophotometery (Performed in Research institute-I at Children hospital of eastren Ontario), and protein with 85% purity was used for immunization.

To assess the ability of pVAX-1-HCV-Core, E1, and E2 vector to express HCV structural proteins *in vitro,* CHO cells were transfected with pVAX-1 core, E1, and E2 vector. CHO cells were transfected by electroporation as described above. Pvax coreE1E2 DNA (50 ul of 1 ug/ul) was mixed with CHO cells (1 million/1 ml suspension). The mixture of the cells and plasmide DNA was exposed to 2 electrical shocks to allow the DNA to enter inside the cells. After the transfection, the cell cultured in IMDM for 3 days and then used to test protein expression.

2.4 Mice:

All mice used in the study were purchased from the Charles River Laboratories (Wilmington, MA) and were from a B6C 3F1 background. Mice were bred in specific pathogen-free conditions at the Animal Care Facilities in the University of Ottawa. Animals were used according to the guidelines of the Animal Care Committee at the University of Ottawa. Mice used for immunization as donor mice were 6 to 8 weeks old and recipient mice were 3 to 6 months old HCV transgenic and non-transgenic mice.

2.5 Production of transgenic mice:

The transgenic mouse model was developed in collaboration with Dr. Rashmi Kothary and his colleagues at Ottawa Health Research Institute using the pronucleous injection method. The linearized plasmid containing HCV genes encoding Core, E1, and E2 proteins was microinjected into the pronuclei of fertilized eggs from a B6C3F1 murine strain

(Charles River Laboratories, Wilmington, MA). Appoximately 2 pL of the DNA in concentration of 2 ng/μL was injected per each egg and the njection buffer was TE with 10 mM TrisHCl and 0.1 mM EDTA. When the pronuclei fused to form the diploid zygote nucleus, the zygote was allowed to divide by mitosis to form 2-cell embryos. The embryos were surgically implanted into the oviducts of pseudopregnant mother, which was prepared by mating female with vectomized male to elicit the hormonal changes needed to make the female uterus receptive. The implanted embryos were developed into pups. 10-20% of the fertilized injected eggs will carry copies of the integrated DNA in their chromosomes. Founder mice harbouring HCV fragments were identified as transgenic via PCR using DNA isolated from tail biopsies according to the DNeasy Tissues kit protocol (Qiagen, Mississauga, ON). Five potential founder mice that demonstrated a high copy number of the transgene were used to establish transgenic lines. Transgenic mice were maintained by breeding with non-transgenic mice to develop a heterozygous colony. All mice were fed ordinary chows, and were maintained in a specific, pathogen-free state and received standard care following guidelines established by the Animal Care Facility at the Faculty of Medicine, University of Ottawa.

2.6 RT-PCR:

Total RNA was extracted from different mouse tissues (liver, spleen, kidney, brain, lung, and heart) using the RNeasy Mini Kit (Qiagen, Mississauga, ON). Reverse transcription of total RNA was performed with Murine leukemia virus (Mul.V) reverse transcriptase (Applied Biosystems, Foster City, CA, USA) and random hexamers (Applied Biosystems, Foster City, CA, USA) to generate cDNA, according to the manufacturer's instructions. The cDNA was amplified by PCR using HCV forward primer (5` ACC ATG AGC ACG AAT

CCT AAA CCTC 3`) and reverse primer (5` TGG TAG GGT TGT GAA GGA ACA CG 3`). RNA extracted from the liver of a non-transgenic littermate was used as a negative control.

2.7 Real-Time RT-PCR Assay:

Total RNA was extracted from frozen mouse livers/ other tissues (weight 20 mg) using the RNeasy Mini Kit (Qiagen, Mississauga, ON). There were four groups of three mice (n=3) of different ages. Each mouse liver or other tissues were tested in triplicate by real time RT-PCR. For the RT reaction, the RNA was reverse transcribed using random hexamers (Applied Biosystems, Foster City, Ca, USA) and MulV Reverse transcriptase (Applied Biosystems, Foster City, Ca, USA), following the manufacturer's instructions. RT was performed for 60 min at 37°C using 1 μg of RNA per reaction in a 20 μl-reaction volume. In order to stop this reaction, the cDNA samples were incubated for 15 min at 72°C. The real time PCR was performed in special optical tubes in 36-well microtiter plates (Perkin-Elmer/Applied Biosystems, Foster City, Ca, USA) with an iCycler (Bio-Rad, Mississauga, ON). Fluorescent signals were generated using the Quantitect SYBR Green PCR kit (Qiagen, Mississauga, ON). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control gene with the following sense and antisense primer sequences: 5' ATG TGT CCG TCG TGG ATC TGA 3' and 5' TTG AAG TCG CAG GAG ACA ACCT 3', respectively. The HCV core gene was analyzed using the following oligonucleotide primers (300 nM): forward 5' ACC ATG AGC AAT CCT AAA CCT C 3' and reverse 5' GCA ACA AGT AAA CTC CAC CAA CGA 3'. The following differentially expressed genes were analyzed using oligonucleotide primers (300 nM): AOX (Acyl-CoA oxidase) sense 5' GAA CTC CAG ATA ATT GGC ACC TA 3' and antisense 5' AGT GGT TTC CAA GCC TCG AA 3', Acaa (acetyl-Coenzyme A acyltransferase) sense 5' CCT GAC TCC TAT GGG GAT

GA 3' and antisense 5' AAT CCC AGC ACG TAA GCA TG 3', and Apo A-4 (apolipoprotein A-IV) sense 5' CTG GTG GCC ATC ACC GGC AC 3' and antisense 5' AGT CCT GGA AGA GGG TAC TGA GC 3'. The Core or other differentially expressed genes and GAPDH genes were amplified using the primers mentioned above and a cDNA template from both transgenic and non-transgenic animals. Target samples were added in individual reactions to a total volume of 50 µl and no cDNA was added to the negative control. For each amplification using real time PCR, the protocol included 15 min at 95°C and 40 cycles of 15 s at 94°C, 30 s at 66°C, and 30 s at 72°C. All PCR experiments were performed with hot start and were run in triplicate. The iCycler software (Bio-Rad laboratories Inc, USA) detected the threshold cycle (C_T) for each amplicon. Normalization was performed using the $2^{-\Delta\Delta C}{}_T$ method [254]. Experimental controls including non-reverse-transcribed RNA samples were performed.

2.8 Immunofluorescence analysis:

Mouse tissues were prepared for immunofluorescence analysis (IFA) as following: transgenic mice and non-transgenic littermates were sacrificed between the ages of 2 to 18 months. (Age groupes were used 2, 4, 6, 12, 14, and 18 months. At least 3 mice by each group were studied). Mice were anaesthetized and then perfused with normal saline followed by 4% paraformaldehyde. Liver, kidney, heart, spleen, lung, salivary glands and brain tissues were surgically removed and placed in 4% paraformaldehyde overnight at 4°C. A piece of certain tissues was placed onto a plastic mould, covered with tissue embedding medium and then frozen in isopentane on dry ice. Frozen tissue sections of 5 µm thickness were cut using a cryostat and placed onto lysine-coated slides. To perform IFA, tissue sections were incubated with blocking buffer (5% normal goat serum and 0.1% Triton X-100 in PBS) for 1

hour at room temperature. Rabbit anti-core E1, E2 polyclonal antibody (prepared in Dr. Diaz-Mitoma's laboratory according to the University of Ottawa Animal Care Facility protocols for antibody production), or anti-core monoclonal antibody (Biogenesis, Kingston, NH), were applied at dilutions of 1:50 and 1:100 respectively, for 1 hour at room temperature. After washing with PBS, FITC-conjugated anti-rabbit IgG (Sigma, Oakville, ON) or FITC–conjugated anti-mouse IgG (Sigma, Oakville, ON) were incubated at a dilution of 1:200 for 1 hour at room temperature. After washing with PBS, the tissue sections were mounted with Vectashield mounting medium (Vector, Burlingame, CA). In addition to using tissues from non-transgenic littermates as negative controls, parallel immunostaining experiments were also performed using pre-immunized rabbit serum, as well as secondary anti-rabbit FITC-labeled antibody alone.

To check the ability of pVAX-1-HCV-Core, E1, and E2 vector to express HCV structural proteins transiently *in vitro,* IFA was also performed for CHO cells transfected with pVAX-1 core, E1, and E2 vector or pVAX-1 vector alone. CHO cells were cultured in IMDM containing 10 % FCS, Pecillline and gentamicine for 3 days until they become fluctuant. A drop of the cell culture was put over the slides that have holes, (10.000 cell/1ul/ hole). The slides were incubated over night in humidified champers. The second day, the slides were washed to remove the unattached cells. The cells were fixed using acetone and stained for IFA as described above.

To assess the lymphocytes migration into intact organs: 1-2 mm thick sections of mouse fresh frozen liver and spleen were fixed in mounting media in a recessed microscope slide and examined under fluorescence microscopy (excitation 491nm and emission 518 nm).

2.9 Histological staining:

Mouse tissues from different age groupes described above, were fixed in 4% paraformadehyde and embedded in paraffin. 5μm thick sections were stained with Hematoxylin and Eosin, Sudan black B or Masson's trichrome staining were performed according to standard methods used in the Department of Pathology and Laboratory Medicine at the Faculty of Medicine, University of Ottawa. Tissue examination was performed, and interpreted according to standard histopathological morphology. To determine the extent of steatosis, a semi-quantitative method was used, in which 1+ represented lipid droplets in sporadic hepatocytes, 2+ was moderate steatosis, in which hepatocytes with lipid droplets were seen clearly in more than 2 of five fields examined, 3+ represented moderate to severe steatosis, in which lipid droplets were extensively distributed in most areas of the liver and 4+ very severe steatosis, in which steatosis was observed everywhere in the liver. Characterization of the tumors was performed according to standard morphological histology. For example, the diagnosis of lymphosarcoma in the liver and nodes was made based on the undifferentiated nature of the cell morphology, which resembled early embryonic cells; the presence of many mitotic figures and the invasiveness represented by a lack of tumor capsule, tumor size and invasion of nodes and liver. In addition, a large portion of normal hepatic tissue was replaced by tumor cells. In contrast, the diagnosis of adenoma was based on cell morphology, including the well differentiated nature of cells with few mitotic figures and the lack of invasion of extra hepatic tissues. Histological examinations was performed in collaboration with Dr. Susantha Gomis and Dr. Lorne Babiuk at Pathology department, University of Saskatoon and Dr. Rudy Mueller at Pathology department, University of Ottawa and interpreted according to standard histopathological morphology.

2.10 Electron Microscopy:

Electron microscopy studies were done according to the standard procedures at the Electron Microscopy Facility at the Children's Hospital of Eastern Ontario in Ottawa. Approximately 0.25 mm thick liver tissue slices were briefly post-fixed in 2% osmium tetroxide in 0.1 M cacodylate buffer dehydrated in graded ethanol and embedded in Spur epoxy resin. Ultrathin 60-70 nm sections were cut using a Leica Ultracut R ultra-microtome, mounted on 200 mesh copper grids and stained with 10% uranyl acetate in 50% methanol, followed by Reynold's lead citrate. Grids were examined and micro-graphed using a JEOL 1010 transmission electron microscope at 60 KV. Liver from 3 transgenic / non-transgenic mice (1 year-old) were used for electron microscoy studies.

2.11 Microarray analysis:

2.11.1 cDNA microarray experiments:

For microarray analysis, total RNA was isolated from frozen liver tissues of HCV transgenic and non-transgenic mice (n=3, 6 month-old) using an RNeasy isolation kit (Qiagen, Mississauga, ON). 10 μg of RNA was reverse transcribed using 400 units SuperScript II reverse transcriptase (Invitrogen, Burlington, ON), 3 μg random hexamer primers (Invitrogen, Burlington, ON), 4 μg Oligo (dT)-18, Cyanine dye-conjugated (Cy3 or Cy5) dUTP (Amersham Pharmacia, Baie d'Urfe, QC). The reaction was incubated for 5 minutes at 65°C, then for 2 hrs at 42°C. To stop the reaction, 4 μl of 50 mM EDTA and 2 μl of 10N Na OH were added to hydrolyze the remaining RNA and added 4 of 5 M acetic acid to neutralize the mixture. The resulting cDNA was labeled with Cy3 for non-transgenic cDNA and Cy5 for transgenic cDNA. The labeled cDNA was combined and collected with the use of Millipore Microcon-30 polymerase chain reaction filters units (Fisher scientific,

Nepean, Ontario). 5 µl of the combined Cy3/Cy5 labeled cDNA sample was added to 80 µl of hybridization solution, which was a 20:1:1 ratio of DIG Easy Hyb (Roche, Mississauga, ON), sonicated calf thymus DNA (sigma-Aldrich, Oakville, ON), and yeast tRNA (Life technologies). This reaction was incubated at 65°C for 2 minutes and was then left to cool to room temperature. The sample was loaded onto double-spotted, two channel mouse cDNA microarray chip (Toronto Microarray Center, ON) and incubated in a hybridization chamber overnight at 37°C. After hybridization, the coverslip was removed in 1× SSC. The microarray was washed three times for 12 minutes each at 50°C in clean slide staining boxes containing pre-warmed 1X SCC, 0.1% sodium dodecyl sulphate (SDS) with gentle agitation. The slide was then rinsed in room-temperature with 1X SCC, 0.1 X SCC, and distilled water. The slide was dried by centrifugation at 500 rpm for 5 minutes and stored in the dark.

Array images were collected for both Cy3 and Cy5 by using a Scan Array XL 4000 fluorescent scanner (Packard Biochip, Montreal, QC) with a 10-µm resolution to detect two 16-bit images corresponding to the Cy3 channels and Cy5 channels. The image intensity data were quantitated and the background was subtracted using QuantArray 3.0 (Packard Bio-chip, CA) software. Data were obtained from cDNA microarrays, consisting of 7 biological replicates. More than 15 000 mouse genes and expressed sequence tags (ESTs) were examined.

2.11.2 Pre-processing, Normalization, and Filtering of microarray data:

Microarray data analysis was performed by Dr. Fazel Family and Dr. Sieu Phan at National Research Council Canada - Ottawa. After quantitation microarray data were processed and analyzed for the identification of differentially-expressed genes using an in-house software suite BioMiner [255]. Prior to normalization, three basic pre-processing

procedures were performed: 1) Background Correction: The background intensity of each spot is subtracted from the foreground intensity. If the foreground intensity is lower than the background intensity, the corresponding channel is marked as "low-intensity" and subject to adjustment in the following Low Intensity Treatment procedure. The two processes in combination effectively retain only channel intensity that is above its background intensity. 2) Low Intensity Treatment: The low-intensity threshold (LIT) is set to 5. For each spot, if only one of the two background-corrected channel intensities is below LIT, the low channel intensity is replaced with LIT. If both channels are below LIT, the spot is marked as "low-intensity-in-both". 3) Bad Spot Handling: Bad spots are spots that (1) have non-numeric entry or missing value, (2) are flagged by the numeric-code 0 by the image quantitation software, and (3) are labeled as "low-intensity-in-both" by the Low Intensity Treatment procedure. Bad spots are removed from the subsequent normalization procedure. They are labeled as "NA" and subjected to deletion or imputation in the down-stream gene-response analyses. Normalization in the context of 2-channel microarray experiments is a transformation to compensate for systematic biases, such as different incorporation of dyes, which exist between the two channels. The print-tip LOESS method was used to correct these effects for each print-tip separately [256]. The method applies the assumptions that most of the genes do not differentially express and that there is no bias towards up- or down-regulation of expression in the experiment.

Following data normalization, the Dominancy Analysis (DA) procedure of BioMiner was performed to eliminate the improper influence of the in consistent replicates in the down-stream analyses such as the calculation of fold-change and p-value. The DA procedure examines each spot across the 7 replicates to determine the dominant behavior of the spot

with regards to gene regulation activity. Basically, the procedure identifies whether a spot is dominantly inclined towards up-regulation, down-regulation, within low-regulation, or noisy (a noisy spot is a spot that the up-down inclination cannot be clearly determined). The noisy and low-regulation spots are filtered from the subsequent gene-response analyses. As for the dominantly inclined towards up- or down- regulation groups, the in-consistent replicates (per spot basis) are replaced with the average of the remaining dominant members of the 7 replicates.

2.11.3 Microarray data analysis:

The filtered and corrected data that resulted from the DA procedure described above were analysed for the detection of differentially-expressed genes. The mean log ratio of intensity for each gene across the 7 replicates was tested using t-test for its significance in difference from 0. For thresholds set for a minimum absolute log2 ratio of 0.6 (which is approximately 1.5-fold) and a p-value of 0.05 or less.

2.12 Immunization of Mice:

Donor mice (n=7) were immunized with HCV vaccine candidate [257] consisting of pVAX-HCV Core, E1 and E2 DNA (100 μg), HCV recombinant core, E1, and E2 polyprotein (25μg) in PBS solution and montanide (50 μl) ISA-51 (Seppic Inc., Fairfield, NJ) as adjuvant. The mice were immunized with 100 μl of the HCV vaccine candidate three times at two week intervals, intramusculary in the quadriceps major. After 3 weeks from tbe third immunization, the mice were boosted twice in two week intervals. Wild-type (non-immunized) mice (n=7) were used as a negative control. After each immunization, the humoral immune response was assessed by ELISA using sera and the cellular immune responses were assessed using peripheral blood mononuclear cells (PBMCs) isolated from

the whole blood after the fourth immunization and using PBMCs isolated from splenocytes after the last immunization. The mice were anesthetized with 50 µl of Somnotal (MTC Pharmaceuticals, Cambridge, ON, Canada), euthanized, and blood and spleens were collected.

2.13 Preparation of lymphocytes from donor mice spleens:

Donor mice were euthanized using anesthetic, and spleens were removed and placed in a sterile PBS. Lymphocytes were prepared as a cell suspension by gently pressing organ segments through a fine nylon mesh using a plastic pipette and then 10 ml of PBS was added to pass it through the mesh. The spleen cell suspensions were depleted of red blood cells using RBC lysis buffer (155mM NH4Cl, 10mM KHCO3, and 0.1mM EDTA). The cellular suspension was washed three times by adding PBS/ 0.1% BSA and centrifuged at 1600 rpm in 4°C for 5 min. The cells were counted and divided to be used for different assays.

2.14 Enzyme-Linked Immunosorbent Assay (ELISA):

To assess the antibody titer against the HCV vaccine, mice were bled at different points of the immunization and the serum was collected. Serum levels of hepatitis C–specific antibodies were measured using the HCV recombinant core, E1, and E2 polyprotein as a capture molecule and a mouse-specific monoclonal antibody–horseradish peroxidase (HRP) conjugate detection system. EIA/RIA Strip Well TM plates (Corning CoStar Inc., New York, NY) were coated with 20 µg/ml recombinant core, E1 and E2 polyprotein dissolved in sterile distilled/ deionized water for 4 hrs and incubated overnight at 4°C. After washing, the plates were blocked with 1% bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO) in PBS for 1 hr at 37°C. Then the plates were washed and dilutions of sera were incubated for 2 hrs at 37°C. Antibodies were detected with a 1/1000 dilution in 1% BSA/PBS of the required

goat anti-species–specific HRP conjugate (IgG H+L: Jackson Immunoresearch Laboratories, West Grove, PA; IgG1, IgG2a: Serotec, Oxford, UK). After incubation, the plates were washed six times with PBS/0.05% Tween-20 (Sigma-Aldrich). O-phenylenediamine dihydrochloride (Sigma-Aldrich) and hydrogen peroxide were used to develop the color reaction. The Optical density (OD) was read at 490 nm after the reaction was stopped with 1 N HCL. An IgG2a monoclonal antibody specific for core protein aa 1-120 (Clone 0126, Biogenesis Ltd., Poole, England) and hepatitis C–negative or pre-immune sera were run in parallel, with all samples tested as negative control. OD values at least 2 standard deviations above the mean OD of the pre-immunization sera were considered positive for an HCV-antibody response.

2.15 IFN-γ intracellular staining:

CTL responses were assessed by measuring the mouse IFN-γ production using intracellular staining. The intracellular procedures were done according to Caltag Laboratories protocol. Briefly, PBMCs isolated from fresh blood or splenocytes from immunized mice were cultured in complete RPMI media in the presence of 10 μg/ml Brefeldin A (Sigma-Aldrich, Oakville, ON) and stimulated with purified recombinant core, E1 and E2 protein (20 μg/ml) prepared as described in section 2.3, core peptides (2 μg/ml) including core peptide 1 (35-44); YLLPRRGPRL, and core peptide 2 (132-140): DLMGYIPLV (Dalton laboratories Inc, Toronto, ON), or recombinant vaccinia vector expressing HCV polyprotein, core, E1, E2, P7 and truncated NS2 (aa 1-966) (rVV- HCV poly) (MOI = 2 PFU/ cell) (NIH, Germantown, MD, USA). Unstimulated or empty vaccinia stimulated cells were used as a negative control. The stimulation of splenocytes with recombinant vaccinia was done as follows: splenocytes were resuspended in serum free

IMDM and aliquoted in 3 x 10^5 cells per tube and incubated with vaccinia virus at a concentration of 2 pfu/cell in a 37°C incubator for 90 min, while agitated every 30 min. The stimulated splenocytes were washed and centrifuged and re-suspended in complete IMDM. Phorbol myristate acetate (PMA) (Sigma-Aldrich, Oakville, ON) at 10 ng/ml and ionomycin (ION) (Sigma-Aldrich, Oakville, ON) at 1 μg/ml stimulated cells were used a positive control. Eighteen hours after incubation at 37°C, the cells were washed with washing buffer containing 2% fecal calf serum (FCS), and 0.01% sodium azide in PBS (all from Sigma). The cells were then surface-stained for 15 min with phycoerythrin-labeled (PE) conjugated rat monoclonal antimouse CD3 (Caltag Laboratories, Hornby, ON), Tri-color (TC) conjugated rat monoclonal antimouse CD4 or CD8 antibodies (Caltag Laboratories, Hornby, ON). The cells were washed as above, fixed and permeabilized using Caltag reagent A and B fixation-permeabilization solutions (Caltag Laboratories). The cells were stained intracellulary with anti-mouse IFN-γ fluorescein isothiocyanate-conjugated (FITC)-labeled antibody (BD Biosciences Mississauga, ON) and incubated for 30 min (in the dark) at 4°C. Following washing, cells were analyzed by fluorescence-activated cell sorting (FACS) caliber flow cytometry (Becton Dickinson, Mississauga, ON). An increase of 0.1% in IFN- γ producing cells over the unstimulated control or empty vaccinia stimulated cells was considered as a positive response to vaccination.

2.16 IFN-γ ELISpot assay:

The ELISpot assay was performed according to Mabtech protocol. Briefly, 96-well microtiter plates were coated with mouse anti-IFN-γ monoclonal antibodies (10 μg/ml in PBS). The cells (250,000/well) were added to the plates and stimulated either with recombinant core, E1 and E2 protein (20 μg/ml), core peptides (2 μg/ml), or recombinant

vaccinia HCV poly (rVV- HCV poly) (MOI = 2 PFU/ cell). Unstimulated or empty vaccinia stimulated cells were used as a negative control. PMA/ION stimulated cells were used a positive control. After 48 hrs of incubation, the cells were removed by washing and a biotinylated antibody against IFN-γ (10 μg/ml in PBS) was added. Then the streptavidin conjugated with alkaline phosphatase enzyme (ALP) was added. Finally, a precipitation substrate for ALP, 5-Bromo-4-Chloro-3'-Indolyphosphate p-Toluidine Salt (BCIP) was added and the plates were incubated until spots emerged at the site of the responding cells. The spots were examined and counted in an image analyzer system. The mean number of specific spot-forming cells (SFCs) was calculated by subtracting the mean number of spots from unstimulated cells or empty vaccinia stimulated cells from the mean number of spots in cells stimulated with core, E1 and E2 or core peptides and rVV- HCV poly.

2.17 Lymphocytes Proliferation assay:

CD4+ T cell proliferation was assessed after labeling the lymphocytes derived from the spleen using succinimidyl ester of carboxyfluorescein diacetate (CFSE) dye. Labeling cells with CFSE was as follow: 10 mM CFSE stock solution was prepared by adding 90 μl Dimethyl Sulfoxide (DMSO) to 500μg lyophilized powder of CFSE dye. The stock solution was diluted in sterile PBS/0.1% BSA to get the desired working concentration of 10 μM. Purified lymphocytes were resuspended to a concentration of 50 million cells per ml in PBS/0.1% BSA before the addition of CFSE dye. An equal volume of 10 μM of CFSE dye was added to the cell suspension in a tube 6 times more than the volume of the cell suspension and mixed well by vortexing. It is important that the cells be well suspended and not aggregated to ensure the uniform labeling with CFSE. The labeled lymphocytes were incubated for 15 min at 37°C. The staining was quenched by adding 5 volumes of ice-cold

complete RPMI followed by incubation on ice for 5 min to stop staining. The cells were washed three times in complete RPMI media and re-suspended in complete RPMI (2 million cells per ml to do proliferation assay and 40 million cells in 150 μl PBS for injection). To make sure that the cells were efficiently labeled, samples of the cell suspension were analyzed by fluorescent microscopy. The proliferation was assessed after stimulation of the cells with core, E1 and E2 proteins (20 μg/ml) or core peptides (2 μg/ml). PMA (10 ng/ml) and ionomycin (1 μg/ml) were added to the cells as a positive control. After adding the stimulant, the cells were incubated at 37° in 5% CO_2 for 4 days. The stimulated cells were then harvested by centrifugation at 1600 rpm for 5 minutes. Cells were surface stained with CD3 TC and CD4 PE (Caltag Laboratories, Hornby, ON) as explained above. The cells were incubated with antibody for 15 minutes at room temperature in dark. After washing the cells with PBS/0.1 azide/5% FCS, the cells were analyzed on FACScaliber flow cytometry.

2.18 Injection of CFSE labelled cells into recipient mice:

CFSE labeled cells from the donor mice (immunized or non-immunized) were pooled. Twenty million CFSE-labelled spleen cells in 75 μl PBS were injected to each recipient mouse (transgenic or non-transgenic) by the tail vein. The recipient mice were bled 24 hrs after the injection and then euthanized one week later. The following tissues were obtained from each recipient mouse: blood, lymph nodes, spleen, thymus and liver.

2.19 Flow cytometry:

Tissues from recipient mice were processed to get cell suspensions by gently pressing them through the cell strainer and collecting them in sterile PBS. The RBCs were lysed from the blood, spleen and lymph nodes. The cells were counted, aliquoted and surface stained as described above with fluorescence-labelled antibodies directed at mouse CD3, CD4 or CD8

for differentiation. (CD3 PE, CD8 TC or CD4 TC) (Caltag Laboratories, Hornby, ON). The cells were then analyzed on FACScaliber flow cytometry.

2.20 Statistical data analysis:

A Student's *t*-test was used to evaluate the significance of the differences in all data.

CHAPTER 3:

Histopathological changes in the liver of a transgenic mouse model expressing genotype 1a hepatitis C virus core and envelope proteins 1 and 2

3.1 INTRODUCTION

Hepatitis C Virus (HCV) constitutes a major cause of chronic liver disease which may progress to liver cirrhosis, and hepatocellular carcinoma. The exact pathological processes of HCV infection are not completely understood. Following an incubation period of 3 to 12 weeks, HCV clinical manifestations are often mild. However, as many as 85% of the infected individuals develop a chronic infection, frequently accompanied with severe long-term liver pathology [87]. HCV infection is also associated with the development of extrahepatic manifestations, such as type II mixed cryoglobulinemia, glomerulonephritis and B-cell non-Hodgkin's lymphoma [238,258,259]. Therefore, understanding the biology of HCV infection is essential. Both direct viral cytotoxicity and immune mediated mechanisms have been suggested as factors in the pathology associated with hepatitis C infection [260]. The lack of an appropriate cell culture system that can support viral replication has hampered detailed analysis of HCV pathogenesis [260]. Combined research efforts with different small animal models will facilitate detailed analysis of HCV *in vivo* and help clarify HCV pathogenesis.

Transgenic mice expressing HCV proteins are useful tools to provide insights into the pathobiology of HCV infection. The majority of HCV transgenic mice have been developed to investigate the role of HCV structural protein expression on liver pathology. Accumulating evidence suggests the direct or indirect involvement of HCV structural proteins in the liver disease associated with hepatitis C infection. These proteins have been expressed either collectively [242,243,251-253] or individually [210,222] under the transcriptional control of either liver specific or generic promoter elements. From several studies, it is clear that expression of HCV proteins, especially core protein, results in steatosis and HCC [209,210].

Also, transgenic mice containing E1 and E2 genes, showed no pathological changes, such as hepatitis or hepatic neoplasia, but they developed exocrinopathy in the salivary and lachrymal glands resembling Sjogren's syndrome [243,244]. Furthermore, HCV core transgenic mice, where core protein was expressed in the enlarged lymph nodes, developed malignant lymphoma [238]. From these studies, it is clear that the expression of core protein and other structural proteins results in the development of the liver steatosis and HCC. Whether this pathology is due the direct effect of protein expression or whether it occurs in combination with host factors leading to chronic liver injury is still unknown.

In the first study of this research thesis, the main aims were: 1) to elucidate the molecular mechanisms of hepatitis C infection pathogenesis by establishing and characterizing of a transgenic mouse model that encodes the HCV structural proteins, core, E1 and E2, and 2) to study the histological and metabolic alterations in the transgenic mice hepatic and extrahepatic sites over a period of time. Therefore, a transgenic mouse model was developed expressing the HCV structural proteins (core, E1, and E2) under the control of a universal CMV promoter. Histological analysis of liver cells demonstrated steatosis in transgenic mice older than 3 months, which was more pronounced with age. These mice became more prone to liver and lymphoid tumor development and hepatocellular carcinoma [261]. This model shared several features of human HCV infection, such as severe hepatopathy characterized by the development of steatosis as well as HCC. Therefore, this animal model could be used to study pathogenesis and novel treatment modalities for HCV infections and liver cancer.

3.2 RESULTS:

3.2.1 *In vitro* expression of the transgene:

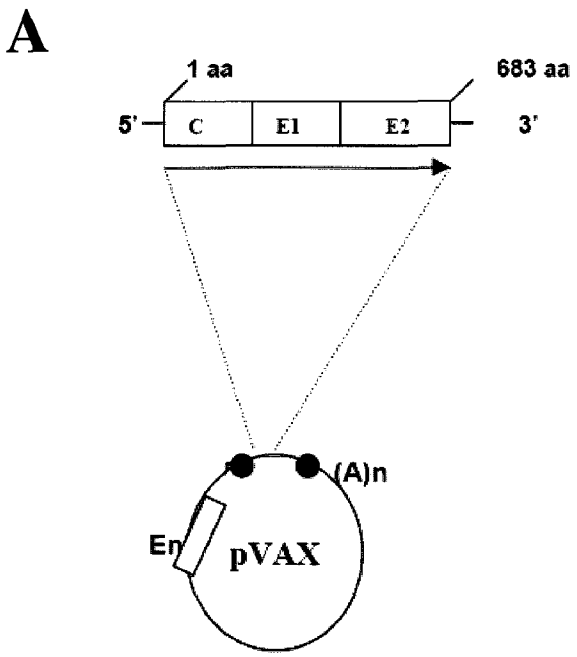
An HCV expression vector containing the coding sequences of core, E1, and E2 proteins was constructed from a PCR product obtained from the serum of a patient infected with genotype 1a HCV. The amplified fragment was inserted into the pVAX-1 vector containing a CMV promoter (Fig. 3.1A). This promoter is the strongest global expression promoter that is able to express the target genes in a variety of mammalian cell types. Thus, CMV promoter was chosen to express the HCV structural genes in different cell types, which will be useful to study the effect of the expression of these proteins in the liver as well as extrahepatic tissues.

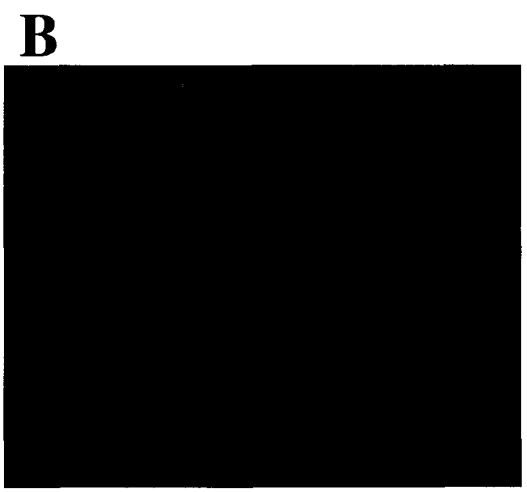
To measure the ability of the CMV promoter to express HCV structural proteins *in vitro*, CHO cells were transfected with pVAX-1 core, E1 and E2 or pVAX-1 as described in section 2.3. CHO cells were cultured for 3 days and then cultured on slides for 24 hrs. cells were washed and fixed to be stained. The cells were analysed by immunolabelling with anti-core monoclonal antibody or with anti-core, E1, and E2 rabbit polyclonal antibody using immunofluoresence analysis. HCV structural proteins were mainly present in the cytoplasm, and no protein expression was detected in the nuclei (Fig. 3.1 C). These cells demonstrated no apparent morphological cellular changes. CHO cells transfected with pVAX-1 alone showing no expression of protein (Fig. 3.1 B).

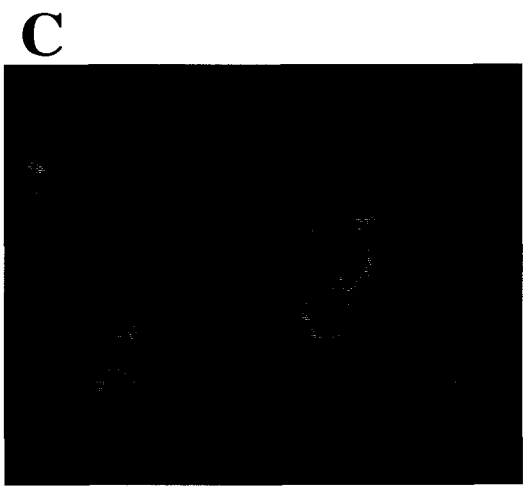
Fig. 3.1 In vitro expression of HCV core, E1 and E2 proteins.

(A) Schematic representation of HCV genome and pVAX-1 plasmid containing HCV core, E1, and E2 genes downstream of a CMV promoter. A fragment containing HCV core, E1, and truncated E2 genes encoding for amino acid residues 1- 683 (2049 nucleotides) was amplified by RT-PCR using RNA obtained from the serum of a patient infected with genotype 1a. The amplified fragment was inserted into the pVAX-1 vector under control of a CMV promoter.

(B and C) *In vitro* expression of HCV core, E1 and E2 proteins in CHO cells. CHO cells were transiently transfected by electroporation with pVAX-1 vector containing core, E1 and E2 or without the inserted gene (50 ul of 1 ug/ul of plasmid was mixed with 1 million CHO cells/ 1 ml suspension). The transfected cells were cultured for 3 days till flucant growth and thencultured again for 1 day on slide holes. The transfected cells were stained using rabbit polyclonal antibodies to core, E1 and E2 as primary antibody, and FITC-conjugated anti-rabbit as the secondary antibody (B) CHO cells transfected with pVAX-1 alone showing no expression of protein. (C) CHO cells transfected with pVAX-1 containing HCV core, E1 and E2 showing expression of proteins in the cytoplasm of the hepatocytes (indicated by arrows). (Magnification power 40x. Scale bar, 50 μm).







3.2.2 Generation of transgenic mice:

pVAX-1 core, E1 and E2 plasmid was used to produce HCV transgenic mice as described in section 2.5. Transgenic mice were identified for HCV transgene expression by PCR using genomic DNA extracted from tail biopsies (Fig. 3.2). Five potential founder mice (F0) that demonstrated a high copy number (More than 500 copies) of the transgene were used to establish transgenic lines. Transgenic mice were maintained by breeding with non-transgenic mice to develop a heterozygous colony, and to ensure that the integrated transgene was successfully transmitted to the offspring. F1 (first generation) and F2 (second generation) heterozygous mice from the five lines were used as models in this study.

3.2.3 Expression of the core, E1 and E2 transgene *in vivo*:

To confirm the expression of HCV core, E1, and E2 transcript in transgenic mice, several tissues were examined for specific mRNA by RT-PCR. Briefly, total RNA was extracted from liver, kidney, spleen, heart, lung, and brain, and examined by RT-PCR. All tissues from transgenic mice showed RNA expression, while non-transgenic mouse tissues did not show transgene RNA expression (Fig.3.3). To assess the expression of HCV- core, E1, E2 proteins in transgenic mouse tissues, immunofluorescence microscopy analysis was performed. The analysis showed that HCV viral proteins were predominantly expressed in the liver of transgenic mice, demonstrating a selective expression in certain tissues (Fig. 3.4A & C). Non-transgenic littermates showed no evidence of HCV structural proteins expression (Fig. 3.4B & D). The liver demonstrated a centrilobular distribution of groups of cells expressing the viral proteins. The expression of the HCV proteins was detected in the cytoplasm of the hepatic cells. HCV viral proteins were not detected in either the heart or the brain, whereas

Fig. 3.2: Successful transmission of the HCV core, E1 and E2 transgene through the transgenic mice colony.

Transgenic mice were analysed for HCV transgene expression by PCR using genomic DNA extracted from tail biopsies. Using HCV specific primers, a DNA band of ~1.2 kb in size was detected by PCR in transgenic mouse genomic DNA (Lane 1, 2, 3 and 4), which indicated that the integrated transgene was successfully transmitted to the offspring. Non-transgenic littermates showed absent of the the transgene (Lane 5). (Pvax-1 core, E1 and E2 plasmid was used as positive control; M is the molecular size marker, 1kb DNA ladder).

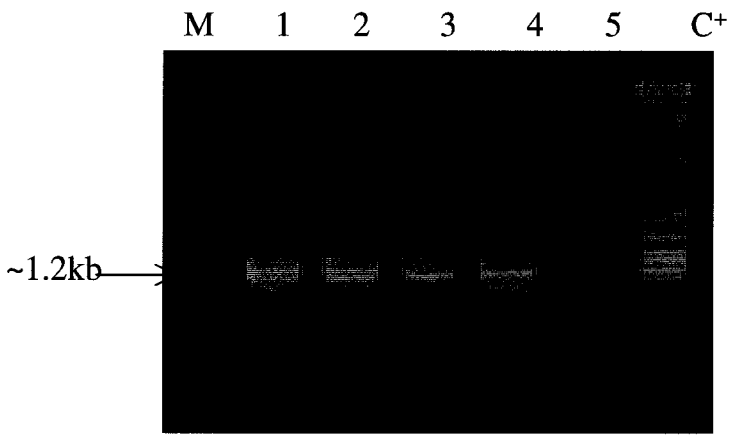


Fig. 3.3: Detection of HCV RNA in different tissues of transgenic mice.

RT-PCR was performed using RNA extracted from different mouse tissues as a template to amplify a 1.2 kb fragment of core, E1 and E2. Lanes 1, 2, 3, 4, 5, and 6 are positive for viral RNA in the tissues of a transgenic mouse corresponding to liver, spleen, heart, kidney, brain and lung respectively. Lane 7 is the RNA extracted from the liver of a non-transgenic mouse used as a negative control. The picture represents what was observed in tissues from at least three transgenic or non-transgenic mice at age of 2- months. (M is the molecular size marker, 1kb DNA ladder).

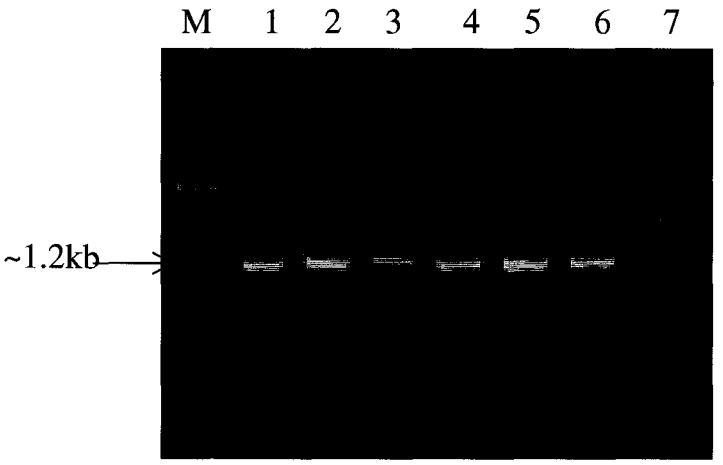
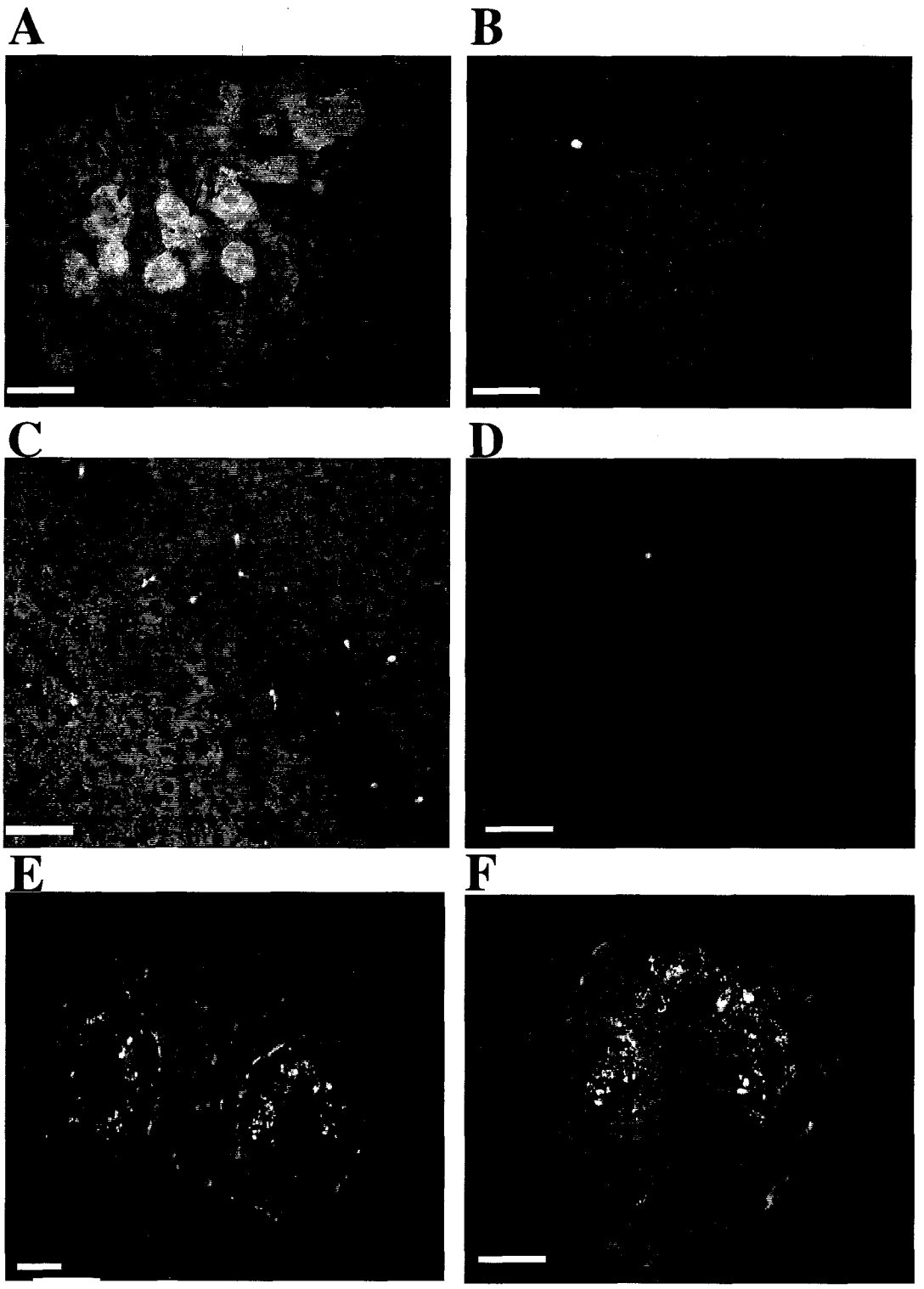


Fig. 3.4: Immunofluorescence staining of HCV structural proteins in frozen formalin-fixed transgenic mice tissues.

Frozen tissue sections were incubated with rabbit anti-core E1, E2 polyclonal antibody for 1 hour at room temperature. After washing, FITC-conjugated anti-rabbit IgG were used as secondary antibodies. (A) Liver section of a 2 month-old transgenic mouse showing the expression of HCV structural proteins in the hepatocyte cytoplasm (arrows). (B) Liver section from a 2 month-old non-transgenic mouse showing no evidence of HCV structural proteins. Propidum iodide was used as a counter stain as indicated by red stain. (C) Liver of one year old transgenic mouse showing an increase in the number of hepatocytes expressing HCV structural proteins in the cytoplasm (arrows). Cells without green fluorescence in the middle of the section are not expressing the protein. (D) Liver section of a one year-old non-transgenic mouse showing no expression of HCV structural proteins. (E&F) Immunofluorescence detection of HCV structural proteins in frozen formalin-fixed sections of salivary glands of a transgenic mouse fetus stained with a rabbit polyclonal antibody to HCV core, E1, E2 proteins. (E) low magnification (20X). (F) high magnification (40X), Proteins are detected in both the epithelial cells layer - arranged as wedges forming an acinus around a central lumen - and in the myoepithelial cell layer surrounding them. (A, B, C, D and F: Magnification 40X. Scale bar, 50 μm) (E: Magnification 20X. Scale bar, 50 μm). The pictures represent what was observed in tissues from at least three transgenic or non-transgenic mice studied.



the spleen cells as well as epithelial cells in the renal microtubules of the kidney demonstrated relatively low levels of viral protein expression (Table 3.1). Tissues from non-transgenic littermates did not show HCV protein expression. Interestingly, when transgenic fetal tissue was analysed by immunofluorescence microscopy to detect the viral proteins, the fluorescent signal was restricted to the salivary glands (Fig. 3.4E & F). Proteins were detected in both the epithelial cell layer, arranged as wedges forming an acinus around a central lumen, and in the myoepithelial cell layer surrounding them. However, salivary glands of adult mice did not express HCV proteins (data not shown). In addition, there was no detectable viral protein expression in fetal livers (data not shown). There was no difference in the distribution of protein expression between the five lines. Core E1 and E2 protein expression was tested in all tissues using both rabbit anti-core, E1, E2 polyclonal and anti-core monoclonal antibodies.

3.2.4 Viral proteins accumulate in hepatocytes with age:

To investigate whether the expression of HCV proteins by transgenic mouse hepatocytes was directly associated with the age of the transgenic mice, comparision study was carriedout in livers from transgenic mice at various ages described in section 2.8. Hepatocyte expression of the HCV proteins was initially detected in transgenic mice at 2-3 months of age (Fig. 3.4A). These hepatocytes were scattered around the central veins in a centrilobular pattern. With increasing age, fluorescent cells appeared contiguously and formed patches of fluorescent hepatocytes. At 12 to 18 months of age, almost all (90%) cells in the liver section expressed the viral proteins (Fig. 3.4C). Livers from non-transgenic mice at any age did not show HCV protein expression (Fig. 3.4B & D). This indicates that HCV proteins accumulate in the liver of transgenic mice with increasing age.

Table 3.1: HCV structural protein expression in various transgenic mouse (6 month-old) tissues as detected by immunofluorescence analysis

Transgenic mouse tissue	Level of protein expression
Liver	++++
Spleen	++
Kidney	++
Lung	+
Heart	-
Brain	-

++++ High level of expression
++ Low level of expression
+ Very low level of expression
- No expression

The expression of HCV proteins in different tissues from transgenic mice (n=3, 6 month-old) was examined by immunofluoresence analysis. The expression of the proteins was semi-quantified as following: about 80% of the hepatocytes/ field showed clearly expression of the protein in 3 fields out of 5 fields examined; 40% of the kidney or spleen cells / field were expressing the protein; 20% of the lung cells / field expressed the protein, 0% cells were detected in the heart and brain in all five fields examined.

3.2.5 Viral RNA is increased with age in transgenic mice:

For further investigation of the increased expression of the viral transgene in older mice, a comparison between 4 age groups of transgenic mice (n=3 / group), aged between 4 and 18 months) was performed using real-time RT-PCR. An increased expression of the HCV RNA transcript was observed in older mice (Table 3.2). In comparison to 4 month old mice, there was a 1.9, 274 and 776 - fold increase in HCV transcript concentration at 6, 14 and 18 months respectively. Thus, similar to transgene proteins, transcript RNA expression increased in the liver of transgenic mice with increasing age.

3.2.6 Differential viral RNA expression in transgenic mice tissues:

To investigate the difference in the expression of HCV transgene in different mouse tissues, real-time RT-PCR was performed with the RNA extracted from various mouse tissues (n = 3/ tissue, at age 6 months). The HCV core, E1, E2 RNA expression was higher in the liver compared to any other tissues examined by real-time PCR (Table 3.3). Hepatic viral RNA expression was increased 100-fold over the brain. In contrast, spleen and kidneys showed approximately the same level of expression of viral RNA which was 0.3-fold of the liver tissue. The level of HCV RNA expressions in the heart and lung were 0.08 and 0.03-fold, respectively, less than the liver. Therefore, HCV RNA expression varied not only according to age, but also according to individual tissues of transgenic mice.

Table 3.2: Relative expression of RNA in the liver of transgenic mice at various ages as detected by real-time RT-PCR.

Age	Mouse No.	HCV Core C_T	GAPDH C_T	ΔC_T(Avg. Core C_T - Avg. GAPDH C_T)	$\Delta\Delta C_T$ (Avg. ΔC_{Tx} – Avg. ΔC_{Txo})	Normalized Core relative to 4M ($2^{-\Delta\Delta CT}$)
18M	4912	30.5	21.3			
	4924	30.0	20.1			
	4908	31.4	22.5			
	Avg.	30.6	21.3	9.3	9.6	776
14M	2574	27.6	16.6			
	2575	28.2	16.6			
	2576	30.3	20.5			
	Avg.	28.7	17.9	10.8	8.1	274
6M	0003	35.9	18.1			
	0021	36.5	19.6			
	0022	37.4	18.3			
	Avg.	36.6	18.6	18.0	0.9	1.9
4M	0136	38.2	18.7			
	0137	37.8	19.5			
	Avg.	38.0	19.1	18.9	0	1

Four age groups of transgenic mice (n =3 /group) were used to study the difference in liver core RNA expression using real-time RT-PCR. The first column represents mouse age group, the second column is the mouse number, the third column is the threshold cycle where the HCV core RNA signal detected, the fourth column is the threshold cycle where the internal gene (GAPDH) is detected, the fifth column (ΔC_T) is the difference between the average threshold cycle of core gene and GAPDH gene, $\Delta\Delta C_T$ method was used to analyze the relative RNA expression between the groups, the sixth column ($\Delta\Delta C_T$) is the difference between the average of the threshold cycle of each age group (ΔC_{Tx}) and 4 M group (ΔC_{Txo}), and the last column is the Normalized Core relative to 4M ($2^{-\Delta\Delta CT}$).
M: Month, Avg: Average, C_T ; Threshold cycle.

Table 3.3. Relative expression of RNA in different tissues as detected by real-time RT-PCR.

Transgenic mouse tissue	HCV core C_T	GAPDH C_T	ΔC_T (Avg. Core C_T- Avg.GAPDH C_T)	$\Delta\Delta C_T$ (Avg. ΔC_T x –Avg. ΔCliver)	Normalised Core Relative to liver ($2^{-\Delta\Delta C T}$)
Liver	25.4	13.00	12.40	0	1.00
Spleen	28.5	14.35	14.15	1.75	0.30
Kidney	25.4	11.20	14.20	1.80	0.29
Heart	26.5	10.45	16.05	3.65	0.08
Lung	29.6	11.45	18.15	5.25	0.03
Brain	30.9	12.40	18.50	6.10	0.01

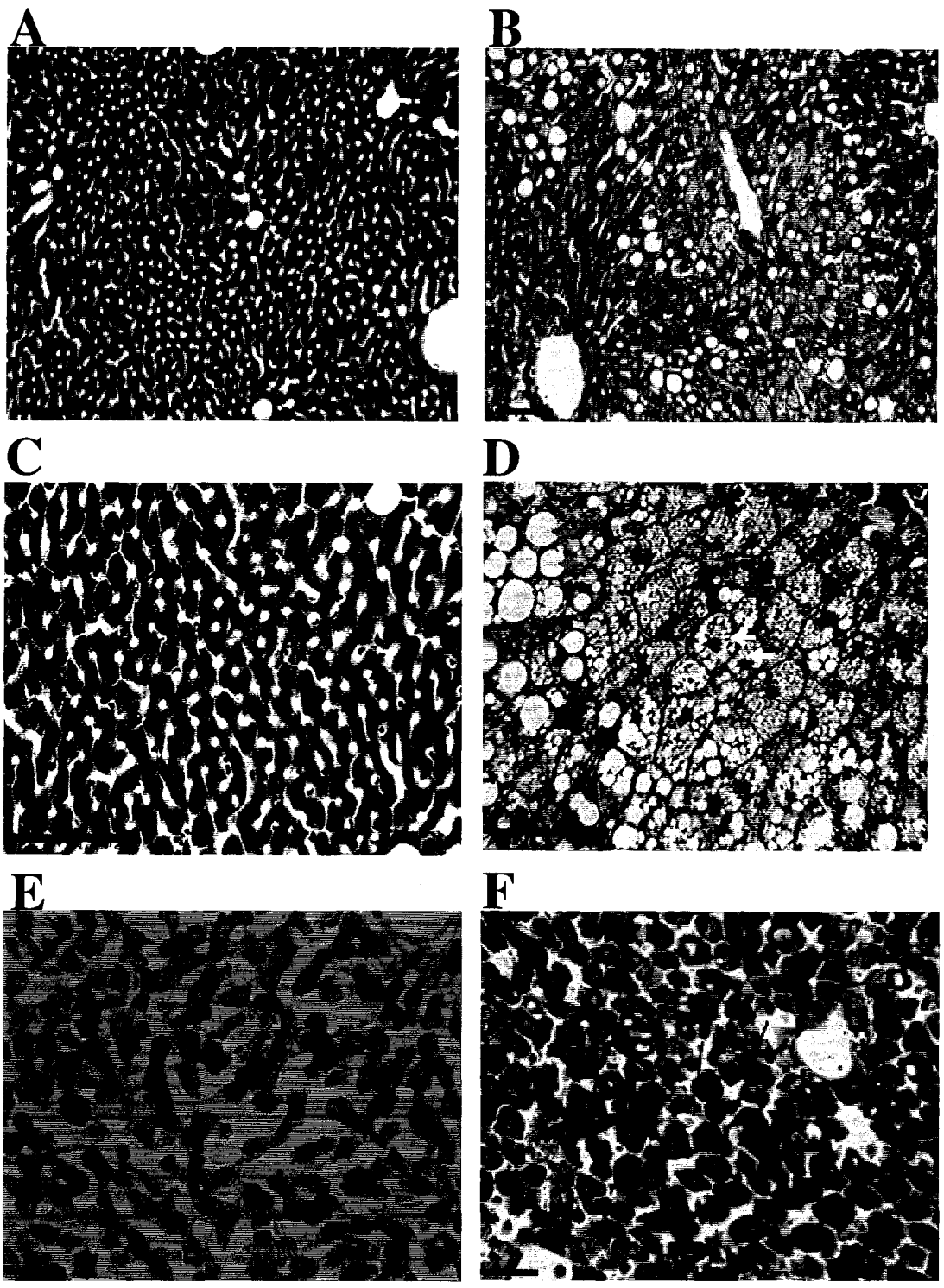
The difference of HCV Core RNA expression in different transgenic mouse tissues using real-time RT-PCR. The first column represents transgenic mouse tissues, the second column is the threshold cycle where the HCV core RNA signal detected, the third column is the threshold cycle where the internal gene (GAPDH) is detected, the fourth column (ΔC_T) is the difference between the average threshold cycle of core gene and GAPDH gene, $\Delta\Delta C_T$ method was used to analyze the relative RNA expression between different tissues, the fifth column ($\Delta\Delta C_T$) is the difference between the average of the threshold cycle of any tissues (ΔC_{Tx}) and liver (ΔC_{Txo}), and the last column is the Normalized Core relative to 4M ($2^{-\Delta\Delta CT}$). n= 3 per each tissue, age = 6 month, Avg: Average, C_T ; Threshold cycle.

3.2.7 Histopathological alterations in HCV transgenic mice:

Post-mortem examination of transgenic mice demonstrated that the gross morphology of organs was normal, with the exception of the livers, which showed hepatomegaly and yellow discoloration. A comparision study between the liver weight and mouse body mass showed that transgenic mice (n=4) had a ratio of liver to body mass of 6.1 +/- 1.6% while the non-transgenic counterpart (n=5) demonstrated a ratio of 4.1 +/- 0.6% ($p<0.01$). To determine if the expression and accumulation of HCV proteins induced morphological alteration in hepatocytes, paraffin-embedded liver sections from all age groupes were stained with hematoxylin and eosin. Transgenic hepatocytes showed several morphological alterations including increased cell volume and disorganization of the cytoplasm. Normal hepatocytes showed a polygonal shape that was well delineated and arranged as hepatic plates (Fig. 3.5A & C). In contrast, transgenic hepatocytes were round and swollen to about 3 times the size of normal cells. There was a loss of hepatic cell borders and hepatic plate arrangement in transgenic hepatocytes as well as non-uniform staining in the nuclei (Fig.3.5B & D). Cytoplasmic vacuolation characteristic of steatosis was prominent in the hepatocytes of transgenic mice at 3 months of age or older. Both microvesicular (accumulation of fine lipid droplets in the hepatocyte cytoplasm) and macrovesicular steatosis (accumulation of large lipid droplet in the cytoplasm of the hepatocytes) was observed. There was a centrilobular distribution of macrovesicular steatosis, while the microvesicular pattern was more generally distributed in the liver.

Fig. 3.5: Hepatic steatosis in HCV transgenic mice.

Mouse livers were fixed in 4% paraformadehyde and embedded in paraffin. 5μm thick sections were stained with Hematoxylin and Eosin staining. Sudan black B was used to stain for lipids. (A & C) are sections from an H&E-stained paraffin-embedded liver from a one year-old non-transgenic mouse. (B & D) are liver sections from a one year-old transgenic mouse showing ballooning of the hepatocytes which are macrovesicular (indicated by green arrows) and microvesicular (indicated by yellow arrows) steatosis. (E & F) Hepatic steatosis in transgenic mice as detected by Sudan Black B staining. (E) 1 year-old non-transgenic mouse liver cryosections (5um) showing no black stained lipid droplets. (F) 1 year-old transgenic mouse liver section showing black stained lipid droplets as indicated by arrows. The nucleus of the cell is stained red. (A, B, E, F: Magnification 20X. Scale bar, 50 μm). (C and D: Magnification 40X. Scale bar, 50 μm). The pictures represent what was observed in tissues from at least three transgenic or non-transgenic mice studied.



In examinaning a total of 109 mice, it was observed that 45.8% of transgenic mice that developed steatosis were male, while 54.2% were female (Table 3.4). The extent of the steatosis became more severe in older mice, which resulted in involvement of more than 90% of hepatocytes at the age of 12 months. Sudan Black staining, a special lipid stain, confirmed that the observed cytoplasmic vacuolation in hepatocytes was in fact lipid deposits in transgenic mice (Fig. 3.5 F). The lipid droplets stain black colour where as the nucleus is counter stained with red colour. There was minimal or no inflammatory cell infiltration in liver sections of transgenic mice. Masson's Trichrome staining, a special staining for fiber and collagen, demonstrated deposits of collagen in portal areas and the collapse of lobular architecture in the liver of older mice (12 to 18 month-old) (Fig. 3.6 F), which may indicate fibrosis formation in older HCV transgenic livers. Histological abnormalities were also found in kidneys of transgenic mice. Microtubular and glomerular epithelial cells were swollen and demonstrated some cytoplasmic vacuolation (data not shown).

3.2.8 Evidence of lymphoproliferative disorders and tumors in transgenic mice:

Several mice developed nodular hyperplasia in liver, spleen and lymph nodes, as well as lymphosarcoma in the liver. Out of 109 transgenic mice investigated, 17 (15.59%) transgenic mice developed different types of tumors. Of these mice, 12 (70.5%) were male suggesting that tumor formation occurred more readily in males compared to females (Table 3.4). Mice which developed lymphoproliferative disorders were 18 months of age or older. Transgenic mice developed different hepatic tumors, including adenomas (29%) (Fig. 3.6 A, B, & C), hemangiosarcoma (10%) (Fig. 3.6 D), hemangiomas (17.6%) (Fig. 3.7 A, B, & C), lymphoid tumors (29%) (Fig. 3.7 D) and hepatocellular carcinoma (23%) (Fig. 3.7 E).

Table 3.4: Steatosis and tumor formation in the five transgenic lines investigated in the study:

Transgenic Line	Transgenic	Total #	Gender	Total #	Steatosis *			Tumor formation	
					*3+	2+	1+	Positive	Negative
Line 1-1	Tg+	53	M	26	13	6	7	6	20
			F	27	10	12	5	2	25
	Tg -	38	M	15	0	0	2	0	15
			F	23	0	0	1	0	23
Line 2-1	Tg+	6	M	3	3	0	0	1	2
			F	3	2	1	0	0	3
	Tg-	15	M	9	0	0	0	0	9
			F	6	0	0	1	0	6
Line 3-1	Tg+	27	M	9	7	2	0	1	8
			F	18	12	6	0	0	18
	Tg-	3	M	3	0	0	0	0	3
			F	0	0	0	0	0	0
Line 4-1	Tg+	5	M	0	0	0	0	0	0
			F	5	3	2	0	2	3
	Tg-	8	M	3	0	0	0	0	3
			F	5	0	1	0	0	5
Line 5-1	Tg+	18	M	12	10	2	0	4	8
			F	6	4	2	0	1	5
	Tg-	12	M	8	0	0	0	0	8
			F	4	0	0	0	0	4

* The extent of steatosis was determined by a semi-quantitative method, in which 1+ represented lipid droplets in sporadic hepatocytes, 2+ was moderate steatosis, in which hepatocytes with lipid droplets were seen clearly in more than 2 of five fields examined, 3+ represented moderate to severe steatosis, in which lipid droplets were extensively distributed in most areas of the liver. M = male, F = female, Tg+ = transgenic, Tg- = non-transgenic.

Fig. 3.6: Development of liver tumors in transgenic mice.

Different liver tumors in transgenic mice (<1 year-old) were stained with H&E stain and examined for their distinct histological appearance. (A & B) Different magnifications of mouse hepatocellular adenomas (indicated by yellow rectangle and arrows), which are typically discrete proliferative lesions with loss of normal lobular architecture, absence of portal areas, and some degree of compression of adjacent normal hepatic parenchyma (indicated by red arrows). This is a discrete basophilic adenoma. Basophilic adenomas are often comprised of closely spaced hepatocytes with small amounts of cytoplasm. (C) Another mouse Hepatocellular adenoma which is typically discrete proliferative lesion (indicated by yellow arrows) with some degree of compression of adjacent normal hepatic parenchyma. Hepatocytes filled with lipid appear in the edge of the adenoma. (D) Liver hemangiosarcomas. The neoplastic cells are generally elongated or spindle-shaped. These neoplasms are typically locally invasive. This mouse has metastasis of hemangiosarcoma into liver, kidney, intestinal lymph nodes. (A: Magnification 1X, Scale bar 250 μm; B & C: magnification 5X, scale bar 100 μm; D; magnification 20X, scale bar 50μm).

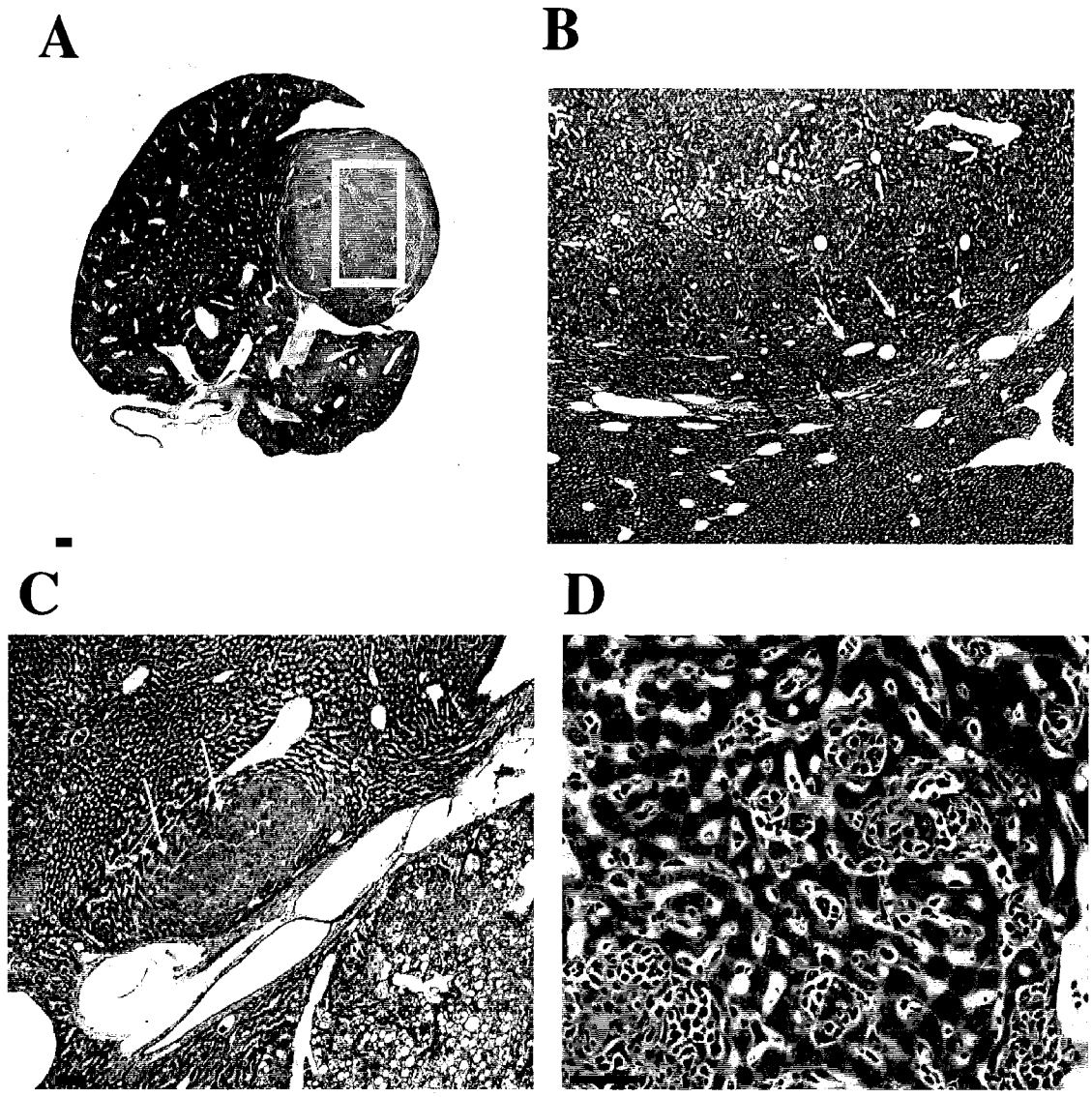
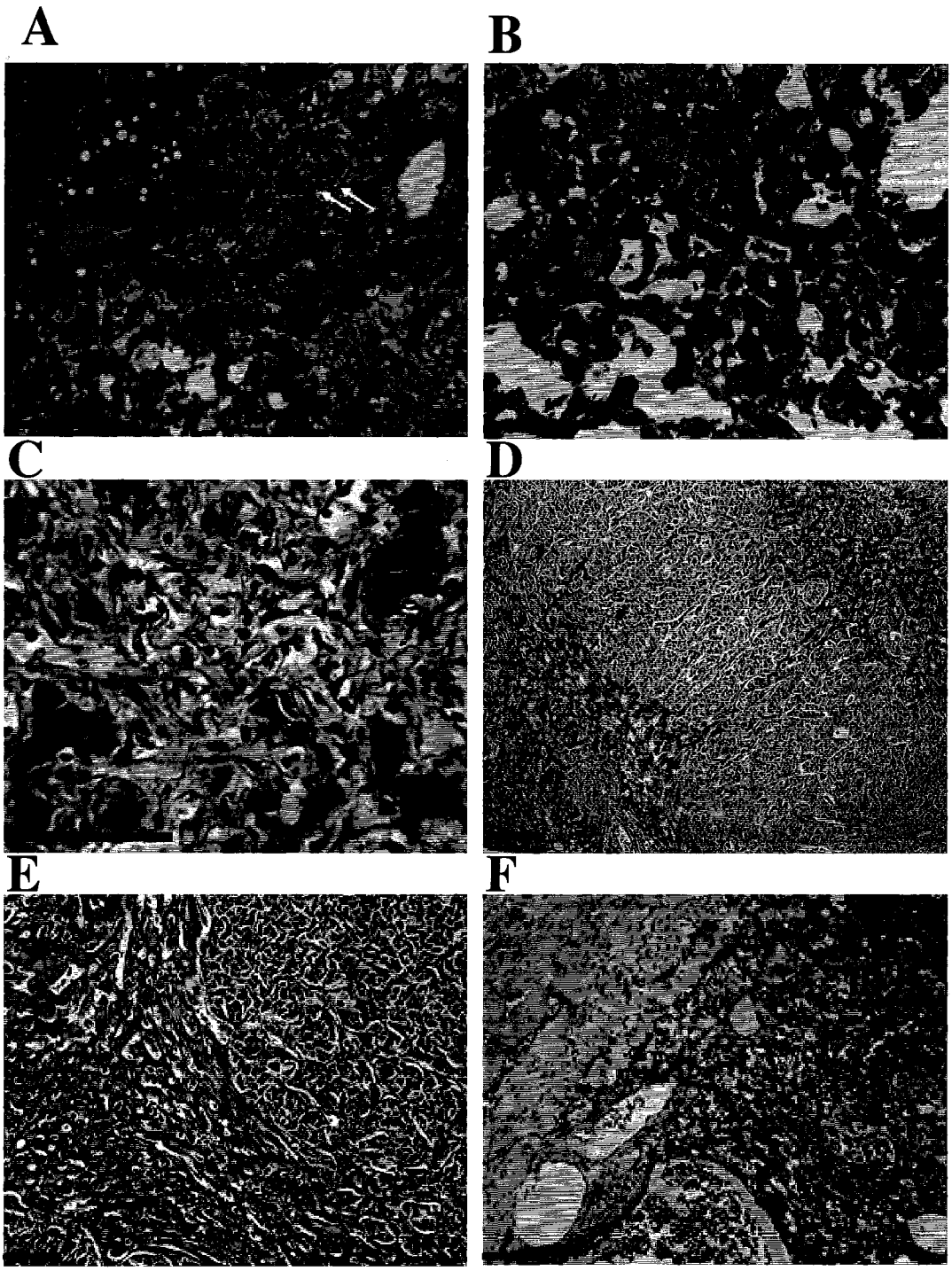


Fig. 3.7: Spectrum of liver tumors in HCV transgenic mice.

(A-B) Hepatic hemangioma in HCV transgenic mice (<1 year-old). H&E-stained paraffin-embedded sections showing early endothelial cell proliferation and peliosis-proliferating endothelial cells (indicated by arrows) invading hepatic sinusoids in the focal area - but there is more focal angiopathy rather than neoplasm, characteristic of benign hemangioma. (A) The edge of the hemangioma. (B) The centre of the hemangioma. (Magnification 40X. Scale bar, 50 μm). (C) Liver hemangioma stained with Masson's trichrome stain showing blue stained collagen trapped between hepatocytes. Red blood cells are shown in light brown colour (Magnification 60X, Scale bar, 50μm). (D) Hepatic lymphoid tumor section stained with H&E stain. Liver tissue infiltrated by a nodular lymphoid mass which is composed of small lymphocytes forming follicles. The lymphoid infiltrate displaced the near hepatic parenchyma. (Magnification 20X, scale bar, 50μm). (E) Trichrome staining of a transgenic liver section demonstrating development of hepatocellular carcinoma. It characterized by polymorphic nuclei and invasion of steatotic liver by anaplastic cells. Cells are arranged in macrotrabecular patterns - where trabeculae are eight or more cells thick (Magnification 20X, scale bar 50μm). (F) Trichrome staining of a liver section in an 18-month old transgenic mouse. Capsular fibrosis involves large lobular areas and multiple lobules, producing approximation of portal fields together, which can still be recognized with a stain for elastic fibres (Magnification 20X, scale bar,50μm).



Hepatic hemangioma was observed in HCV transgenic mouse mice (<1 year-old). It is characterized by early endothelial cell proliferation, peliosis and proliferating endothelial cells invading hepatic sinusoids in the focal area. There is more focal angiopathy rather than neoplasm, characteristic of benign hemangioma (Fig. 3.7 A, B, & C). Interestingly, lymphoid tumors developed in the liver of HCV transgenic mice, which indicate an association between the HCV proteins expression and the formation of lymphoid tumors. The microscopic analysis of lymphoid liver tumor showed that the tissues were infiltrated by a nodular lymphoid mass which was composed of small lymphocytes forming follicles. This lymphoid infiltrate displaced the closest hepatic parenchyma (Fig. 3.7 D). The mice with liver lymphoid tumors also developed tumors in the spleen and in cervical lymph nodes as well. Furthermore, some HCV transgenic mice developed hepatocellular carcinoma at age more than 1 year.. Microscopically, HCC showed polymorphic nuclei cells and invasion of steatotic liver by anaplastic cells (Fig. 3.7 E). Cells are arranged in macrotrabecular patterns, where trabeculae are eight or more cells thick, differentiating it from an adenoma. All corresponding tissues from non-transgenic littermates did not demonstrate tumors or any evidence of lympoproliferative disorders.

3.2.9 Subcellular abnormalities in transgenic hepatocytes:

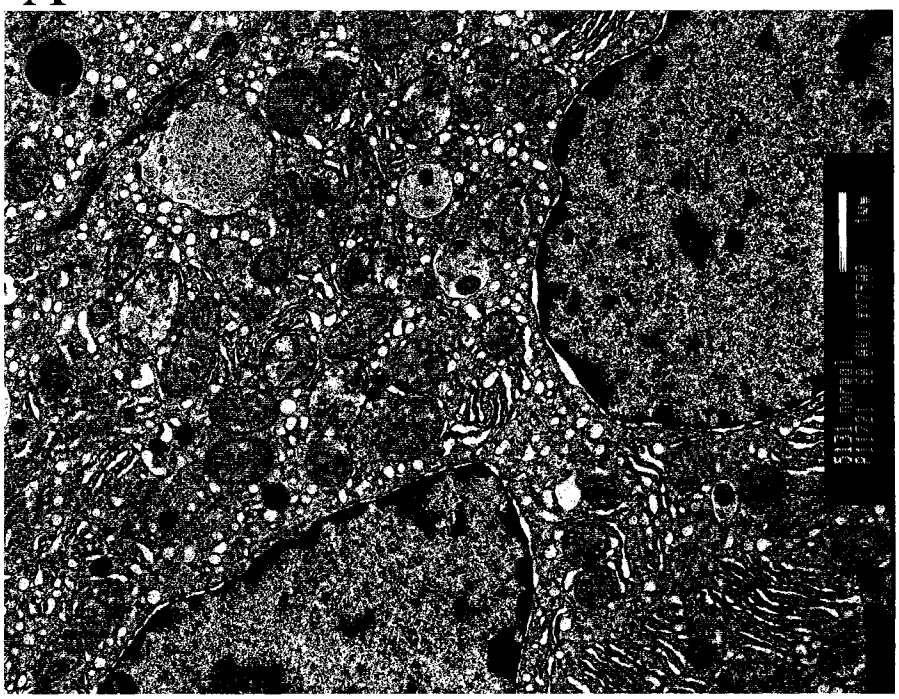
In order to examine the livers in more details, electron microscopy analysis was carried out. Several abnormalities in the intracellular organelles of hepatocytes from transgenic mice were evident (Fig. 3.8 A & B). A loss of the rough endoplasmic reticulum pattern was observed as well as swelling of mitochondria and loss of mitochondrial cristae. Additionally, the nucleus had lost the normal organization of chromatin, which appears

uncondensed and uneven in contrast to a normal nucleus. These abnormalities were observed in 3 mouse livers that were investigated in 1 year old mice.

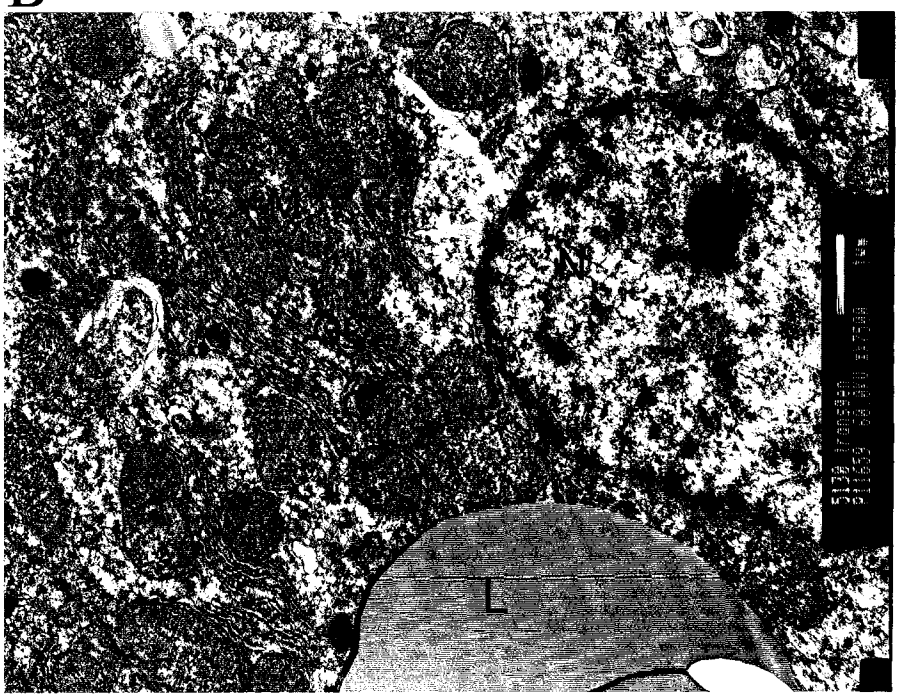
Fig. 3.8: Subcellular abnormalities in transgenic hepatocytes.

Electron microscopy analysis of normal (A) and transgenic mouse liver (B) was performed. In the transgenic, extensive abnormalities are observed in the hepatocyte organelles, with abnormal mitochondrial shape, swelling and loss of cristae as well as chromatin disorganization and lipid droplets in the cytoplasm (magnification 7500x). (L: lipid, M: Mitochonderia, N: Nucleus, GC: Golgi complex). The pictures represent what was observed in one mouse liver out of three one year old mice.

A



B



3.3 DISCUSSION

The purposes of this thesis was to elucidate the role of HCV structural proteins in hepatitis C pathogenesis by establishment and characterization of a transgenic mouse model that encodes the HCV structural proteins (core, E1 and E2) and studying the histopathological changes in the transgenic mice over a period of time. The structural proteins, core, E1, and E2, of HCV were expressed in a transgenic mouse model using a universal promoter to elucidate the effects of HCV on hepatic and extra-hepatic tissues. Initially, the Pvax-1 core, E1 and E2 vector was used to transfect CHO cells, in order to confirm proper expression of the viral proteins *in vitro*. The results showed a high level of protein expression from this vector. The HCV-transgenic mice expressed RNA encoding the structural proteins in all examined tissues. However, in adult mice viral proteins were selectively expressed in the liver, but detected in low concentrations in both the kidney and the spleen. The differential expression of viral proteins in murine tissues suggests that viral protein expression may be controlled via translational mechanisms. The translational activity of the CMV promoter could be affected by some cellular factors which cause diffent protein expression in different tissues. Immunohistochemistry suggested that the level of viral proteins in the liver increased with age, which could be due to effect of some cellular factors in hepatocytes associated with increasing age or could be a polyclonal expansion of the hepatocytes that express the protein with increasing age. The difference in the protein expression according to age was also investigated in the level of RNA by real time RT-PCR, which was performed in transgenic mice livers of different age groups and in various tissues.

The expression of the HCV structural proteins in the kidneys of transgenic mice was associated with histological abnormalities including swollen microtubular and glomerular

epithelial cells. It has been shown that chronic infection with HCV can lead to the immune complex syndromes of cryoglobulinemia and membranoproliferative glomerulonephritis [101,102,107]. Also hepatitis C is a complicating factor among patients with end-stage renal disease and renal transplants [262]. When fetal transgenic mice were examined, the expression of the viral protein was only seen in salivary glands. This expression was not observed in adult transgenic mice. Several studies have shown that HCV can replicate in several tissues other than the liver, such as lymphocytes and salivary glands [238,243,244]. This may have important implications for the pathogenesis of HCV infections. There is a strong association of salivary gland lesions, lymphocytic capillaritis, and lymphocytic adenitis with chronic hepatitis C. HCV infection of lymphocytes may be related to HCV-associated lymphoma and/or autoimmune disorders. Similarly, salivary gland infection may be associated with Sjogren's syndrome [244,263].

The HCV structural proteins caused profound structural abnormalities in the liver. Progressive steatosis, mitochondrial swelling and nuclear abnormalities became more severe with age. This hepatopathy was more widespread in the liver of older mice. Eventually there was increasing fibrosis, and liver cancer in older mice. Thus, this transgenic model of HCV represents many of the liver abnormalities observed in human infection. Moriya *et al.* (1997) described an HCV transgenic mouse model that expressed only the core protein. Their results showed that mice 16 months of age developed hepatic tumors, first appearing as adenomas with fat droplets in the cytoplasm. Additionally, they demonstrated neoplasia development within the adenoma, presenting in a "nodule-in-nodule" formation. The authors concluded that the HCV core protein may have a primary role in hepatocellular carcinoma development in transgenic mice and suggested that steatosis may develop as early as 3 months of age.

Similarly the transgenic mouse model used in this study developed steatosis as early as 3 months and developed adenoma and carcinoma after one year of age. In contrast to the HCV core transgenic mice; this transgenic model expressed the three HCV structural core, E1 and E2 proteins.

The three HCV structural proteins are important for the viral life cycle, specifically in the entry and formation of the viral capsid [21,37]. The significance of including core, E1 and E2 as a polyprotein in this mouse model allows for the analysis of the impact of these three proteins as a complex in the liver over the life span of the transgenic mice. While younger transgenic mice have no detectable HCV proteins, at 3 months of age an increasing number of hepatocytes expressing these proteins around central veins was observed. Viral protein expression and steatosis appeared in a similar timeframe. Transgenic mice did not develop steatosis until they were at least 3 months of age with both severe micro and macro-vesicular steatosis eventually developing. Several studies suggested that the accumulation of lipids in hepatocytes is indicative of a disturbance in the lipid metabolism that may be caused by the accumulation of viral proteins and it is likely related to defects in mitochondrial and peroxisomal fatty acid oxidation and biosynthesis [178,209,237,264,265]. The results presented in my studies demonstrated aggravation of liver abnormalities with increasing age. Previous studies have demonstrated that steatosis is associated with a decreased anti-oxidant effect in the aging liver. Similarly, a study performed by Lerat *et al.* (2002) also indicated that steatosis increased with age. However, their mouse model rarely developed steatosis before 10 months of age.

A cross-sectional study by Kumar *et al.* (2002) suggested that the HCV genotype 3 but not genotype 1 was cytopathic and induced hepatic steatosis in HCV infected patients.

However, the mouse model I studied expressing HCV genotype 1a caused severe steatosis, which demonstrates that this genotype might also involved in the development of this abnormality at least in mice. Both Rubbia-Brandt *et al.* (2000) and Adinolfi *et al.* (2001) suggested that steatosis in patients infected with HCV genotype 1 is not of viral origin. However, the model described in this study suggested that HCV genotype 1a may induce steatosis and it is of viral origin. Steatosis caused by obesity was ruled out in this study, as the transgenic mice were not obese. More compelling evidence included that the non-transgenic littermates did not show steatosis.

In contrast to earlier studies by Lerat *et al.* (2002) using an albumin liver specific promoter and Moriya *et al.* (1997) using an exogenous promoter, the results of this present study reflect the activity of a CMV promoter that has a universal transcriptional regulator. Thus, protein expression was illustrated in extra-hepatic tissues. After establishing the presence of HCV in older mice, fetal transgenic mice were analyzed. In fetal mice, HCV structural proteins were detected only in the salivary glands. Koike *et al.* (1995) confirmed this in another study. In contrast to Koike *et al.* (1995), there was no sialadenitis present in our transgenic mouse model. However, Koike *et al.* (1995) developed mice that expressed only E1 and E2. In addition, the expression of HCV proteins in the salivary glands of older mice seemed to be lost, which may explain the lack of sialadenitis in this model. The expression of HCV structural proteins in extra-hepatic tissues was unstable in this study. Therfore these transgenic mice may not be as useful for characterizing extra-hepatic sites of pathogenesis associated with persistent HCV infection.

Several mice older than 18 months of age demonstrated lymphoproliferative disorders with nodular hyperplasia and lymphoma developing in the liver. A recently described

transgenic model, expressing HCV core, developed lymphomas and liver adenomas in mice 20 months of age or older [238]. Similarly, HCV patients may develop two types of lymphoproliferative disorders, cryoglobulinemia and Non-Hodgkin's B-cell lymphomas in the liver [258,259]. Clonal expansion of B-cells has been detected in bone marrow, blood and liver of HCV-infected patients. One study suggests that HCV E2 could play a pathogenic role by stimulating a strong humoral response and the clonal expansion of B-cells, resulting in lymphoproliferative disorders [259]. However, the transgenic mice used in this study are tolerant to the transgenes and chronic lymphocyte stimulation may therefore originate by other mechanisms. It is likely that the core protein may be involved in oncogenicity and lymphoproliferation by interfering with some cell growth functions. Thus, the study of this model may be useful to define the pathogenesis of HCV-associated lymphoproliferative disorders.

Recent publications have illustrated that HCV confers oncogenic potential to hepatocytes [93,266-271]. In addition, HCC tumorigenesis in patients infected with HCV is correlated with steatosis [268,272,273]. Transgenic mice with steatosis displayed dysplastic growth of cells evolving into tumor formation. Furthermore, transgenic mice showed deposition of collagen and progressive fibrosis [251]. These transgenic mice expressing core with a liver specific serum amyloid P regulator did not demonstrate neither steatosis, nor hepatic tumors after 18-24 months. Therefore, the other two structural proteins of HCV (E1 and E2) used in this study may be a requisite for liver tumor development. Although the underlying mechanism of HCC progression in HCV transgenic mice remains to be understood, our transgenic mice are suitable models to elucidate this role.

Murine models are critical for understanding HCV pathogenesis and to explore the molecular interface between HCV, the liver and other tissues. Recently transplantation of healthy human hepatocytes that carry a plasminogen activator transgene (Alb-uPA) into SCID mice was carried out [248]. This model may be useful in acute studies of antiviral activity against HCV. In summary, expression of HCV core, E1 and E2 in the transgenic mice liver may contribute to the development of liver diseases including steatosis, fibrosis and liver tumors including lymphoid tumors and HCC. These transgenic mice showed alterations in the morphology of the mitochondria, which indicate an injury of mitochondria due to HCV structural proteins expression. However, further studies are required in order to explain the role of the structural proteins in mitochondrial injury and more generally in affecting their functions in lipid metabolism. The histopathological changes presented in the liver of HCV transgenic mice due to structural proteins expression, could be indicative of the alterations induced by these proteins in some liver biological processes. Therefore, in the following section, I will study these liver abnormalities in transgenic mice using complementary DNA (cDNA) microarray analysis. Thus, the effect of the HCV structural protein expression in the global gene expression in the transgenic mouse livers will be the next objective of my work.

CHAPTER 4

Disturbance of novel hepatic gene expression pathways induced by HCV structural proteins in a transgenic mouse model

4.1 INTRODUCTION

DNA microarrays provide a powerful technique for investigating the changes in gene expression characteristic of different physiological and pathological conditions. By using this technique, the expression levels of thousands of genes can be measured in a single experiment. Microarray analysis has been used in a variety of systems, including studies in whole organisms. It has been used to compare the gene expression profile in cardiac and skeletal muscles of mice [274], and it was also used to demonstrate the biological process in Drosophila development [275]. Recently, microarray studies have been used to study the virus-host cell interactions for some viral infections, for example. human immunodeficiency virus, human cytomegalovirus, and human papillomavirus [215,276-278]. These studies showed that HIV-1 infection causes differential changes in genes involved in T-cell signaling, subcellular trafficking and transcriptional regulation, while human papillomavirus type 31 change the expression of interferon-inducible genes and Stat-1. The analysis in these viral infections has helped in the identification of several important genes involved in viral infection and pathogenesis.

DNA microarray analysis is an attractive tool to characterize the HCV-host interactions, which could elucidate the mechanisms controlling HCV persistent infection and progression. Previous studies used microarrays to analyze liver biopsy samples from chimpanzees with acute-resolving HCV infections [227,279]. These studies demonstrated remarkable changes in gene expression in the IFN-stimulated genes (ISGs), including IFNα/β., which is initial cell response that limits HCV replication in the liver. Microarray technique was not only used in acute-resolving HCV infection, but it was also used to study intrahepatic gene expression during chronic hepatitis C virus infection in chimpanzees [128].

These chronically infected animals showed a sustained elevation in ISGs, which indicate an ongoing response to IFNs. Some of the gene changes were unique for individual animals, which may be related to infection with different viral genotypes, age, and/or duration of infection. Also, hepatic gene expression profiling has been used to discriminate responders and non-responders to IFN-α plus ribavirin therapy of chronic hepatitis C infection [280]. This study demonstrated up-regulation of IFN-responsive genes predicts nonresponse to exogenous IFN therapy.

In addition, microarray analysis was valuable in the identification of potential markers in livers with progressive fibrosis, cirrhosis, and HCC associated with chronic HCV infection [281-283]. It has also been used to identify specific gene-expression profiles associated with a risk for liver carcinogenesis during HCV infection. Differentially expressed genes in HCV-positive patients were analyzed by cDNA microarrays. It showed that specific gene-expression signatures, such as some oncogenes, in non-cancerous liver tissue may help to predict the risk for developing HCC [284]. Another study in livers of HCV-infected patients identified genes that are associated with fibrosis progression and hepatocarcinogenesis [285]. The expression of genes involved in the cell cycle and oncogenesis were increased in patients with HCC.

In vitro microarray analysis studies demonstrated that the HCV core protein, expressed in human hepatocellual carcinoma (HepG2) cell line, changes the expression profiles of genes involved in molecular transport, signal transduction, regulation of transcription and protease activity [286,287]. Another study, using HepG2, demonstrated that HCV core protein induces expression of genes regulating immune evasion and anti-apoptosis in hepatocytes [288]. This study suggested that HCV core expression caused decrease in the

expression of some chemo-attractants which lead to suppression of inflammatory response and trafficking of macrophages and neutrophils to the site of HCV infection, and it also increased the expression of anti-apoptosis factors such as PAK2 [288]. It has been shown that HCV core protein promotes immortalization of primary human hepatocytes by causing changes in the expression of genes involved in several cellular pathways, including cell growth regulation, immune regulation, oxidative stress, and apoptosis. This study showed that HCV core protein increases the expression of IL-6, gp130, leptin receptor, and STAT3 [289]. Thus, these studies indicate that DNA microarrays are a useful tool to investigate the spectrum of changes in liver gene expression during the course of HCV infection.

Little is understood regarding the dynamic of host gene expression resulting from persistent HCV infection. To better elucidate the changes in host gene expression characteristics of pathological changes observed in the HCV transgenic mouse model, cDNA microarray analysis was performed in the livers of HCV transgenic mouse model expressing HCV structural proteins. As I explained in chapter 3, this HCV transgenic mouse model showed severe hepatopathy characterized by the development of steatosis as well as liver and lymphoid tumors [261]. In this study, differential gene expression in transgenic and non-transgenic mice expressing core, E1 and E2 proteins were analyzed using cDNA microarrays. The differential expression of these genes in HCV transgenic mice may help us understanding the mechanisms of histopathological changes observed in transgenic mouse lives including steatosis, fibrosis and tumor formation. This will be useful to elucidate the HCV pathogenesis in chronic liver disease.

4.2 RESULTS:

4.2.1 Differentially-expressed gene identification

15 600 genes were filtered by removing bad spots (spots that have low intensity and non-numeric value by the image quantitation software) and applying dominancy analysis (analysis used to eliminate the improper influence of an inconsistent replicate) procedures to detect the differentially-expressed genes. The *t*-test was used to identify differentially expressed genes (P value $\geq$ 0.05 and fold change $\geq$ 1.5). Differences in 394 genes were identified to be statistically significant, with 196 genes (142 corresponding to known genes) identified as up-regulated (Appendix I) and 198 genes (164 corresponding to known genes) identified as down-regulated (Appendix II). These 306 known genes were further clustered by applying GO Annotations search or KEGG Pathway Links search. GO terms search were performed for the 306 differentially-expressed genes from the GO Database (Table 4.1). Genes were then clustered according to their behavior, function, and structure using GO annotations. For example, Bcl2-associated athanogene 4 (Bag4) (up-regulated), transcription factor Dp1 (Tfdp1) (down-regulated) and B-cell receptor associated protein 31 (Bcap31) (down-regulated) belong to the apoptosis biological process.

A search was also performed for pathway links from the KEGG database, which is a collection of manually drawn pathway maps representing knowledge on the molecular interaction for metabolic pathways, genetic information processing, cellular processing, human diseases, etc. Among the 306 differentially-expressed genes, 207 have not been reported in this database. The remaining 99 genes distribute among 102 pathways. For example; 5 of the differentially-expressed genes are involved in peroxisome proliferator-activated receptors (PPAR) signaling pathway, a signaling system of fatty acid derivatives.

Table 4.1: Some of differentially expressed genes in HCV transgenic livers described in the study according to GO classification:

Biological Process GO Term	Clone ID	MGI ID	Symbol	Description
GO:0006631 Fatty acid metabolism				
	H3134C07	**MGI:2148491**	**Acaa1 (Up)**	Acetyl-Coenzyme A acyltransferase 1
	H3119H04	**MGI:1921455**	**Utp14b (Dn)**	Acyl-CoA synthetase long-chain family member 3
	H3018A12	**MGI:1330812**	**Acox1 (Up)**	acyl-Coenzyme A oxidase 1, palmitoyl
GO:0006869 Lipid transport				
	H3131B04	**MGI:98254**	**Scp2 (Dn)**	Sterol carrier protein 2, liver
	H3043A10	**MGI:88049**	**Apoa1 (Dn)**	Apolipoprotein A-I
	H3120B05	**MGI:88054**	**Apoc2 (Up)**	apolipoprotein C-II
	H3067H01		**Osbpl3 (Up)**	Oxysterol binding protein-like 3
	H3025G09	**MGI:88051**	**Apoa4 (Up)**	Hypothetical LOC403343
GO:0006412 Protein biosynthesis				
	H3122C10	**MGI:1333818**	**Rps7 (Dn)**	ribosomal protein S7
	H3130B01	**MGI:1915141**	**Rpl4 (Dn)**	Ribosomal protein L4
	H3130A12	**MGI:1915141**	**Rpl4 (Dn)**	Ribosomal protein L4
	H3117C09	**MGI:98073**	**Rpl7 (Dn)**	Ribosomal protein L7
	H3129B12		**Eef2 (Dn)**	Eukaryotic translation elongation factor 2
	H3129C02		**Eef2 (Dn)**	Eukaryotic translation elongation factor 2
	H3155E03	**MGI:2385325**	**Rpl21 (Dn)**	ribosomal protein L21
	H3130H12	**MGI:1351605**	**Rpl3 (Dn)**	Ribosomal protein L3
	H3112F09	**MGI:1351605**	**Rpl3 (Dn)**	Ribosomal protein L3
	H3011F03	**MGI:1351605**	**Rpl3 (Dn)**	Ribosomal protein L3
	H3112F11	**MGI:1351605**	**Rpl3 (Dn)**	Ribosomal protein L3
	H3124F09	**MGI:1927099**	**Rplp1 (Dn)**	ribosomal protein, large, P1
	H3011E01	**MGI:1350927**	**Rpl8 (Dn)**	Ribosomal protein L8
	H3115D02	**MGI:105381**	**Rpsa (Dn)**	ribosomal protein SA
	H3115D03	**MGI:105381**	**Rpsa (Dn)**	ribosomal protein SA
	H3125H12	**MGI:98159**	**Rpsa (Dn)**	ribosomal protein S6
	H3036C09		**Eef1a1 (Dn)**	eukaryotic translation elongation factor 1 alpha 1

	H3123C07	**MGI:1096881**	**Eef1a1 (Dn)**	eukaryotic translation elongation factor 1 alpha 1
	H3020D07	**MGI:1096881**	**Eef1a1 (Dn)**	eukaryotic translation elongation factor 1 alpha 1
	H3126A06	**MGI:1096881**	**Eef1a1 (Dn)**	eukaryotic translation elongation factor 1 alpha 1
	H3133G05	**MGI:1096881**	**Eef1a1 (Dn)**	eukaryotic translation elongation factor 1 alpha 1
	H3115D01	**MGI:105381**	**Rpsa (Dn)**	ribosomal protein SA
	H3115G05	**MGI:1913739**	**Rpl35 (Dn)**	ribosomal protein L35
	H3036C07	**MGI:105943**	**Rpl10 (Dn)**	ribosomal protein 10
	H3138D12		**Eefla1 (Dn)**	Eukaryotic translation elongation factor 1 alpha 1
	H3112B11	**MGI:1924058**	**Rpl18a (Dn)**	Ribosomal protein L18A
	H3115D04	**MGI:105381**	**Rpsa (Dn)**	Laminin receptor 1 (ribosomal protein SA),
	H3118C05	**MGI:102854**	**Rpl5 (Dn)**	Ribosomal protein L5
	H3113A02	**MGI:1917438**	**Eif2c2 (Up)**	eukaryotic translation initiation factor 2C, 2
GO:0016055 Wnt receptor signaling pathway				
	H3122C01	**MGI:894659**	**Cog5 (Dn)**	
GO:0019886 Antigen processing, exogenous antigen via MHC class II				
	H3109E03	**MGI:95921**	**H2-DMb1 (Dn)**	histocompatibility 2, class II, locus Mb1
	H3028F04	**MGI:88564**	**Ctsl (Dn)**	Cathepsin L
GO:0006955 Immune response				
	H3015C01	**MGI:1350933**	**Bcap31 (Dn)**	B-cell receptor-associated protein 31
	H3067G10	**MGI:1929492**	**Arl6ip2 (Up)**	ADP-ribosylation factor-like 6 interacting protein 2
	H3044D11		**C4bp (Up)**	Complement component 4 binding protein
GO:0000165 MAPK cascade				
	H3035C08	**MGI:2144624**	**Map2k3 (Up)**	Mitogen activated protein kinase kinase 3
	H3069H04	**MGI:2149960**	**Rasa2 (Up)**	RAS p21 protein activator 2
	H3058C09	**MGI:96646**	**Jun (Up)**	Jun oncogene
	H3139G09	**MGI:87881**	**Prkcb1 (Dn)**	protein kinase C, beta 1
	H3030A07	**MGI:1339984**	**Pak2 (Up)**	P21 (CDKN1A)-activated kinase 2
GO:0006915 Apoptosis				
	H3079D08	**MGI:1914634**	**Bag4 (Up)**	BCL2-associated athanogene 4
	H3015C01	**MGI:1350933**	**Bcap31 (Dn)**	B-cell receptor-associated protein 31
	H3146G01	**MGI:101934**	**Tfdp1 (Dn)**	Transcription factor Dp 1

Dn: down- regulated, Up: upregualted

PPARs are lipid-activated transcription factors that belong to the steroid/thyroid/retinoic acid receptor super-family. All their characterized target genes encode proteins that participate in lipid homeostasis [290,291]. These 5 differentially-expressed genes include: Acyl-Coenzyme A oxidase 1 (Acox1) (up-regulated), apolipoprotein A-IV (Apoa4) (up-regulated), malic enzyme, (Mod1) (up-regulated), apolipoprotein A-I (Apoa1) (down-regulated), and sterol carrier protein 2 (Scp2) (down-regulated).

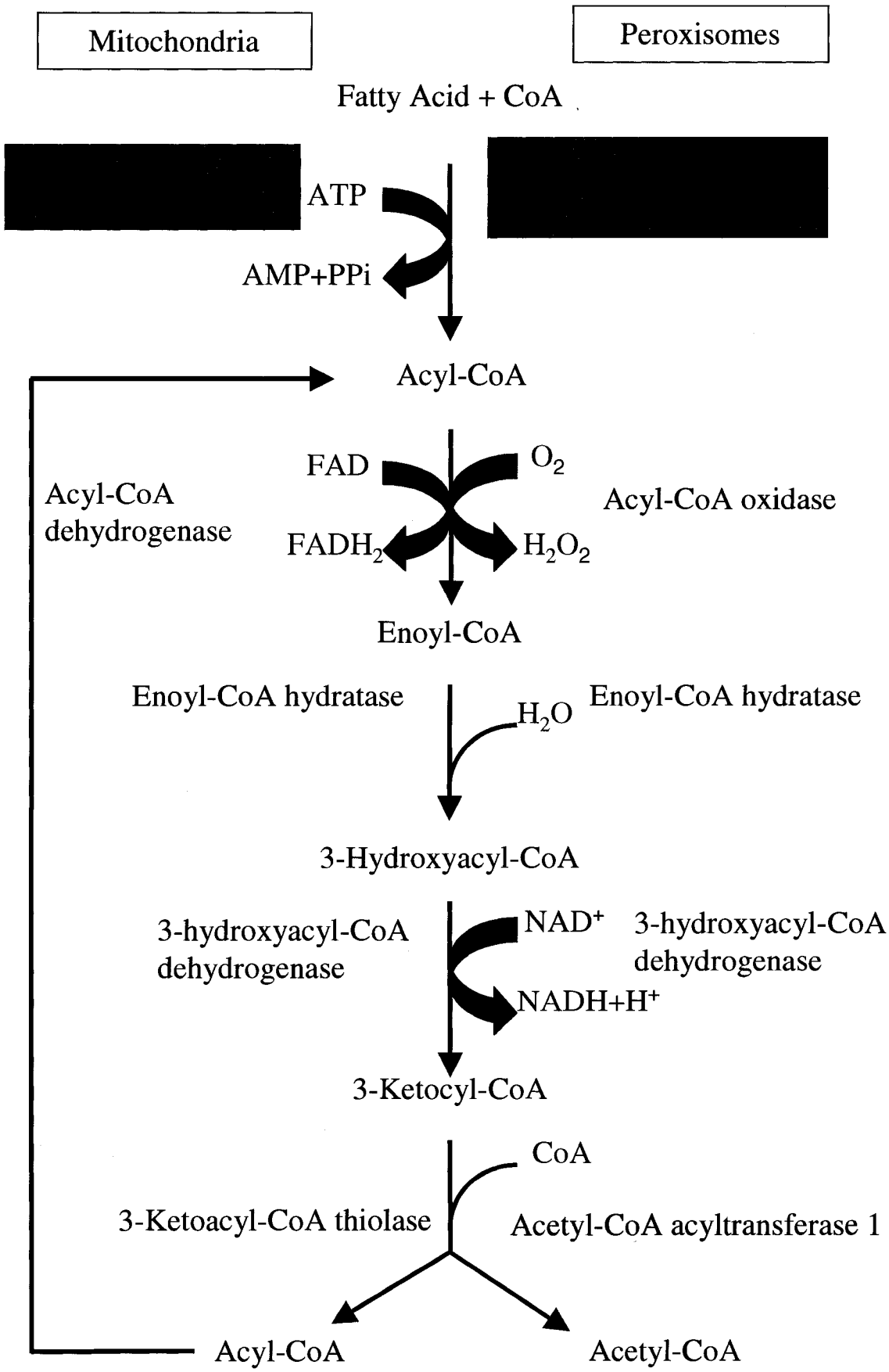
I will illustrate the results derived from the GO annotation search of some genes (Table 4.1) depending on GO to clarify the involvement of the differentially expressed genes in several key biological functions in the liver of the HCV transgenic mouse model.

4.2.2 Differentially expressed genes involved in hepatic lipid metabolism:

Several differentially expressed genes are involved in lipid metabolism. These include: Acyl-CoA synthetase long-chain family member 3 (Acsl-3 or Utp14b), which is down regulated in transgenic livers, and Acetyl-Co-enzyme A acyltransferase 1 (Acaa1) [Synonym: Peroxisomal 3-oxoacyl-CoA thiolase A (Pktaa)], and Acyl-Co A oxidase 1, palmitoyl (Acox1) [Synonym: Acyl-CoA oxidase (AOX)], which are up regulated in transgenic mice (Fig. 4.1). These three enzymes play an essential role in fatty acid metabolism. Acyl-CoA synthetase long-chain family member 3 (Acsl-3) has both catalytic activity and ligase activity to form long-chain-fatty-acid. It converts a relatively inert fatty acid molecule into an activated form (fatty acyl-CoAs) that can be utilized by numerous metabolic pathways, including fatty acid β-oxidation [292]. Fatty acyl-CoAs can be degraded for energy production or incorporated into triacylglycerol for storage. Acyl-Co A oxidase 1 (AOX) enzyme is a rate limiting enzyme in peroxisomal fatty acids β-oxidation, a metabolic

Fig. 4.1: Schematic representation of fatty acid β-oxidation pathway in mitochondria and peroxisomes:

Fatty acids are activated by acyl-CoA synthetase to form fatty acyl-CoA, which enter the β-oxidation pathway. Very long chain fatty acids oxidation occurs in peroxisomes (Right side), while oxidation of short, medium and long chain fatty acids occurs in mitochondria (Left side). Acyl-CoA synthetase long-chain is downregulated in HCV transgenic mice (blue color), Peroxisomal enzymes, Acetyl-Co-enzyme A acyltransferase and Acyl-CoA oxidase, are up regulated in HCV transgenic mice (yellow color)



oxidation of a very long-chain fatty acids in peroxisomes. It has acyl-CoA oxidase activity, by which it catalyzes the first step of fatty acid β-oxidation, the oxidation of very long chain acyl-CoA thioester to give the corresponding trans-2-enoyl-CoAs [293]. Similarly, acetyl-CoA acyltransferase 1 (Acaa1) is involved in peroxisomal fatty acid β-oxidation metabolism. It catalyzes the transfer of the acyl group from acyl-CoA to Acetyl-CoA to form oxoacyl-CoA, the last step of fatty acid β-oxidation [294]. Thus, the expression of HCV structural proteins in the liver of transgenic mice induces alterations in gene expression of three of the most important enzymes involved in fatty-acid metabolism. The down regulation of Acsl-3 gene in the transgenic liver might cause accumulation of fatty acids (as triglycerides) without proceeding to the metabolic pathways. This may explain the hepatic steatosis observed in transgenic mice.

4.2.3 Differentially expressed genes involved in lipid transport:

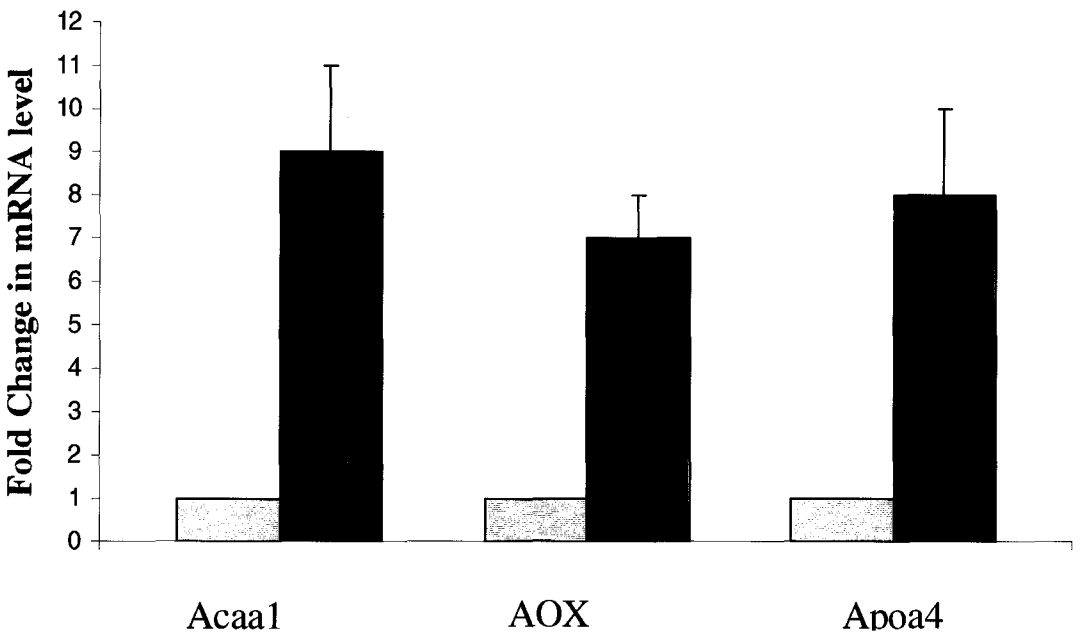
Some of the differentially expressed genes are involved in the transport of lipids. These genes include: liver sterol carrier protein 2 (Scp2) and apolipprotein A-1 (Apoa1), which are down regulated; apoliprotein A-IV (Apoa4), apolipoprotein C-II (Apoc2), and oxysterol binding protein-like 3 (Osbpl3), which are up regulated genes in the transgenic mice. Scp-2 is an intracellular non-specific lipid binding protein. It is involved in the transport and metabolism of cholesterol and it also participates in acyl-CoA metabolism [295]. This protein is believed to participate in these activities by forming a stoichiometric complex with sterols. Apolipoprotein A-I (apoa1) is the major apolipoprotein of high-density lipoproteins (HDL) and has an important role in the regulation of the stability, lipid transport, and metabolism of HDL particles [296]. It transports cholesterol from tissues to the liver for excretion which is called reverse cholesterol transport [297]. It mediates the transfer of

cholesterol from extra-hepatic tissues to HDL particles which in turn shuttle cholesterol to the liver for excretion from the body in the form of bile salts or free cholesterol. Apoliprotein A-IV (Apoa4) is associated with chylomicrons, and is secreted only by the small intestine in humans [298]. In rodents, both the small intestine and the liver secrete apoa4; however, the small intestine is the major organ responsible for circulating apoa4 [299]. Of all the apolipoproteins associated with chylomicrons secreted by the small intestinal epithelial cells (enterocyte), apoa4 is the only one that is stimulated by the absorption of fat [299,300]. Since apoa4 is one of the elements of chylomicrons, it is therefore important in the transport of dietary triglycerides, phospholipids, cholesterols and cholesterol esters absorbed by enterocytes to other tissues such as hepatocytes [300]. Apoc2 protein participates in lipoprotein metabolism and lipid transport from the intestine to the liver. Apoc2 is also a regulator of lipoprotein lipase (LPL) and it may inhibit LPL-mediated hydrolysis of triglycerides and modulates the metabolism of triglyceride-rich lipoproteins [301]. Osbpl3 protein is involved in lipid transport, but its molecular function is still unknown. Mod1 is involved in the oxidative decarboxylation of malate with the concomitant production of pyruvate. This is also may help to explain hepatic steatosis in transgenic mice.

To verify the microarray results for these genes, I performed real time-RT-PCR analysis for some of the genes involved in lipid metabolism and lipid transport; including AOX, Acaa1 and Apoa4. Real time-RT-PCR assays were performed using the GenAmp 5700 Sequence Detection System. Data were normalized to an internal control (GAPDH) and $\Delta\Delta$CT method was used to obtain the relative expression level for each gene. In comparison to non-transgnic mice, there was a 9, 7, 8 – fold increase in the RNA concentration of Acaa1, AOX, and Apoa4 genes, respectively, in the transgenic mice (Fig. 4.2).

Fig. 4.2: Confirmation of upregulation of three differentially expressed genes involved in lipid metabolism and transport by real-time RT-PCR.

Three differentially expressed genes (Acaa1, AOX, and Apoa4) involved in lipid metabolism and transport were examined by real-time RT-PCR using gene-specific primers. Assays were performed in triplicate with SYBR Green I dye using the GenAmp 5700 Sequence Detection System. The relative levels of Acaa1, AOX, and Apoa4 mRNAs in transgenic livers (n=3, age = 1 year) quantitated and normalized to the levels in non-transgenic livers (n=3, age = 1 year). GAPDH used as an internal control. ΔΔCT method was used to obtain the relative mRNA levels for each gene. (Yellow column represents non-transgenic mice, Blue column represents transgenic mice, and error bars represent 3 livers)



4.2.4 Differentially expressed genes involved in protein biosynthesis:

Twenty eight genes involved in protein biosynthesis were down regulated in the transgenic livers. These genes include: ribosomal proteins and eukaryotic translation elongation factors which are important for ribosomal biogenesis and protein synthesis. The ribosomal proteins, in conjunction with rRNA, make up the ribosomal subunits involved in protein translation. For example; S7, S10, and L18a, are required for ribosome binding to initiate transcription. Most of the other ribosomal proteins are involved in the structural integrity of the ribosome. In addition, some eukaryotic translation elongation factors were down regulated. These include eukaryotic translation elongation factors 1 alph 1 and 2 (Eef1α 1 and Eef2). Eef1α 1 protein promotes the GTP-dependent translocation of the nascent protein chain from the a-site to the p-site of the ribosome, which is selectively interact with guanosine triphosphate and it may function in chain elongation during polypeptide synthesis at the ribosome.

4.2.5 Gene involved in Wnt receptor signaling pathway:

High mobility group (HMG) box transcription factor 1 (HBP1 or Cog5), is down regulated in the HCV transgenic model. HBP1 is a unique member of the HMG-box transcription family. HBP1 was originally identified as an interactor with the retinoblastoma protein (RB) and p130 growth and tumor suppressor genes [302,303]. It also regulates the expression of several genes involved in cell cycle regulation, including cyclin D1, N-myc, c-myc and others [303-306]. Furthermore, HBP1 was identified as an inhibitor of the Wnt

oncogenic pathway [306]. The Wnt signaling pathway describes a complex network of proteins that have roles in embryogenesis and cancer, and involved in normal physiological processes in adult animals [307].

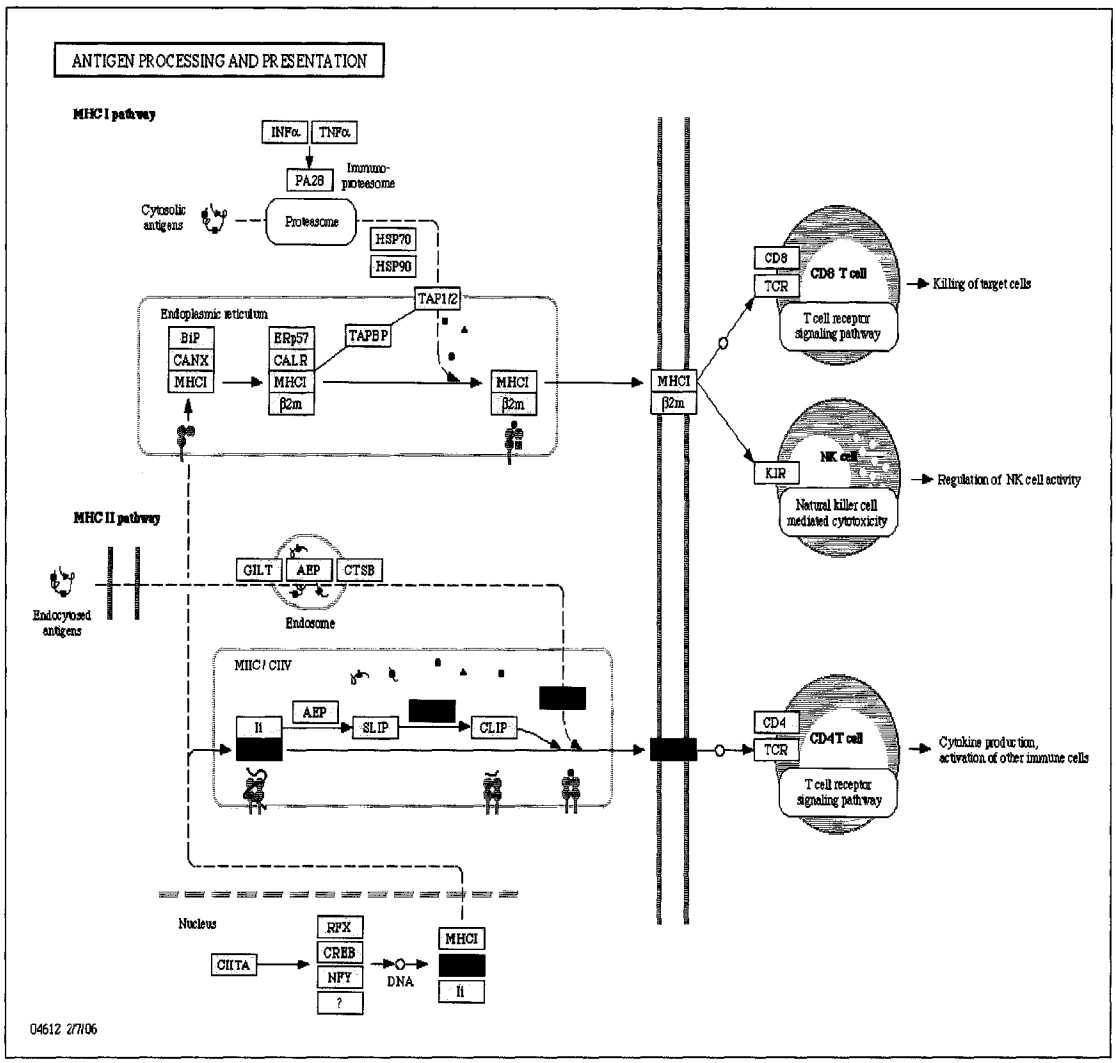
4.2.6 Differentially regulated genes involved in antigen processing and presentation:

There was significantly down regulation of three important genes involved in the Major Histocompatibility Complex class II (MHC II) pathway of antigen presentation. These genes include; Histocompatibility 2, class II antigen A, alpha (H2-Aa) Cathepsin L (Ctsl), and MHC class II, locus Mb1 (H2-DM b1) (Fig. 4.3). Since HCV is a chronic viral infection, down-regulation of these immune genes may have a link in the pathogenesis of this viral disease. The MHC is a set of molecules displayed on cell surface that are essential for antigen presentation and lymphocyte recognition, which play a critical role in protection against infection. The MHC class II pathway allows for the presentation of exogenous antigens in the endosomal compartment [308,309]. Antigen presentation mediated by this exogenous pathway is fundamental to the life of a CD4+ T cell, as it is essential for its development, peripheral maturation and acquisition of effector function, and maintenance. MHC class II molecules compose of alpha and beta (α/β) heterodimers, which are assembled in the ER. The heterodimers are properly folded by chaperone molecules, such as the invariant chain (Ii) [309]. Ii is a type II membrane glycoprotein that forms homotrimers that are capable of binding three MHC II α/β heterodimers in the ER [310]. Within the cytoplasmic tail of the Ii is a signal motif that allows for the targeting of MHC II molecules to the endosomal pathway [311,312]. A portion of the luminal region of the Ii, called CLIP (class II-associated Ii peptide), is placed in the peptide-binding groove of MHC II heterodimers.

Fig. 4.3: Schemetic diagram identifying down-regulated genes involved in antigen processing and presentation in HCV transgenic mice:

Genes involved in antigen processing and presentation through MHC class I and MHC class II pathways are shown in green color, as provided in the KEGG pathway database. HCV down-regulated genes include; Histocompatibility 2, class II antigen A, alpha (MHC II / H2-Aa) Cathepsin L (CTSL/ Ctsl), and MHC class II, locus Mb1 (HLA-DM/ H2-DM b1) and are shown in blue color.

(Modified from http://www.genome.jp/kegg/pathway/mmu/mmu04612.html)



The presence of CLIP in the groove inhibits premature peptide loading [313]. The maturation of MHC class II in the endosomal compartments is followed by acidification of these compartments, which causes the activation of endosomal/lysosomal proteases known as cathepsins [314]. The activated cathepsins process Ii which leaves CLIP seated in the peptide cleft. The MHC-like molecule H2-M (DM) then facilitates the exchange of CLIP for a highly diverse array of both exogenously and endogenously derived peptides [315-317], after which the peptide-loaded MHC class II molecules are transported to the cell surface, ready for CD4+ T-cell recognition [318]. All together, the endosomal pathway of antigen presentation is a highly synchronized process and any impairment in the genes regulate this pathway is critical.

Histocompatibility 2, class II antigen A, alpha (H2-Aa) gene expression was significantly downregulated in the HCV transgenic mouse model. This gene encodes the alpha-1 domain of the MHC-II. MHC II molecules are composed of two polypeptide chains, alpha and beta. Alpha polypeptide folds into two separate domains; alpha-1 and alpha-2, while beta polypeptide folds into beta-1 and beta-2. Alpha-1 domain is essential for antigen presentation because it binds the beta-1 domain to form the antigen binding cleft, where the antigen peptide anchors [319]. MHC class II, locus Mb1 (H2-DM b1) gene is one of three genes of murine MHC class II gene family [320]. In addition to the MHC class II molecules; there are two other class II-like proteins which are produced from the class II region of the MHC, HLA-DM (DM) and HLA-DO (DO) (called H2-M, or H2-DM and H2-O in mouse). The DM protein is a resident of the endosomal/lysosomal system within antigen presenting cells [321]. The function of DM protein is well known. It supports the peptide loading of MHC-II molecules in the endosomal/lysosomal system by catalyzing the release of CLIP

peptides in exchange for more stably binding peptides [317,322]. The Cathepsin L (Ctsl) gene was also down-regulated in HCV transgenic mice. Ctsl is a lysosomal protease required for the degradation of Ii, the first step in the endosomal pathway of antigen processing and presentation. Once cathepsin is activated, the Ii is degraded leaving CLIP seated in the peptide cleft.

4.2.7 Differentially expressed genes involved in immune response:

Other genes involved in the immune response that were differentially expressed in the HCV transgenic model include; B-cell receptor-associated protein 31 (Bcap31) (downregulated), ADP-ribosylation factor–like 6 interacting protein 2 (Arl6ip2) (upregulated) and complement component 4 binding protein (C4bp) (upregulated). BAP31 is a resident integral protein of the endoplasmic reticulum membrane, and it regulates the export of other integral membrane proteins to the downstream secretory pathway. It regulates MHC class I traffic from endoplasmic reticulum to the Golgi complex [323]. Therefore, it acts as a putative chaperone/quality control factor that regulates the fate of integral membrane proteins in the ER membrane. ADP-ribosylation factor–like 6 interacting protein 2 (Arl6ip2), is one of the ADP-ribosylation factors-like proteins (ARLs), which are important factors in intracellular membrane trafficking [324]. Complement component 4 binding protein (C4bp) is a complement protein that has regulatory functions for the C4b-mediated reactions, thereby inhibiting the classical pathway of complement [325-327].

4.2.8 Differentially expressed genes involved in MAPK cell signaling pathway:

HCV transgenic mice demonstrated significant alteration in some of the MAPK signaling pathway genes (Fig.4.4). These genes include; RAS p21 protein activator 2

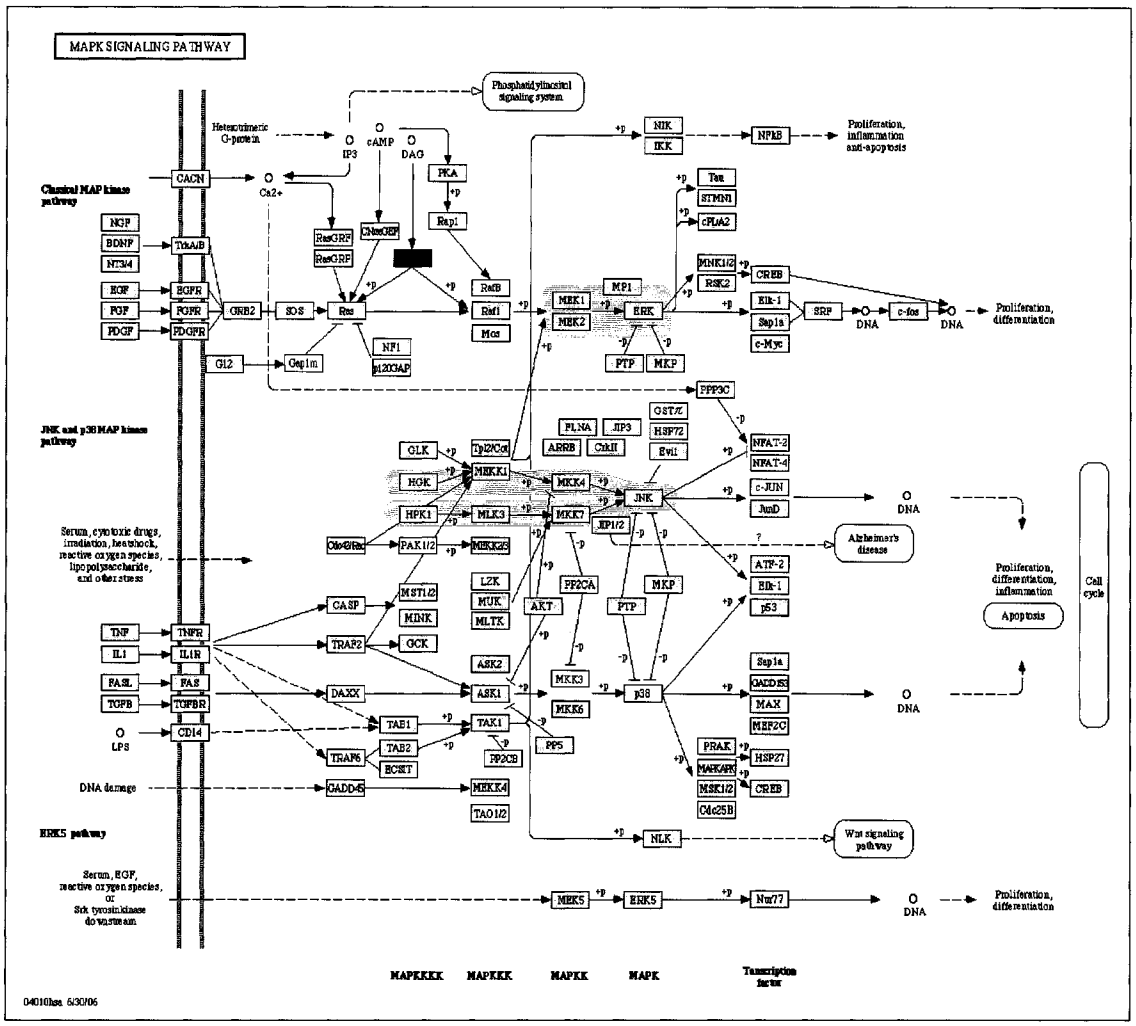
(Rasa2), p21-activated kinase 2 (Pak2), Jun oncogene (Jun), and mitogen activated protein kinase kinase 3 (Map2k3 / MKK3) which were upregulated. In contrast, protein kinase C, beta 1 (prkcb1/ PKC-βI) were downregulated. The MAPKs pathway regulates cell proliferation, differentiation, motility, and survival. P21-activated kinase 2 (Pak2) is one of the p21-activated kinases (Paks) family which are serine/threonine kinases that activate the stress-activated JNK and p38 MAPK cascade [328,329]. Mitogen activated protein kinase kinase 3 (Map2k3 / MKK3) is a MAPKK that have been reported to specifically activate p38 MAP kinase. This activation is critical for p38 MAP kinase *in vivo*, because targeted disruption of MKK3 gene expression completely inhibits the ability of Type I IFNs to induce p38 activation [330,331].

Jun oncogene (c-Jun or AP-1) is the cellular homologue of v-jun, the transforming oncogene of the avian sarcoma virus 17 [332]. c-Jun encodes one major component of the activator protein 1 (AP-1), a transcription factor complex. AP-1 regulates the expression of multiple genes essential for cell proliferation, differentiation and apoptosis [333]. RAS p21 protein activator 2 (Rasa2) is one of the guanosine triphosphatase-activating proteins (GTPase-activating proteins) or GAPs family. The best-known function of this family is as negative regulator of the Ras/MAPK-signaling pathway, which mediates cellular growth, differentiation, and proliferation from various receptor tyrosine kinases on the cell surface [334,335]. Protein kinase C, beta 1 (prkcb1/ PKC-βI) is down regulated in the HCV transgenic mice. It is a member of Protein kinase C (PKC) family, which are serine/threonine kinases that play key, distinct roles in major cellular functions in a variety of cell types. This kinase could also be activated by phosphatidylserine (PS) and diacylglycerol (DAG) in a Ca^{2+}-dependent manner as well as by tumor-promoting phorbol esters such as PMA.

Fig.4.4: Schematic diagram of MAPK signaling pathway showing differentially expressed genes in HCV transgenic mice.

Genes involved in the MAPK signaling pathway are shown (green color), as provided in the KEGG pathway database. Down-regulated gene; protein kinase C (PKC) is shown in blue color. Up-regulated genes: RAS p21 protein activator 2 (Rasa2 , p21-activated kinase 2 (Pak2), Jun oncogene (c-Jun), and mitogen activated protein kinase kinase 3 (MKK3), are shown in yellow color.

(Modified from http://www.genome.ad.jp/kegg/pathway/hsa/hsa04010.html)



It has been elucidated that PKCs play an important role in the signal transduction pathway in various cells. PKC-βI is one of the conventional PKCs which are activated in a diacylglycerol (DAG) - and calcium-dependent manner.

4.2.9 Differentially expressed gene involved in Apoptosis:

HCV transgenic mice showed alteration in genes associated with apoptosis, including: Bcl2-associated athanogene 4 (Bag4), which was upregulated, and transcription factor Dp1 (Tfdp1) and B-cell receptor associated protein 31 (Bcap31), which were downregulated. Bcl2-associated athanogene 4 (Bag4/ Sodd) is a member of the BAG family of proteins, which are molecular chaperone regulators that affect diverse cellular pathways. All BAG proteins share a conserved motif, called the BAG domain or BD, which binds the heat shock proteins 70 (Hsp70/Hsc70) and modulates their activity as molecular chaperones [336]. The ability of Hsp70 proteins and BAG proteins, including BAG4, to suppress apoptosis have been reported [337]. Transcription factor Dp1 (Tfdp1) binds to E2F transcription factor to form E2F/DP complexes which activate or repress the transcription of E2F target genes, depending on the association of the hypophosphorylated form of the retinoblastoma protein (pRB) family member. This complex then regulates cell cycle progression from G1 to S phase [338]. Thus, E2F-1/Dp complex appears to mediate both cell proliferation and apoptosis. B-cell receptor-associated protein 31 (Bcap31 / BAP31) is an integral protein of the ER membrane involved in the transfer of selected membrane proteins from the ER to downstream compartments of the secretory pathway [339,340]. In addition, Bcap 31 has been shown to be an important target of caspase-8 in Fas-mediated death pathways [341-343]. Thus the down regulation of this gene might be expected to affect a variety of cellular functions.

4.3 DISCUSSION:

The expression of HCV structural proteins in transgenic mouse livers induces alterations in genes involved in fatty acids metabolism and lipid transport, MHC-II pathway of antigen processing and presentation, protein biosynthesis, carcinogenesis, apoptosis, etc. Furthermore, some of the differentially expressed genes were associated with important cell signaling pathways such as MAPK pathway and Wnt oncogenic pathway. Alterations in the expression of these genes in the HCV transgenic mice could explain histopathological changes observed in the liver of HCV transgenic model, such as hepatic steatosis, liver tumor formation and impairment of antigen presentations.

4.3.1 Differentially expressed genes involved in lipid metabolism and transport:

Chronic HCV infection is often associated with an abnormal condition known as hepatic steatosis [176,344,345]. Several *in vitro* studies have suggested that HCV proteins cause alteration of lipid metabolism and transport leading to hepatic steatosis [346,347]. In particular HCV core protein has been reported to induce hepatic steatosis by several mechanisms [209,222,224]. HCV core protein interferes with the assembly of VLDL by decreasing the level of microsomal triglyceride transfer [178]. Also, it has been observed that core protein interact with apoa2, which is a major component of HDL [177,265]. Moreover, HCV core induces steatosis due to mitochondrial toxicity and to production of reactive oxygen species [209,224]. However, the detailed mechanisms of how HCV proteins interfere with lipid metabolism and transport, and the resulting hepatic steatosis remain to be explained. In this study microarray analysis was used to elucidate the mechanisms by which HCV structural proteins cause abnormalities including hepatic steatosis in transgenic mice.

Several genes associated with lipid metabolism and transport, were differentially expressed in the HCV transgenic mouse livers. Fatty acids are stored in the form of triacylglycerols primarily within adipocytes of adipose tissue and in response to energy demands, the fatty acids of stored triacylglycerols can be mobilized for use by peripheral tissues. Fatty acids must be activated in the cytoplasm before being oxidized in the mitochondria and peroxisomes. Acsl-3 enzyme plays an important role in the transport of fatty acids into cells and their subsequent activation by thioesterification to CoA, which are fundamental processes required for entry of fatty acids into the metabolic stream in mitochondria [348,349]. Since, Acsl-3 is an important transporter and activator of long-chain and very long chain fatty acids into cells, the down regulation of Acsl-3 gene expression in the HCV transgenic livers may affect the uptake of fatty acid by the cells, which might cause accumulation of fatty acids (as triglycerides) without proceeding to the metabolic pathways. It has been shown that hepatic accumulation of triglycerides is principally driven by fatty acid overload or accumulation [350,351]. This may explain steatosis in transgenic livers.

Acox1 and Acaa1 enzymes are important in very long chain fatty acid β-oxidation in peroxisomes. Acox1/AOX enzyme is essential in the first step of β-oxidation. It oxidizes the long chain acyl-CoA thioester to give the corresponding trans-2-enoyl-CoAs [293]. Acaa1 enzyme is involved in the last step of fatty acid β-oxidation, at which it catalyzes the transfer of the acyl group from acyl-CoA to Acetyl-CoA to form oxoacyl-CoA [352]. Fatty-acid β-oxidation is the most important oxidation reaction for fatty acids and occurs both in the peroxisome and mitochondria in eukaryotic cells. Peroxisomal β-oxidation does not contribute to energy production directly, but is required for the initial oxidation of very long chain fatty acids, which can not be directly oxidized by mitochondria. Most of the chain-

shortened products are transported from the peroxisome to mitochondria for complete degradation. In the peroxisome there are two separate β-oxidation pathways; the classical peroxisome proliferator-inducible pathway and the non-inducible pathway [353]. Acox1 and Acaa1 enzymes are members of the classical peroxisome proliferator-inducible pathway. The up-regulation of these two enzymes in the HCV transgenic mouse livers may lead to increase in their enzymatic activities. It has been reported that increase in the activities of these enzymes is associated with proliferation of liver peroxisomes which would affect the fatty acids metabolism [354]. Another study using the same HCV transgenic model, demonstrated a proliferation of liver peroxisomes (Soare *et al*, unpublished data). This is in conjunction with the up-regulation of these peroxisomal enzymes suggests that HCV alters the lipid metabolism by affecting the expression of these enzymes. In addition, over-expression of peroxisomal fatty acyl-CoA oxidases may have a role in carcinogenesis. Over-expression of fatty acyl-CoA induces an increase in the intracellular H_2O_2, apoptosis, and cell transformation, including tumor formation [355,356]. Thus, the upregulation of these peroxisomal enzymes in the HCV transgenic mouse livers could explain, in part, liver tumor formation associated with HCV infection.

The HCV transgenic model showed alterations in several genes involved in lipid transport. Scp-2 protein, which is down regulated in HCV transgenic model, is a non-specific lipid binding protein that play an important role in the transport and metabolism of cholesterol [295]. A study in mice lacking the Scp-2 gene showed peroxisome proliferation, hypolipidemia, impaired body weight control, and neuropathy. Beside these abnormalities, catabolism of methyl-branched fatty acyl CoAs was impaired [357]. In addition, Scp2 plays also an important role in hepatic cholesterol metabolism, biliary lipid secretion, and

intracellular cholesterol distribution [358]. Apoa1 is the major apolipoprotein of HDL and has an important role in reverse cholesterol transport [296,297]. The down regulation of Scp-2 and apoa1 genes in the HCV transgenic mouse model could decrease their lipid transport activity and more lipids will be accumulated inside the hepatocytes leading to hepatic steatosis.

On the other hand, several genes involved in lipid transport were upregulated in the HCV transgenic model. Apoa4 is important in the transport of dietary triglycerides, phospholipids, cholesterols and cholesterol esters absorbed by enterocytes to other tissues such as hepatocytes [300]. Thus, the up regulation of Apoa4 gene in the HCV transgenic mouse model may increase the transport of lipid from enterocytes to hepatocytes and may lead to lipid accumulation in the liver. This might contribute to hepatic steatosis associated with HCV proteins expression in the liver. Some studies have shown that HCV core protein localizes to fat droplets [359-361] as well as mitochondria [362] . Since apoa4 protein binds to lipids and renders them water soluble in the form of lipoproteins [363], it may be a likely candidate for core protein interaction. On the other hand, HCV has been described as a lipid-containing virus; in the plasma of HCV infected patients there is a heterogeneous viral density distribution which is partially due to the binding of HCV to LDL, VLDL, and to a minor degree to HDL [364]. Apoc2 protein also participates in lipoprotein metabolism, and lipid transport from the intestine to the liver. It inhibits lipoprotein lipase (LPL) mediated hydrolysis of triglycerides and modulated the metabolism of triglyceride-rich lipoproteins [301]. Since Apoc2 is an inhibitor for LPL enzyme, which mediates triglyceride hydrolysis, the upregulation of this gene in transgenic mice may cause accumulation of triglycerides in hepatocytes. This could also be a contributor to the steatosis observed in the transgenic

mouse livers. It is important, therefore, to emphasize that several *in vivo* observations regarding HCV infection might be related to these findings. Firstly, a characteristic of HCV infection is the presence of liver steatosis; it is plausible that this steatosis could arise, at least in part, from direct effects of HCV proteins on lipid metabolism and transport.

4.3.2 Down-regulation of genes involved in protein biosynthesis:

Several genes encode ribosomal proteins and eukaryotic translation elongation factors were down regulated in HCV transgenic livers. These proteins are important for ribosomal biogenesis and protein synthesis. The 5` noncoding region of HCV RNA genome contains an IRES, which interact with 40S subunit of the ribosome for initiation of viral proteins translation [61,365]. The interaction with 40S subunit is followed by the joining of the 60S ribosomal subunit to form functional 80S complex. This interaction requires some ribosomal proteins to form the binary complex. It was initially reported that ribosomal proteins (S9 ans S5) are essential for the binding of IRES and 40S subunit [366,367]. Moreover, it was observed that the ribosomal proteins (S2, S3, S10, S15, S16, S18 and S27) are linked with 40s subunit of the ribosome [368]. Also, S3, S5, S7, and S18 can bind to the HCV IRES out of the ribosome in cell extract [369]. However, the relationships between these interactions and initiation of HCV RNA translation are as yet not clear. A ribosomal protein L18a, a constituent of 60S subunit, was also shown to interact with HCV IRES RNA, which also might influence the HCV IRES mediated translation [370]. S7, S10, and L18a, are required for ribosome binding to initiate transcription and they are down regulated in the transgenic mice. Most of the other ribosomal proteins, down regulated in our HCV transgenic livers, are involved in the structural integrity of the ribosome. In addition, some eukaryotic translation elongation factors, such as Eef1α 1, were down regulated. Down-regulation of several

ribosomal protein genes and transcription regulator genes may reflect reduced metabolic activities in transgenic mice.

4.3.3 Alterations of genes associated with Wnt oncogenic pathway:

HBP1 transcriptional factor is down regulated in HCV transgenic livers. It have been reported that HBP1 regulates the expression of several genes involved in cell cycle regulation, including cyclin D1, N-myc, c-myc and others [303-306]. HBP1 may also block the proliferation of terminally differentiated tissues, and inhibits G1 progression in cell and animal models [303,371,372]. In addition, HPB1 is a target of the p38 MAPK pathway. p38 MAPK activity contributes to cell cycle inhibition by increasing HBP1 and other G_1 inhibitory factors by regulating protein stability [373]. Moreover, the repression of superoxide production through the NADPH oxidase contributes to the observed cell cycle inhibition by HBP1 [374]. Furthermore, HBP1 was identified as an inhibitor of the Wnt oncogenic pathway [306]. Impairment in the regulation of Wnt pathway at any of its components (β-catenin, and axin), were reported to be associated with numerous cancers [375]. It was also reported that mutations in the Wnt/β catenin pathway is associated with HCC [376,377]. Taken together, these studies indicate that HBP1 is a tumor suppressor by inhibiting G1 progression and/or major oncogenic signaling pathways such as Wnt oncogenic pathway. Since it has been shown that impairment of Wnt signalling (due to β-catenin mutations) is significantly associated with HCC in HCV infection [378], down regulation of HBP1 gene in the HCV transgenic mice, may be associated with increased susceptibility for liver tumor formation in our model.

4.3.4 Down-regulation of important genes of antigen processing and presentation:

Several genes involved in the MHC class II pathway of antigen presentation are significantly down regulated in the HCV transgenic mouse livers. Histocompatibility 2, class II antigen A, alpha (H2-Aa) gene encodes the alpha-1 domain of the MHC-II, which is very essential for antigen presentation [319]. MHC class II, locus Mb1 (H2-DM b1) gene is MHC class II-like protein (HLA-DM/ DM) and it is a resident of the endosomal/lysosomal system within antigen presenting cells [379]. It supports the peptide loading of MHC-II molecules in the endosomal/lysosomal system by catalyzing the release of CLIP peptides in exchange for more stably binding peptides [317,322]. Numerous studies have shown the essential role of DM protein in normal presentation of MHC-II restricted antigen to CD4+ T cells [322,380-382]. In addition, this molecule is crucial for the normal maturation of T-dependent humoral immune responses *in vivo* [383]. Cathepsin L (Ctsl) protein is also required for antigen processing and presentation. It degrades Ii, leaving CLIP seated in the peptide cleft.

Several studies suggest the impairment of antigen presentation in chronic HCV infection. For example, Dendritic cells recovered from chronically HCV infected patients or chimpanzees were unable to present antigen to immune cells [155,384,385]. Thus, the downregulation of H2-DM b1, Ctsl, and H2-Aa genes, three important components of MHC class II pathway, in transgenic mice could be an important clue to explain the impairment of antigen presentation observed in chronic HCV infection.

4.3.5 Differentially expressed genes involved in immune response:

Microarray analysis showed differential expression of important genes involved in the immune response. These genes include: B-cell receptor-associated protein 31 (Bcap31), which is downregulated, ADP-riboylation factor–like 6 interacting protein 2 (Arl6ip2) and

complement component 4 binding protein (C4bp), which are upregulated. BAP31 is integral protein of the ER membrane and it regulates MHC class I traffic from ER to the Golgi complex [323]. This regulation has important implications for maintaining the integrity of many cell surface functions. Defects in this process are well known to contribute to numerous diseases, such as cystic fibrosis, leukocyte adhesion deficiency, and familial hypercholesterolaemia [339,340]. BAP31 is also an important target of caspases in certain apoptosis pathways. It acts as a substrate of caspase-8 [343,386]. Thus, down regulation of this gene might be expected to influence a variety of cellular functions.

C4bp, a regulator of classical complement pathway, was down-regulated in transgenic mouse livers. C4bp inhibits the classical pathway of complement in at least three ways. Firstly, it is an essential cofactor to the serine protease factor I (FI) in the degradation of C4b in the classical complement pathway [325,326]. Secondly, the C4b degradation in turn inhibits the formation of the C4b2a complex (C3 convertase); and thirdly it accelerates its decay [327], a key step in producing an inflammatory response [387]. This could be important in avoiding deposition of inflammatory complexes on tissues and maintaining tolerance for self-antigens, thereby providing protection against inflammation and autoimmunity. Thus, C4bp is important to control undesirable consequences of complement activation such as excessive activation on a single target, and activation on self. Thus, the up-regulation of C4bp gene in the HCV transgnic mice is probably to maintain tolerance to the HCV transgene, which is a self-antigen in this model.

4.3.6 Differentially expressed genes involved in MAPK cell signaling pathway activation:

The microarray results demonstrated significant alterations in important genes involved in MAPK signaling pathways. These genes include; Rasa2, Pak2, Jun, and MKK3, which were upregulated, and PKC-βI, which was downregulated.

To date, five distinct groups of MAPKs have been characterized in mammals: extracellular signal-regulated kinases (ERKs), c-Jun amino-terminal kinases (JNKs), p38 MAPK [388]. ERK1 and ERK2 are preferentially activated in response to growth factors and phorbol esters, while the JNK and p38 kinases are more responsive to stress stimuli [389]. Each family of MAPKs is composed of a set of three evolutionarily conserved, serially acting kinases: MAPK, MAPKK, and MAPKKK. The MAPKKKs are often activated through phosphorylation and/or as a result of their interaction with a small GTP-binding protein of the Ras/Rho family in response to extracellular stimuli [390]. MAPKKK activation leads to the phosphorylation and activation of a MAPKK, which then stimulates MAPK activity through double phosphorylation on threonine and tyrosine residues. Once activated, MAPKs phosphorylate target substrates on serine or threonine residues in specific manner [330,391].

Pak2 activates the stress-activated JNK and p38 MAPK cascade by behaving as MAPKKKK and mediating the Rac1 and/or Cdc42 (Rac1/Cdc42)-induced activation of p38 MAPK [392]. Thus, upregulation of Pak2 gene might results in activation of p38 MAPK pathway. MKK3 is MAPKK that specifically activate p38 MAPK. This activation is critical for p38 MAPK *in vivo*, because targeted disruption of MKK3 gene expressipn completely inhibits the ability of Type I IFNs to induce p38 activation [330,331].

c-Jun gene encodes one major component of the activator protein1 (AP-1), a transcription factor complex consisting of a homodimer and heterodimers of members of the Jun family (c-Jun, JunB, and JunD) and heterodimers of the Jun and Fos (c-Fos, FosB, Fra1, and Fra2) families. AP-1 regulates the expression of multiple genes essential for cell proliferation, differentiation and apoptosis [393]. The activity of AP-1 is regulated at least in part by the activation of JNK and MKK7 [394]. It has also been suggested that the activation of NF-κB is regulated by some upstream MAPKs that also regulate JNK activation in the cells [395]. Constitutive activation of endogenous AP-1 is required for tumor formation in avian and mammalian cell transformation systems [396], and it also occurs in distinct human tumor cells [397], suggesting that AP-1 plays an important role in human oncogenesis.

RAS p21 protein activator 2 (Rasa2) acts as negative regulator of the Ras/MAPK-signaling pathway [398,399]. Ras p21 GAP and the other three known GAPs: GAP-NF1, GAPM1, and GAP1-like protein- change the active GTP-bound Ras to the inactive GDP-bound form [400-402]. Mutations that make Ras constitutively active and resistant to GAPs are often found in malignant tumors[403]. Inactivating mutations of the GAP proteins, in turn, can also lead to inordinately active Ras signaling and tumorigenesis [404,405]. Rasa2 protein is upregulated in the HCV transgenic mouse livers, which might indicate that HCV structural proteins do not activate MAPKs signaling pathway via Ras. Since activation of Ras/ MAPKs signaling pathway was shown to be associated with tumorigenesis, therefore the liver tumors formed in our HCV transgenic mouse model may not correlated to Ras oncogenic potential. PKC-βI is downregulated in the HCV transgenic mice. It is required for cell growth and for the G2-M transition of the cell division cycle [406]. Also, it mediates a protein kinase cascade which includes the BCK1, MKK1/MKK2 and MPK1 kinases [407].

The observation that 4 important genes involved in p38 MAPK pathway were upregulated in HCV transgenic mice indicates that HCV structural proteins may activate p38 MAPK pathway. This is likely to be biologically significant because of the role of p38 MAPK in the regulation of cell cycle progression [408]. Indeed, defects in the p38 MAPK pathway are associated with tumorigenesis [409]. Because of the importance of MAPK pathways in controlling cellular functions, destruction of MAPK signaling cascade has severe, pathological consequences. The MAPK pathways have been shown to be modulated by many viruses, such as reovirus, rabies virus, Epstein-Barr virus, and hepatitis B virus, which lead to important inference for viral pathogenesis [410-412]. Currently, many studies focus on HCV-related, complicated, clinical phenomena on the basis of cellular signaling events. These studies suggested that HCV proteins interfere with MAPK signaling cascade, which might help to explain chronic hepatitis, liver cirrhosis, carcinomas, and IFN resistance. It has been reported that ERK signaling pathway was activated in cell lines expressing HCV core protein [413]. Transgenic mice expressing HCV core protein and given ethanol showed activation of ERK and p38 MAPK signaling pathways [414]. Furthermore, HCV core protein expression activates ERK, JNK, and p38 MAPKs and increases cell proliferation. This was accompanied by an activation of c-Jun and ATF-2 [415]. Additionally, HCV NS5A was reported to inhibit transcription factor-activating protein-1 function by disturbing the ERK pathway [416]. More recently, it has been documented that the interaction of the HCV E2 protein with CD81 and LDLR on target cells resulted in the activation of ERK and p38 MAPK signaling pathways, which was accompanied by enhancement of cell proliferation and production of IFN-γ and interleukin-10 (IL-10) cytokines [417,418]. Therefore, the

interaction of the HCV proteins with the MAPK signaling pathways is an essential key for understanding the pathogenesis of HCV infection.

4.3.7 Alterations of important genes involved in Apoptosis:

Apoptosis is a programmed cell death that plays an essential role in many physiological processes such as; embryological development, tissue homeostasis, and immune cell education [419]. Impairment of apoptosis regulation is implicated in the pathogenesis of many diseases [420,421]. Furthermore, apoptosis represent a crucial host defense mechanism against pathogens. For instance, virus infected cells become suicidal to limit viral replication [422]. Also, some of apoptosis regulator proteins contribute to the inflammatory responses to infection. For example, caspases are critical effectors of apoptosis and some are responsible for activation of pro-inflammatory cytokines such as pro-Interleukin-1β and pro-Interleukin-18 [423]. Some caspase activation proteins are also involved in induction of NFκB family transcription factors, which regulate expression of numerous genes important for inflammatory and immune responses [424]. Thus, apoptosis is associated with many important physiological processes.

HCV transgenic livers showed alteration in genes involved in apoptosis. Bcl2-associated athanogene 4 (Bag4/ Sodd) gene expression was up-regulated in HCV transgenic mice. It was demonstrated that BAG4 protein plays a role in suppression of apoptosis [337]. BAG4 may also a play a role as a cellular "adaptor''. It has been reported that Bag4 engages HSC70 to tumor necrosis factor receptor 1 (TNFR1) and death receptor 3 (DR3) [425,426], which cause conformational changes that prevent receptor signaling in the absence of ligand. Thus, the BAG4 protein appears to play an important role in suppression of apoptosis and its upregulation in HCV transgenic mice suggests that HCV structural protein expression may

influence apoptosis processes. Transcription factor Dp1 (Tfdp1) binds with E2F transcription factor to form E2F/DP complexes which activate or repress the transcription of E2F target genes and mediate both cell proliferation and apoptosis [338]. B-cell receptor-associated protein 31 (Bcap31 / BAP31) has been shown to be important target of caspase-8 in Fas-mediated death pathways [341-343]. Caspase 8 cleaves Bcap31 and generates p20 Bcape 31, which inhibit pro-apoptotic ER-mitochondrial pathway via pros-survival BCL-2 [427,428]. Thus the down regulation of this gene in our HCV transgenic model might be expected to affect a variety of cellular functions.

I confirmed the the expression of some genes involved in lipid metabolism and lipd transport; including Aox1, Acaa and Apoa4, by Real-time RT-PCR. However, the validation of the rest of the genes by Real-time RT-PCR is still in-progress.

Taken together, the data presented in this study demonstrated that expression of HCV structural proteins in transgenic mouse livers induces alterations in the expressions of genes involved in several important biological processes such as lipid metabolism and transport, antigen processing and presentation, protein biosynthesis, carcinogenesis, apoptosis, etc. Additionally, some of the differentially expressed genes were associated with important cell signaling pathways such as MAPK pathway and Wnt oncogenic pathway. Changing the expression of these genes in the HCV transgenic mice successfully explained important abnormalities associated with HCV transgenic model and HCV infection, such as hepatic steatosis, impairment of antigen presentations, and carcinogenesis. Thus, applying microarray analysis to this HCV transgenic mouse model could be helpful to understand the mechanisms of HCV pathogenesis in the liver.

CHAPTER 5

Study of immune-mediated liver cell damage in HCV infection using HCV transgenic mice.

5.1 INTRODUCTION

Immune responses to HCV play an important role at various stages of viral infection. There is emerging evidence that the ability of acutely HCV-infected patients to control the primary HCV infection depends on the vigorous cellular immune reaction to the virus [429]. In chronic phase of infection, immune responses determine the rate of progression of disease both by limiting viral replication and by contributing to immunopathology. Livers from HCV chronically-infected individuals show T cell infiltration in this organ, however these cells are not HCV specific and are unable to eradicate the virus [430]. These liver-infiltrating lymphocytes are associated with liver damage in chronic hepatitis C infection via mechanisms that are not well understood [431]. There are several immune evasion mechanisms which might explain the ability of the virus to escape immune responses and establish a persistent infection such as virus mutation, primary T cell response failure, impairment of antigen presentation, suppression of T cell function, impairment of T cell maturation and tolerogenic environment of the liver [181]. However, the immunological basis for the inefficiency of the cellular immune response in chronically infected patients is not well understood.

Cellular immune responses play a critical role in liver damage during hepatitis C infection. HCV-specific CD4+ T cells are involved in the eradication of virus in acute infection but their responses are weak and insufficient in chronic hepatitis [141]. However, there is no clear evidence showing that CD4+ T cells play a direct role in the liver injury observed during chronic hepatitis infection. CD4+ T cells activate the CD8+ cytotoxic T lymphocyte (CTL) response and eradicate the virus-infected cells either by inducing apoptosis (cytolytic mechanism) or by producing IFN-γ which suppresses the viral

replication (non-cytolytic mechanism) [147]. Enhanced hepatocyte apoptosis mediated by immune responses leads to liver damage in chronic hepatitis [182]. HCV-specific CD8+ CTL responses are compromised in most patients who fail to clear the infection. In addition, these cells have a diminished capacity to proliferate and produce less INF-γ in response to HCV antigens [183]. Thus, CD8+ T cell responses are inadequate to clear the chronic infection and instead they mediate liver damage associated with chronic HCV infection.

The mechanisms responsible for immune-mediated liver damages associated with HCV are currently poorly understood. One of the important mechanisms for liver damage is that the HCV-activated T cells express Fas ligand at the cell surface which will bind with the Fas receptor on the surface of hepatocytes and initiate Fas-mediated signaling which may lead to cell death [184]. It has been shown that HCV core protein increases the expression of Fas ligand on the surface of liver infiltrating T cells leading to the induction of hepatic inflammation and liver damage [185,186]. Another important mechanism of liver damage is through CD8+ T cell-mediated cytolysis. Previous studies have demonstrated that CD8+ T cells can kill the target cells *in vivo* by cytolytic mechanisms mediated by perforin or requiring IFN-γ [187,188]. This may also involve additional molecule such as TNF-α [189]. Therefore the level of cytolytic activity or expression of cytolysis mediators from the infiltrating lymphocytes could be a determinant for induction of liver mediated damage.

It is still controversial whether the liver damage associated with hepatitis C infection is due to the virus or due to the immune response. In the previous study the direct effect of viral structural proteins in the pathogenesis of HCV infection was investigated. HCV transgenic mouse model that expresses the HCV structural proteins, core, E1 and E2 predominantly in the liver was developed [261]. This model showed hepatopathy including hepatic steatosis

and liver tumors. One of the limitations of this transgenic mouse model is that the role of the immune response in the liver damage can not be studied because these transgenic mice are immunotolerant to the transgene. Because of this limitation the study the role of immune responses in liver damage associated with chronic HCV infection is difficult. Thus a model of liver immune-mediated cell damage was developed by means of adoptive transfer of spleenocytes from HCV immunized mice into HCV transgenic mice. This experimental model might be useful to study the immune-mediated liver injury in these transgenic mice. It may also be used to study the kinetics of immune cells that have antiviral specificity.

5.2 RESULTS:

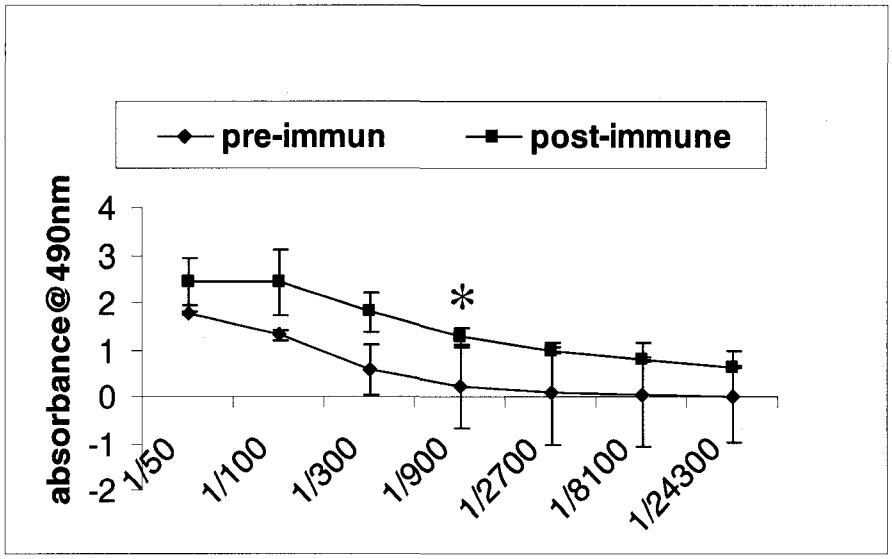
5.2.1 Immune response in HCV-immunized donor mice:

In this study, I used the HCV transgenic mouse model to analyze the kinetics of immune cells featuring an antiviral immune response against hepatitis C in adoptive transfer experiments after immunization with an HCV vaccine candidate. Previously it was shown that mice immunized with a combinations of a candidate HCV vaccine consisting of recombinant HCV core, E1, and E2 DNA plasmid and rHCV polyprotein and the adjuvant ,montanide, demonstrated significant humoral and cellular immune response [257]. In this study the same strategy was used to immunize wild type mice (donor mice), in order to transfer their immune cells to HCV transgenic mice. Mice immunized with a combined HCV vaccine consisting of DNA (HCV core, E1, and E2), protein and the adjuvant montanide A51 showed humoral and cellular antiviral immune responses. ELISA results demonstrated a significant increase in the antibody titer against an HCV immunogen. After the third immunization, there was a significant increase in total IgG at 1:900 antibody titer (P value > 0.05), IgG1 at 1:100 antibody titer (P value > 0.05), and IgG2a at 1:100 antibody titer (P value > 0.01) (Fig. 5.1). Similarly, in response to HCV antigens CD4+ T cell proliferation was demonstrated by CFSE staining. After the last immunization the splenocytes were cultured in the presence of core, E1 and E2 polyprotein or core peptides. There was an increase in the proliferation response of the immunized mice splenocytes when they were stimulated with HCV Core, E1, and E2 protein or core peptides, as indicated by the decrease in the CFSE stain intensity. As the cells proliferate, the cell population shifts to a lower intensity due to the decrease of staining in the cell membranes of proliferating cells (Fig. 5.2).

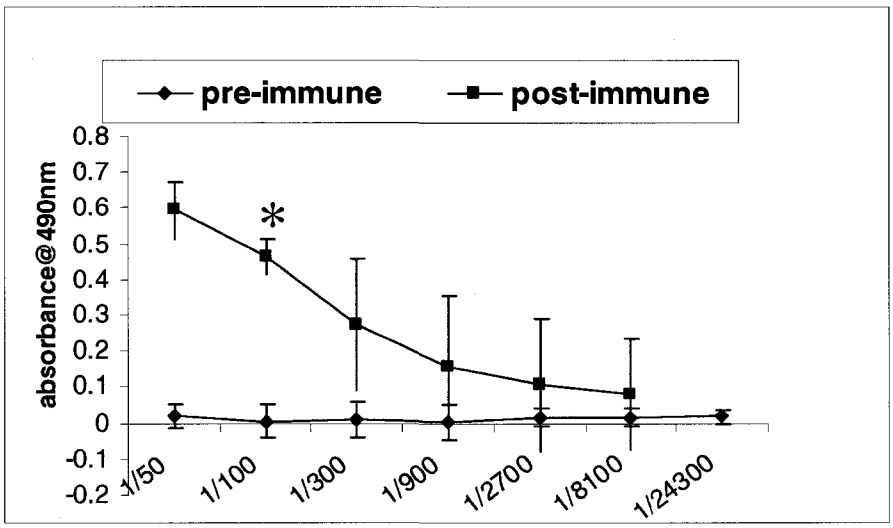
Fig. 5.1: Humoral immune responses of the donor mice immunized with HCV immunogens detected by ELISA.

Mice (n = 7) were immunized with HCV immunogens containing HCV plasmid DNA, HCV recombinant polyprotein and montanide adjuvant. After immunization, serum samples were collected, serially diluted and tested for reactivity with recombinant HCV core, E1 and E2 protein. Pre-immunized sera were used as a baseline. After the third immunization, immunized mice had significant increase in total IgG (A) at 1:900 antibody titer (*P value < 0.05), IgG1 (B) at 1:100 antibody titer (*P value < 0.05), and IgG2a (C) at 1:100 antibody titer (*P value < 0.01),

A



B



C

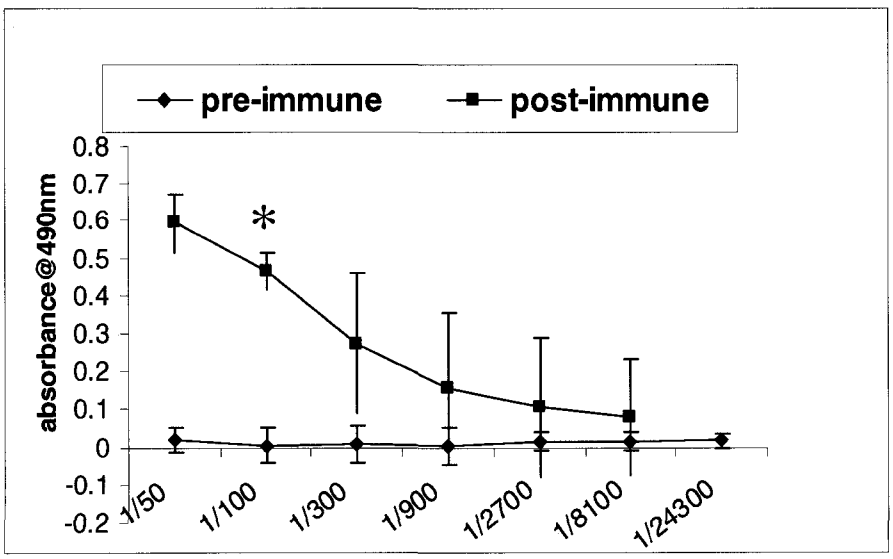
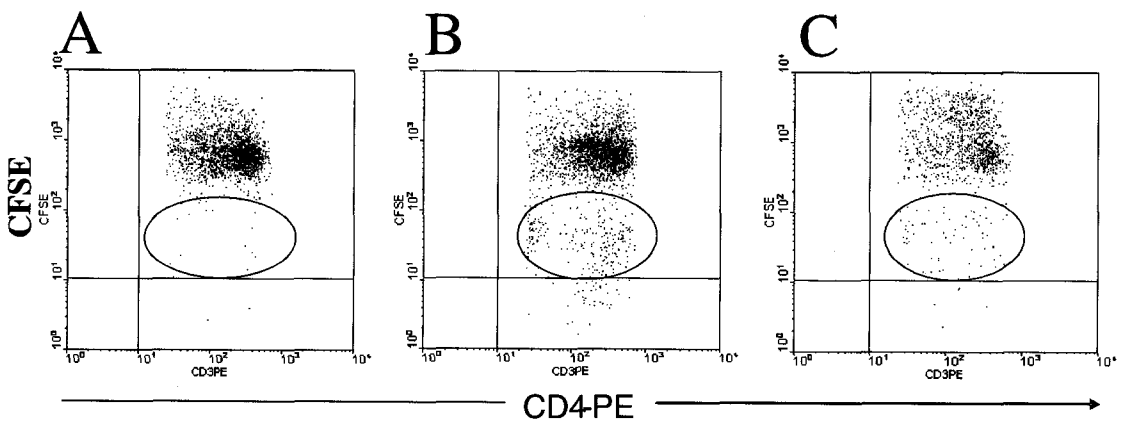


Fig. 5.2: Proliferation response by CD4+ T cells of HCV-immunized mice.

The spleen cells from immunized mice were stained with CFSE dye and incubated with different stimulants for 4 days. Cells were stained for surface markers using anti-CD3 and CD4- antibodies and tested using flow cytometry. (A) Unstimulated cells showing no proliferation, (B) CE1E2 protein-stimulated cells showing proliferation of the cells which is indicated by the shift of fluoresecence in the cell population (circle), (C) Core peptide stimulated cells showing proliferation. Daughter cells contain half the fluorescent intensity of the parent cell. The picture represents proliferation of CD4+ T cells in one mouse out of 7 immunized mice.



CD8+ T cell cytolytic activity was demonstrated by INF-γ production using intracellular staining and ELISPOT. IFN-γ antibody production was significantly higher in immunized mice compared to controls (Fig. 5.3). About 1.5% of the CD8+ T cells produced IFN-γ when they were stimulated with HCV core peptide and 1.75% of the cells produced IFN-γ when they stimulated with vaccinia encoding HCV recombinant proteins (vaccinia HCV poly) (Fig. 5.3 C&D). Unstimulated (A) or empty vaccinia (B) stimulated cells were used as a control. These results were confirmed by IFN-γ ELISPOT (Fig. 5.4).

5.2.2 Flow cytometric analysis of recipient mice tissues:

Splenocytes from the immunized (n=7) or non-immunized wild mice (n=7) were collected and labeled with CFSE dye before the adoptive transfer with the goal of studying the splenocyte kinetics in the HCV transgenic mice and to indirectly evaluate the immune response generated after HCV vaccination. The labeling of mouse donor splenocytes with CFSE was confirmed by immunofluorescent microscopy and flow cytometry (Fig. 5.5). These CFSE labeled cells were injected intraveinously into recipient mice (transgenic mice (n=7) or non transgenic mice (n =7). The celle were tracked in the mice blood *in vivo* after 24 hours by collecting blood sample from recipient mice and analyze them using flow cytometry. Seven days after the adoptive transfer, recipient mice were euthanized. The location and number of transferred cells were tested by flow cytometry for blood, lymph nodes, spleens and livers.

All groups of recipient mice had similar percentages of donor CD4+ and CD8+ T cells at 24 hours post-adoptive transfer, indicating that all groups received similar amounts of donor splenocytes (Fig. 5.6A). Seven days after the adoptive transfer, the percentage of the donor

Fig. 5.3: CTL response by CD8+ T cells in HCV immunized mice as demonstrated by IFN-γ intracellular staining.

Two weeks after the last HCV vaccine immunization, cultured splenocytes were unstimulated (A), stimulated with empty vaccinia (B), core peptide (C), or vaccinia HCV poly (D). Cells were cultured for 18 hrs in the presence of brefeldin A then stained intracellularly with anti-IFN-γ antibody and surface stained with anti-CD3 and anti-CD8 antibodies to be analyzed by flow cytometry. Percentages in the upper right quadrant represent the frequency of CD3+CD8+ T lymphocytes expressing IFN-γ. The picture represents CTL response of CD8+ T cells in one mouse out of 7 immunized mice.

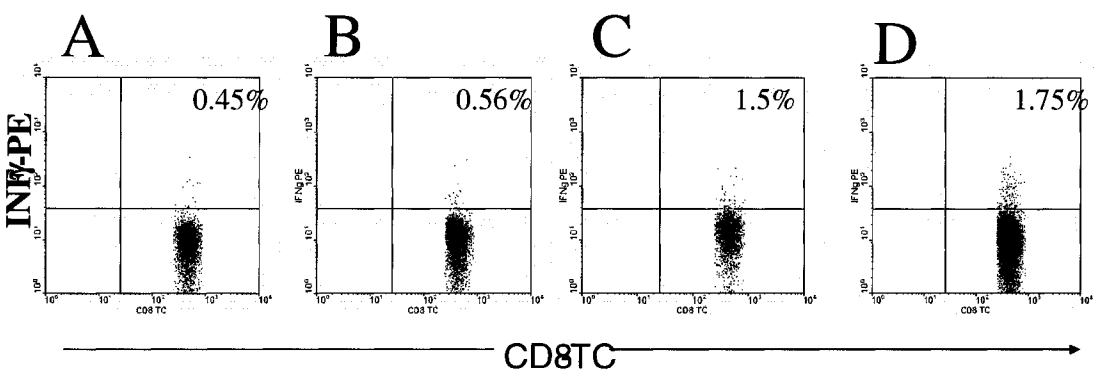


Fig. 5.4: Detection of CD4+ and CD8+ T lymphocyte responses to HCV vaccine in immunized mice using IFN-γ ELISPOT assay.

ELISPOT counts (spot-forming units [SFUs]/1 × 10^6) in response to core, E1 and E2 protein, Core peptides, or vaccinia HCV poly. Spot forming cell (SFC) frequencies are shown after subtraction of background with unstimulated cells or empty vaccinia stimulated cells. Cells were incubated with core, E1 and E2 protein, Core peptides, or vaccinia HCV poly for 48 hrs before measuring IFN-γ ELISPOT responses. Spot forming cell (SFC) frequency per million cells is indicated for each immunized and non-immunized donor mice (After 5 immunization). (Number 1 is non-immunized mouse; Number 2-6 are immunized mice).

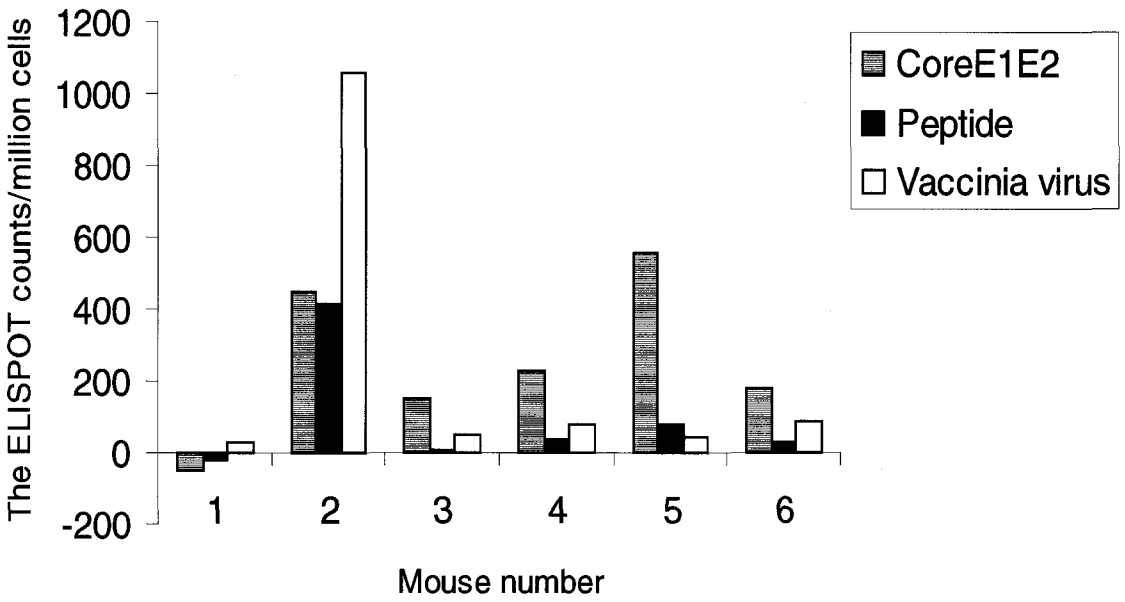
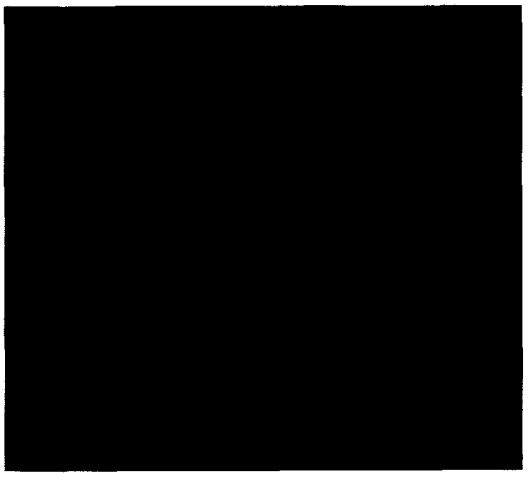


Fig. 5.5: Immunofluoresent analysis of CFSE labeled splenocytes before injection.

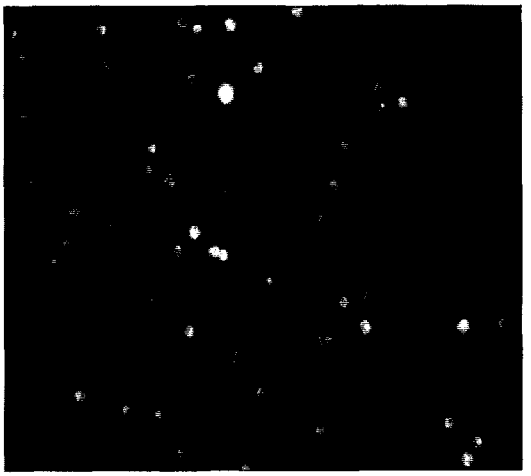
A drop from cell suspension of CFSE labeled splenocyte was spread over a slide and covered with a cover slip and examined under fluorescent microscopy.

A) CFSE unlabeled splenocytes showing no CFSE staining. B) CFSE labeled splenocytes showing green fluorescent cells.

A



B



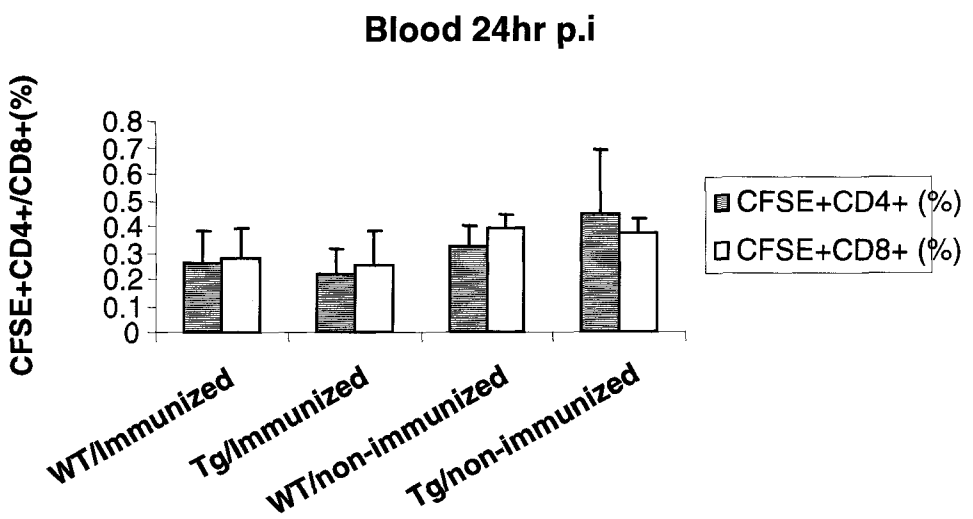
CD4+ and CD8+ T cells in the blood differed between the recipient mice receiving immunized and non-immunized donor cells (Fig. 5.6 B). There was a significant increase in the percentage of donor T cells in the blood of wild type mice receiving immunized donor cells. In contrast, there was a significant decrease in the percentage of donor T cells in the blood of transgenic mice received immunized donor cells. In fact, among the groups of mice studied, the transgenic animals had the lowest percentage of donor T cells in the blood. There was no significant difference of donor cell percentages in the groups receiving cells from non-immunized donors.

A higher percentage of donor T-cells from the non-immunized groups homed to the spleen as compared to the immunized animals. There was a four to ten-fold increase in the number of CD4+ and CD8+ T cells in the spleens of mice receiving non immunized donor cells (Fig. 5.7A). The donor cells from immunized animals homed to the lymph nodes of the wild type mice only. The percentages of CD4+ and CD8+ T cells in the non-transgenic recipient mice lymph nodes were significantly higher than the transgenic mice when they received cells from immunized donor mice (Fig. 5.7 B). The proportion of CD8+ T cells was higher than CD4+ T cells in lymph nodes of these wild type recipients of immunized donor mice. There was no difference between the transgenic and non-transgenic recipient mouse groups when they received transfers from non-immunized donors. This may be due to a defect in the homing receptors of the T cells in the transgenic mice lymph nodes. In contrast to non-transgenic mice, donor cells from immunized mice homed to the liver of transgenic mice as demonstrated by a three-fold increase in both CD4+ and CD8+ T cells compared to the other groups of recipient mice (Fig. 5.8). This may indicate a trapping mechanism for T-cells in transgenic mice livers due to the dominant expression of the HCV transgene.

Fig. 5.6: Flow cytometric analysis of recipient mouse blood 24 hours and 7 days post-adoptive transfer.

(A) The percentage of CFSE CD4+ and CD8+ T cells in the blood of the recipient mice 24 hrs post-injection. The X axis indicates the donor and recipient mouse groups (n=7). The Y the percentage of the CFSE+ CD4+ or CD8+ T cells. (B) The percentage of donor CD4+ and CD8+ T cells in the blood seven days after the injection. The cells were surface stained with anti-CD3 and anti-CD4 antibodies or anti-CD3 and anti-CD8 and analyzed by flow cytometry. (P value > 0.001). (Tg = Transgenic, WT = Wild type)

A



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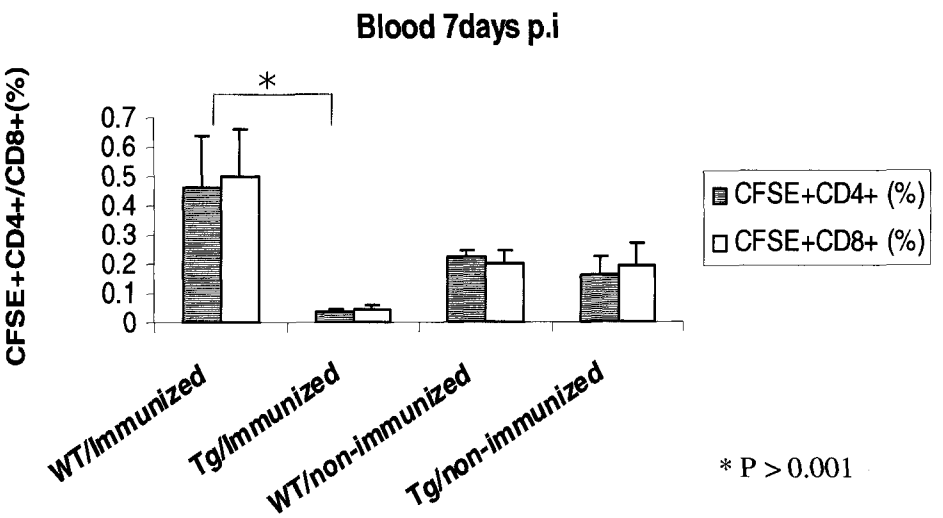
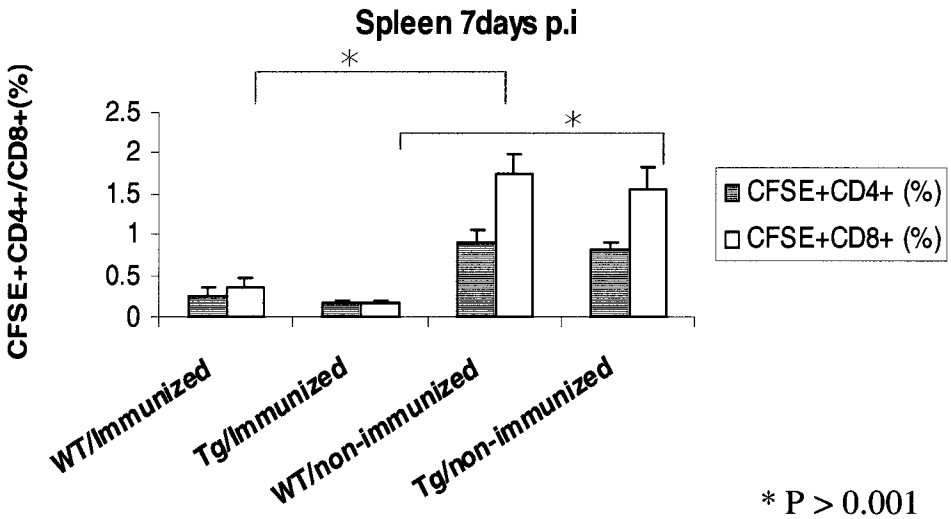


* P > 0.001

Fig. 5.7: Flow cytometric analysis of recipient mouse spleens and lymph nodes.

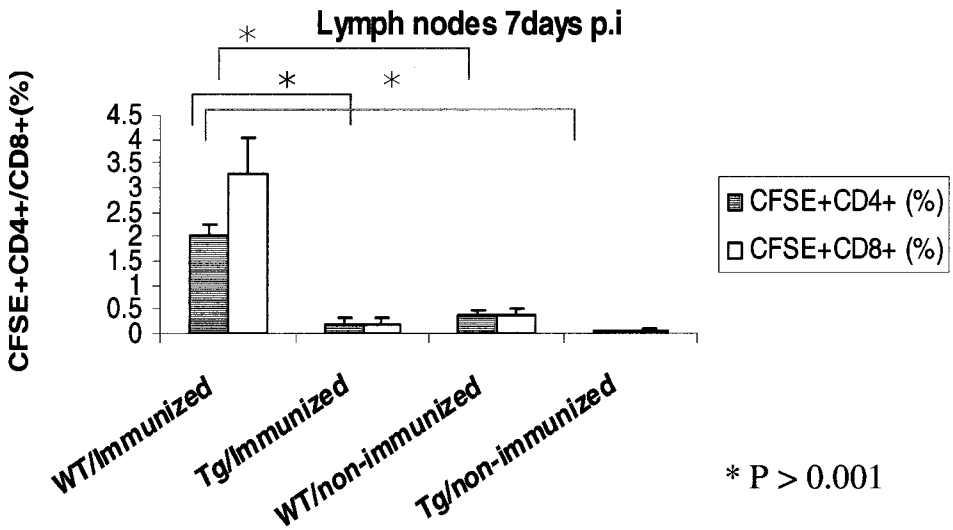
(A) The percentage of CD4+ and CD8+ T cells in the spleens of mice receiving immunized and non immunized donor cells. (B) The percentage of CD4+ and CD8+ T cells in the lymph nodes of the recipient mice. The cells were surface stained with anti-CD3 and anti-CD4 antibodies or anti-CD3 and anti-CD8 and analyzed by flow cytometry. (P value > 0.001). (Tg = Transgenic, WT = Wild type)

A



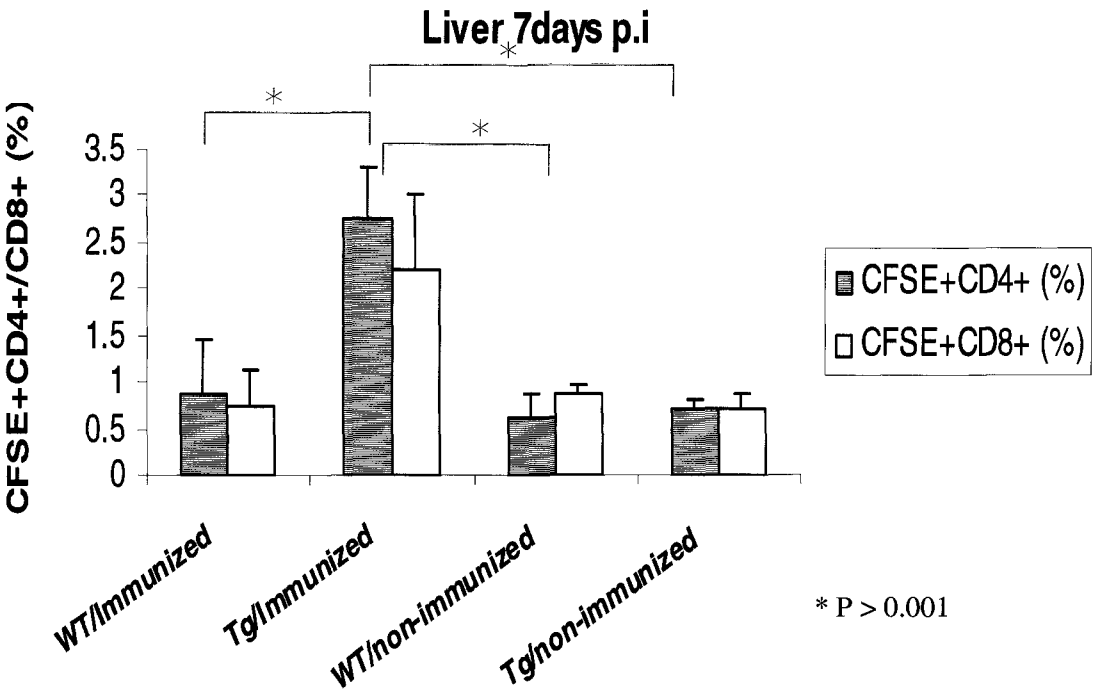
* P > 0.001

B



* P > 0.001

Fig. 5.8: Flow cytometric analysis of recipient mouse livers. The percentage of CD4+ and CD8+ T cells in the livers of mice receiving immunized and non immunized donor cells was detected by FACS. The cells were surface stained with anti-CD3 and anti-CD4 antibodies or anti-CD3 and anti-CD8 and analyzed by flow cytometry. (Tg = Transgenic, WT = Wild type)



Proliferation of CFSE-labeled transferred cells was not observed in the blood or any other tissues 7 days after the transfer, ie; there were no different T cells population with different CFSE intensity.

5.2.3 Immunofluorescence analysis and histological changes in the livers of recipient mice:

Immunofluoresence analysis of the liver sections of the transgenic mice showed infiltration of high number of the CFSE labeled cells, when they received transfer from immunized mice (Fig. 5.9 A). H&E staining of the liver sections for the same group of recipient mice showed infiltration of lymphocytes beside the histological changes, such as steatosis, due to the expression of transgene (Fig. 5.9 B). Interestingly, the infiltrating cells were concentrated in the areas where steatosis was observed. On the other hand, the transgenic mice receiving cells from non-immunized donors showed few CFSE labeled cells on the liver sections and no cells infiltration was observed in the H&E stained liver section (Fig. 5.10). The non-transgenic mice showed no histological changes and no infiltration of CFSE labeled cells, whether they received transfers from immunized (Fig. 5.9 C, D) or non-immunized mice (Fig. 5.10 C, D).

Fig. 5.9: Histological alterations in livers from transgenic and non-transgenic mice injected with CFSE-labeled splenocytes from immunized mice.

(A) Immunofluorescent analysis of frozen liver sections (5μm in thickness) of a transgenic mouse showing CFSE labeled cells scattered over all the liver section. The fluorescent cells are indicated by arrows. (B) H&E stained liver section of transgenic mouse showing steatosis. There is infiltration of lymphocytes in the liver which is concentrated close to hepatic steatosis (indicated by arrows). (C) Immunofluorescence analysis of frozen liver sections (5μm in thickness) of non-transgenic mouse showing no CFSE labeled cells over the liver section. (D) H& E staining of liver section of non-transgenic mouse showing normal histology of the liver and no lymphocyte infiltration. (Magnification power 20X). The pictures represent one mouse liver out of 7 donor mice.

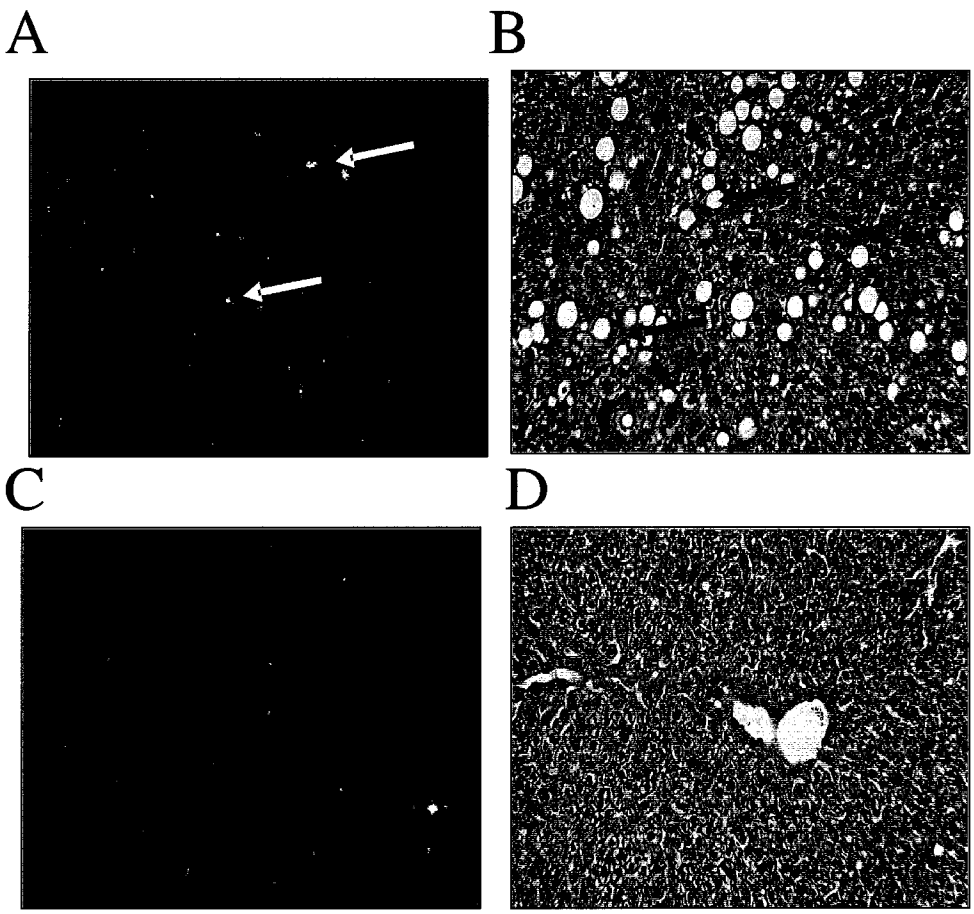
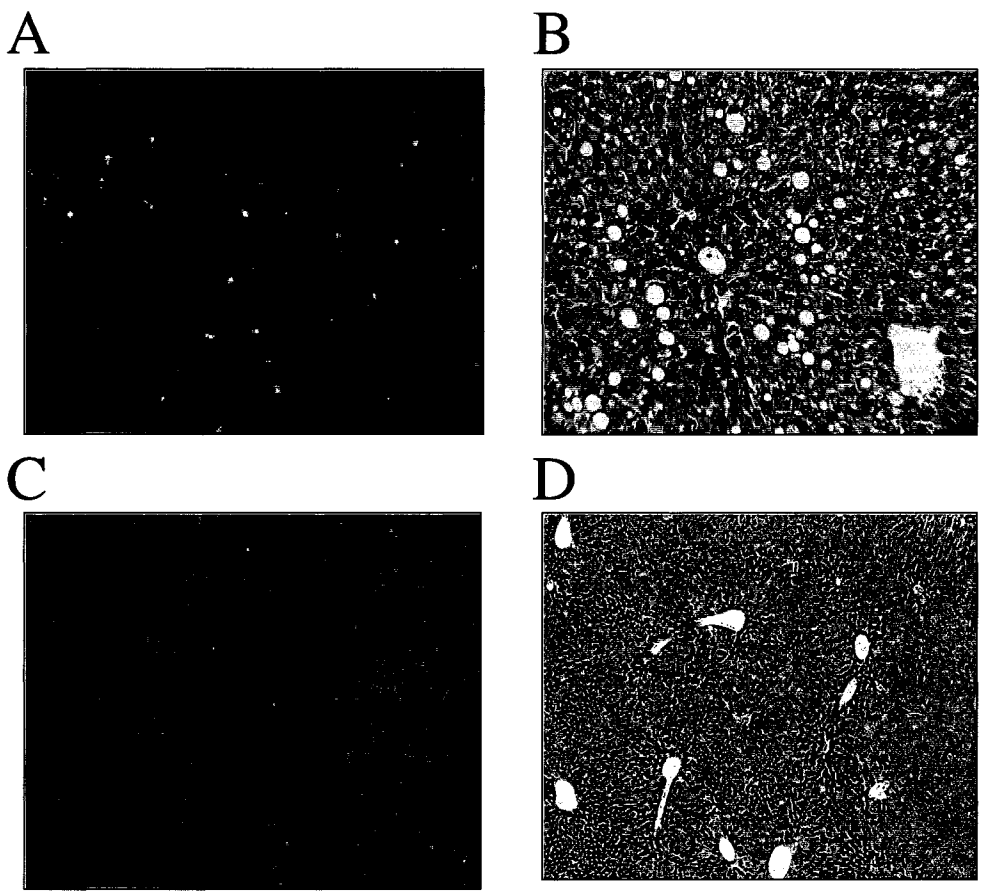


Fig. 5.10: Histological alterations in livers from transgenic and non-transgenic mice injected with CFSE-labeled splenocytes from non-immunized mice.

(A) Immunofluorescent analysis of frozen liver sections (5μm in thickness) of a transgenic mouse showing few CFSE labeled cells scattered over all the liver section. (B) H&E stained liver section of transgenic mouse showing steatosis. There is no infiltration of lymphocytes in the liver. (C) Immunofluorescence analysis of frozen liver sections (5μm in thickness) of non-transgenic mouse showing no CFSE labeled cells over the liver section. (D) H& E staining of liver sections of non-transgenic mouse showing normal histology of the liver and no lymphocytes infiltration. (Magnification power 20X). The pictures represent one mouse liver out of 7 donor mice.



5.3 DISCUSSION:

Chapter 3 of my research thesis indicated that HCV transgenic mice developed liver steatosis, hepatopathy and tumor formation due to HCV protein expression [261]. In this study, the role of immune responses in HCV pathogenesis described by using a model of an adoptive transfer from HCV immunized mice to HCV transgenic mice. As previously showed [257] and confirmed in this study, mice immunized with a combination of a candidate HCV vaccine (consisting of recombinant HCV core, E1 and E2 DNA plasmid, recombinant HCV polyprotein and montanide) demonstrated significant humoral and cellular antiviral immune responses. In order to confirm the specificity of the antiviral immune response and to assist the immune response in liver damage associated with hepatitis C infection, the spleenocytes from the immunized mice were transferred to HCV transgenic mice. Seven days after the adoptive transfer, the transferred cells were tracked in different mouse tissues using flow cytometric analysis. There was a significant decrease in the percentage of CFSE-labeled CD4+ and CD8+ T cells in the peripheral blood of transgenic mice having received cells from immunized donors, while the non-transgenic mice maintained a high percentage of the transferred T cells in their blood. This indicates that injected cells migrated from the peripheral blood and homed to different mouse organs. T cells from HCV immunized mice selectively homed to transgenic mouse livers, which is likely due to the recognition of HCV transgene preferentially expressed in this organ.

The immune response against pathogens depends on the ability of lymphocytes to migrate to organs where the pathogen's antigens are present. Here the lymphocyte migration to various organs of recipient mice was studied. The lymphocytes derived from HCV immunized mice homed to HCV transgenic livers where the HCV antigens are

predominantly expressed. In contrast, the lymphocytes from non-immunized/naive mice homed to the spleen of donor mice. Naive cells home to the spleen, while lymphocytes from immunized donors homed preferentially to the lymph nodes in the non-transgenic recipients. These cells are likely activated and perhaps recognize different homing receptors than lymphocytes from naïve animals.

The CD4+ and CD8+ T cells from immunized mice frequently display activation markers. Although activated cells are more likely to migrate to the liver, more cells from immunized animals homed to this organ than cells from non-immunized animals, suggesting immune specificity against the viral antigens. Generally, during adoptive immune response, two types of antigen-experienced T cells are produced. Short-lived effector T cells, which will home to the sites where the pathogen is present, and long-lived memory T cells, which could provide protection against the pathogen that they have encountered during the previous immune responses [432]. In this study, I speculate that lymphocytes from immunized mice included both effector T cells, which produced interferon gamma, and memory T cells. Both effector and memory T cells derived from immunized mice, could home to the liver of transgenic mice, whereas another fraction of memory T cells may stay in the lymph nodes. However, this type of phenotypical characterization of the adoptively transferred cells was not performed in this study. HCV-activated T cells homed to the lymph nodes of non-transgenic mice because there was no specific target in the non-transgenic animals. Increased knowledge on the mechanisms that regulate lymphocyte homing could have clear applications in designing more effective immunotherapeutic regimens for HCV.

There is strong evidence for the important role of both virus-specific CD4+ and CD8+ T cells in HCV virus clearance as well as in mediating liver cell damage in chronic hepatitis C

infection [180,433]. The two major mechanisms of T-cell mediated lysis are perforin-granzyme-mediated cytotoxicity and Fas-mediated cytotoxicity. Both mechanisms can kill the infected cells directly or by bystander killing which were demonstrated to be important in causing hepatic injury [434]. The Fas-Fas ligand system is reported to be associated with the killing of hepatocytes in patients chronically infected with hepatitis C virus. The expression of Fas ligand was up-regulated in the hepatocytes of patients with chronic hepatitis [435,436]. Liver infiltrating lymphocytes express Fas ligand which will bind with the Fas receptor on the surface of hepatocytes and initiate Fas-mediated cell death [184,437]. In previous studies it has been shown that CD8+ T cells can kill the targets *in vivo* by cytolysis mechanisms mediated by perforin and TNF-α [187] or required IFN-γ [188,434]. However, the ability of the adoptively transferred cells to cause lysis of the hepatocytes expressing the transgene will be elucidated in future studies.

There are several experimental models of immune-mediated liver damage in chronic hepatitis. Adoptive transfer models using transgenic animals expressing HBV proteins in hepatocytes have previously been described. These mice develop tolerance to virus-encoded proteins, but infusion of non-tolerant T cells will cause liver inflammation [188,438,439]. Recently, similar adoptive transfer experiments using HCV transgenic mice have been reported. In one study HCV-specific CTLs were injected intravenously into HCV transgenic mice [440]. In contrast to these results, their transgenic mice did not show any pathology in the liver. Furthermore, adoptive transfer of spleenocytes with strong CTL activities did not induce any hepatocellular injury. They attributed this to the low-level expression of HCV proteins in transgenic mice livers which was inadequate to encourage homing of these HCV-specific T cells into the liver. In accordance with this study, a report showed that HCV E1

protein specific CTLs transferred into interferon regulatory factor-1 (IRF-1) negative CD8-deficient transgenic mice expressing the HCV structural genes on hepatocytes, had an elevation of serum ALT as well as histological damage of the liver tissue [441]. However, transgenic mice used in this study were different from our study. These mice only transiently expressed the protein in their liver, while the transgenic mice in my study expressed HCV proteins permanently.

In summary, in this study I was able to adoptively transfer non-tolerant T cells into transgenic mice expressing the HCV transgene predominantly in hepatocytes. The adoptive transfer experiments results in rapid and selective accumulation of the activated T cells in the liver of the transgenic mice but not in the spleen or lymph nodes of the transgenic mice. In this study we did not detect the fate of these transferred cells but these cells may have the potential to have an antiviral effect and may also result in liver injury to the liver. This study may provide a good model to study the mechanisms of hepatic injury during chronic hepatitis C infection and may potentially help with the development of immunotherapeutics for HCV infection.

CHAPTER 6

CONCLUSIONS, GENERAL DISCUSSION, SIGNIFICANCE AND FUTURE DIRECTIONS

The molecular and physiologic mechanisms of HCV pathogenesis are not well understood. Studies on HCV pathogenesis have been hampered by the lack of appropriate cell culture systems and adequate animal models to support HCV replication. My research effort has been centered in developing a transgenic mouse model to provide insights into the pathobiology of HCV infection. This model provides a useful tool to elucidate the role played by HCV proteins in chronic hepatitis. The HCV structural proteins (core, E1 and E2) play important roles in the virus life cycle, but as discussed through out this thesis, they also play a vital role in the causation of biochemical and subcellular structural alterations that may lead to liver disease.

The structural proteins of HCV were expressed in the transgenic mouse model using a universal promoter to elucidate the effects of HCV on hepatic and extra-hepatic tissues. I was able to confirm that the HCV-transgenic mice expressed mRNA encoding the viral proteins in all examined tissues. However, the viral proteins were selectively expressed in the adult liver, but detected in low concentrations in both the kidney and the spleen. The differential expression of viral proteins in murine tissues suggests that viral protein expression may be controlled via translational mechanisms. The translational activity of the CMV promoter could be affected by some cellular factors which cause diffent protein expression in different tissues. Immunohistochemistry suggested that the level of viral proteins in the liver increased with age, which could be due to effect of some cellular factors in hepatocytes that found with increasing age or could be a polyclonal expansion of the hepatocytes that express the protein which increased with age.

Interestingly, the transgenic mice developed histological abnormalities in the liver, including steatosis and fibrosis, which are relevant clinical manifestations seen in HCV-

infected individuals. These structural abnormalities, in parallel to increasing viral protein expression, became more pronounced with animal age and eventually more severe manifestations of liver disease such as increasing fibrosis, and formation of liver tumors including HCC and lymphoid cancer occurred [261]. In addition, electron microscopy analysis demonstrated several abnormalities in hepatocyte intracellular organelles of transgenic mice including a loss of the rough endoplasmic reticulum pattern, swelling of mitochondria, and loss of mitochondrial shape and of their cristae. All these abnormalities indicate profound mitochondrial injury in the transgenic hepatocytes. The expression of the viral protein in extrahepatic tissues could be helpful to elucidate the mechanisms of extrahepatic disease manifestations of HCV infection, such the increased prevalence of lymphoid tumors in patients infected with HCV. Put together, these results suggest that HCV structural proteins cause the histological alterations seen in HCV infection, perhaps by interfering with biological processes in hepatocytes, such as lipid metabolism, cell cycle and oncogenesis.

To examine how the HCV structural proteins could interfere with biological processes in the liver, the second goal of my research thesis aimed to investigate the spectrum of changes in the global liver gene expression in the HCV transgenic mouse model. cDNA microarrays were analyzed to try to understand the dynamic of host gene expression resulting from persistent expression of HCV proteins in the liver. Three hundred and ninety four genes were identified to be differentially expressed at a statistically significant level, with 196 genes (142 corresponding to known genes) were identified as up-regulated and 198 genes (164 corresponding to known genes) were identified as down-regulated. These 306 differentially-expressed known genes were further clustered by applying Gene Ontology

(GO) Annotations search. The data presented in this study showed that HCV structural proteins in transgenic mouse livers induced alterations in the expression of genes involved in many important biological processes such as lipid metabolism and transport, antigen processing and presentation, protein biosynthesis, carcinogenesis and other pathways. Several of the differentially expressed genes were associated with cell signaling pathways such as the MAPK and Wnt oncogenic pathways. The differential expression of these genes in the HCV transgenic mice could explain important abnormalities associated with HCV infection, such as hepatic steatosis, impairment of antigen presentation and carcinogenesis. However, these observations will require further clarification and detailed experimental analysis.

Hepatic steatosis in these transgenic mice was developed in the same timeframe of the viral protein expression, which indicate the direct effect of HCV strudctural protein expression in lipid accumulation. Furthermore the morphological changes in mitochondria indicated a disturbance in their function. These results are in consistence with several studies which suggested that the accumulation of lipids in hepatocytes is indicative of a disturbance in the lipid metabolism that may be caused by the accumulation of viral proteins and it is likely related to defects in mitochondrial and peroxisomal fatty acid oxidation and biosynthesis [178,209,237,264,265]. Microarray studies done on the HCV transgenic mouse livers showed a disturbance in the expression of important genes involved in lipid metabolism and lipid transport. The Acsl-3 gene was down regulated in the transgenic mouse lives. Acsl-3 activates inert fatty acids to active form and move them from their storage sites to the mitochondria or peroxisomes to be metabolized [442,443]. Therefore, down regulation of this gene may lead to the accumulation of fatty acids (as triglycerides) without proceeding

to the metabolic pathways and lead to hepatic steatosis. Acox1 and Acaa1 enzymes, members of the classical peroxisome proliferator-inducible pathway, were up-regulated in HCV transgenic mouse livers. This may lead to increase in their enzymatic activities. It has been shown that increase in the activities of these enzymes is associated with proliferation of liver peroxisomes which would affect the fatty acids metabolism [354].

Hepatic steatosis in the transgenic mice can also be explained by disturbance in the expression of genes involved in lipid transport. Scp-2 protein, which is down regulated in HCV transgenic model, is a lipid binding protein that is important in the transport and metabolism of cholesterol [295]. In addition, Apoa1, the major apolipoprotein of HDL and has an important role in reverse cholesterol transport [296,297], was also down-regulated. The down regulation of Scp-2 and apoa1 genes in the HCV transgenic mouse model could decrease their lipid transport activity and more lipids will be accumulated inside the hepatocytes leading to hepatic steatosis. Other genes involved in lipid transport were down regulated, such as Apoa4 and apoc2. these two proteins are important in the transport of dietary triglycerides, phospholipids, cholesterols and cholesterol esters absorbed by enterocytes to other tissues such as hepatocytes [300]. Furthermore, Apoc2 inhibits lipoprotein lipase (LPL) mediated hydrolysis of triglycerides and modulated the metabolism of triglyceride-rich lipoproteins [301]. Thus, the up regulation of Apoa4 and apoc2 genes in the HCV transgenic mouse model may increase the transport of lipid from enterocytes to hepatocytes and may lead to lipid accumulation in the liver. Therefore, liver steatosis in transgenic mice could arise, at least in part, from direct effects of HCV proteins on lipid metabolism and transport.

The expression of HCV structural proteins in the liver of transgenic mice led to formation of liver tumors including HCC and lymphoid cancer [261]. The microarray analysis conducted in this model showed differential gene expression in some important genes involved in tumor formation. The up-regulation of AOX and Acaa1 enzymes genes in the transgenic mouse livers may have a role in carcinogenesis. Over-expression of fatty acyl-CoA induces an increase in the intracellular H_2O_2, apoptosis, and cell transformation, including tumor formation [444,445]. Furthermore, transgenic mouse liver showed decrease in the expression of HPB1 gene, which was identified as an inhibitor of the Wnt oncogenic pathway [306]. The upregulation of many genes, such as Pak2, c-Jun, and MKK3, involved in MAPK signaling pathways could also explain tumor formation in transgenic livers. for example, c-Jun is cconstitutive activation of endogenous AP-1 is required for tumor formation in mammalian cells [446], and it also occurs in distinct human tumor cells [447]. Rasa2 gene was down regulated. It acts as negative regulator of the Ras/MAPK-signaling pathway [448,449]. Decrease in the expression of this gene may explain the tumorgenesis in this model. Therefore, liver tumor formation in transgenic mice could arise from effects of HCV proteins on these genes.

In addition to the suggested role played by HCV structural proteins in the pathogenesis of HCV infection, the host immune response may also be involved. Many studies suggest the importance of the immune response in liver injury associated with chronic hepatitis C. Therefore, the third goal of this research thesis aimed to study the role of the host immune response in the liver damage associated with HCV infection. Since constitutive gene expression typically induces tolerance to the HCV transgene, examination of the physiologic functions of the transgene under the condition of host immune response is

limited. Thus, I developed a model of liver immune-mediated cell damage by means of adoptive transfer of splenocytes from HCV immunized mice into HCV transgenic mice. Splenocytes from HCV immunized mice were labeled with CFSE dye and adoptively transferred into HCV transgenic and non-transgenic recipient mice. The transferred cells were tracked *in vivo* in the blood, spleen, lymph nodes and liver of recipient mice by flow cytometry. Seven days after the adoptive transfer, flow cytometry was used to track the CFSE labeled CD4 and CD8 T cells in the blood and other tissues. I observed that there was a significant decrease in the percentage of CFSE-labeled T cells in the peripheral blood of transgenic mice having received cells from immunized donors, while the non-transgenic mice maintained a high percentage of the transferred T cells in their blood, which indicated the migration of the transferred cells from the peripheral blood to different mouse organs. Interestingly, a high percentage of T cells from HCV immunized mice selectively homed to transgenic mouse livers, which is likely due to the recognition of HCV transgene preferentially expressed in this organ. On the other hand, less T cells homed to the spleen or lymph nodes of the transgenic mice. Furthermore, the percentage of T cells in the non-transgenic recipient mice lymph nodes was significantly higher than the transgenic mice when they received cells from immunized donor mice. There was no proliferation of the CFSE transferred cells in any mice tissue. The histological analysis of the liver sections from the transgenic recipient mice, which received transfer from immunized donors, showed infiltration of lymphocytes. These infiltrating cells were concentrated in the areas where steatosis was seen. However, in this study I did not detect the fate of these transferred cells. It is not yet known whether these cells may have an antiviral effect or whether they could result in liver inflammation and injury to the liver. This study may provide a good model to study

the mechanisms of hepatic injury mediated by immune responses in chronic hepatitis C infection and may potentially help with the development of an immunotherapy for HCV infection.

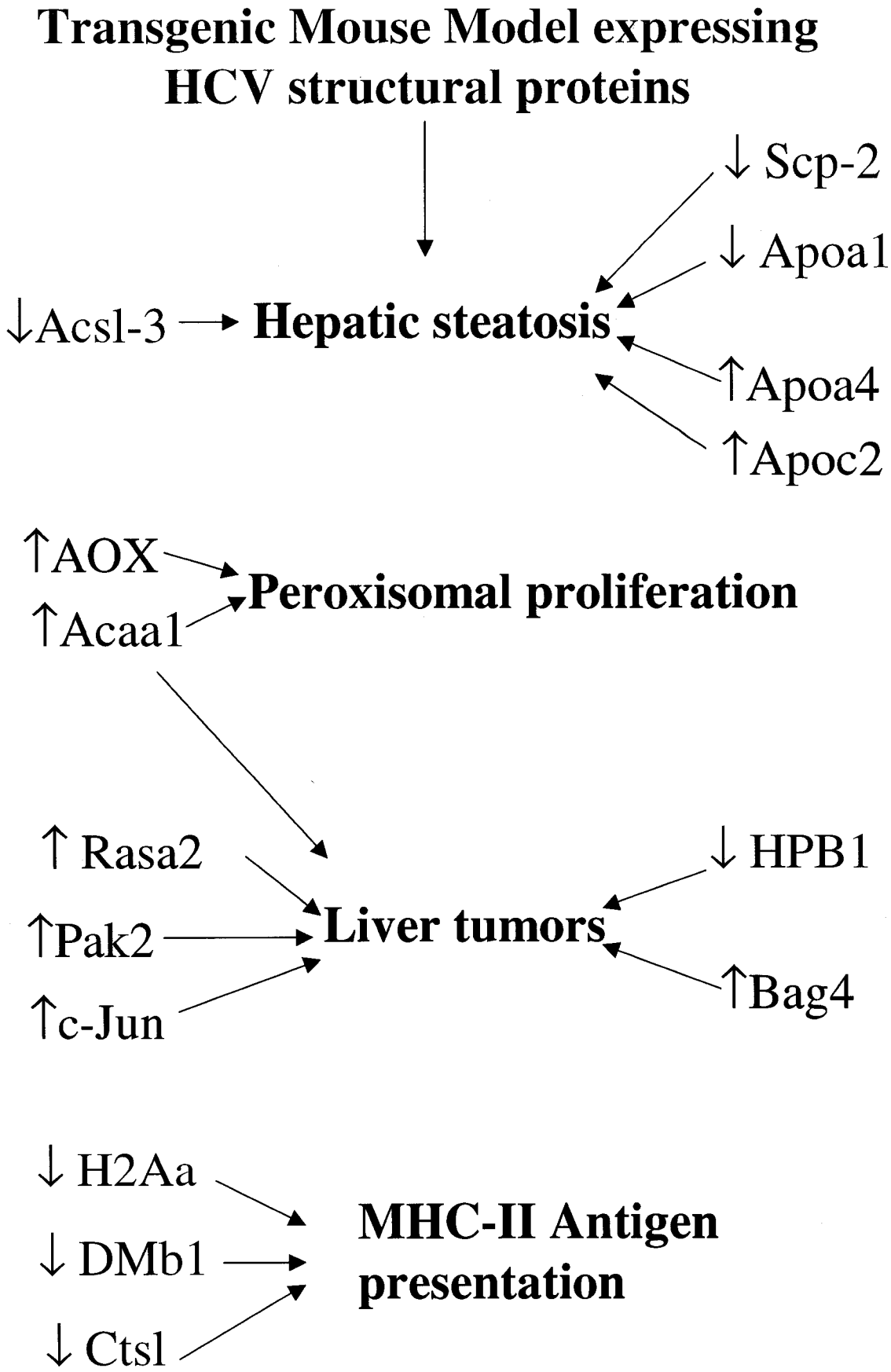
Further studies in this transgenic model are required in order to explain whether the mitochondrial structural changes in transgenic liver are indicative of the impairment of mitochondrial function and oxidative injury due to production of ROS. In addition, the impairment of dendritic cell function observed in HCV infection need to be clearly explained on the basis of down regulation of important MHC II pathway genes. The exact mechanisms that lead to malignant transformation in the transgenic animal model needs to be further studied. In the adoptive transfer experiment, the transferred cells have the potential to cause an antiviral effect or induce liver injury. However, these potential effects need to be elucidated.

In summary, the HCV transgenic mouse model presented in my research thesis is a suitable model to elucidate the role of HCV structural proteins in the pathogenesis of HCV (Fig. 6.1). However, in this transgenic model only the effect of structural protein expression was studied. HCV non-structural proteins are also important in the disease mechanisms of HCV chronic infection. From the studies presented, it is clear that expression of HCV proteins results in steatosis and HCC. These histopathological changes were associated with alterations in liver expression of many genes involved in different biological processes, such as lipid metabolism and transport, oncogenesis, immune responses...etc. The presence of HCV transgene from birth cause that these transgenic mice are immunotolerant to these proteins which limit the study of HCV- related immune mediated mechanisms of liver injury. In the third study this limitation was overcame by adoptive transfer of immunized cells to

transgenic mice. The cells from HCV immunized mice were selectively trapped into the liver of the transgenic mice where the transgene expressed predominantly. Thus, this animal model could play a pivotal role in elucidating the molecular mechanisms of chronic hepatitis and development of HCC during HCV infection. Understanding the mechanisms of HCV chronic infection and carcinogenesis are important for designing future means of treatment and prevention of HCC.

Fig. 6.1: Model of hepatitis C pathogenesis in transgenic mouse model expressing HCV structural proteins

Transgenic mice expressing HCV structural proteins in their livers showed histopathological changes including hepatic steatosis and liver tumor formation. These changes could be explained by decreased (↓) or increased (↑) of the expression of genes involved in these cellular processes. Hepatic steatosis could be explained by disturbance in the expression of genes involved in lipid metabolism and transport such as: decreased Acyl-CoA synthetase long-chain family member 3 (Acsl-3), decreased sterol carrier protein 2 (Scp2), decreased apolipprotein A-1 (Apoa1), increased apoliprotein A-IV (Apoa4), and increased apolipoprotein C-II (Apoc2). Liver tumors could be explained by disturbance in the expression of genes such as increased p21-activated kinase 2 (Pak2), increased Jun oncogene (c-Jun), increased RAS p21 protein activator 2 (Rasa2), increased Bcl2-associated athanogene 4 (Bag4), and decreased High mobility group (HMG) box transcription factor 1 (HPB-1). Increased Acyl-Co A oxidase 1 (AOX) and increased Acaa1 acetyl-CoA acyltransferase 1 (Acaa1) could cause peroxisomal proliferation and tumor formation. Decreased in the expression of genes involved in MHC II pathway of antigen presentation including; Cathepsin L (Ctsl), MHC II, locus Mb1 (H2-DM b1) and MHC II antigen A, apha (H2-Aa), could explain impairment of MHC II antigen presentation in chronic hepatitis C infection.



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APPENDICES

Appendix I: Differentially up-regulated genes in HCV transgenic mouse livers:

Clone ID	MGI ID	Symbol	Description	With Dominancy Correction	
				Fold-change	p-value
H3134C07	MGI:2148491	Acaa1	Acetyl-Coenzyme A acyltransferase 1	0.728	0.000
H3025G09	MGI:88051	Apoa4	Hypothetical LOC403343	0.694	0.000
H3018A12	MGI:1330812	Acox1	Acyl-Coenzyme A oxidase 1, palmitoyl	0.687	0.000
H3030C12	MGI:2139341	E130013N09Rik	RIKEN cDNA E130013N09 gene	0.603	0.000
H3123B09	MGI:1353452	Aldh3a2	Aldehyde dehydrogenase family 3, subfamily A2	0.753	0.000
H3111C08		Elovl5	ELOVL family member 5, elongation of long chain fatty acids (yeast)	0.709	0.000
H3022F06	MGI:97043	Mod1	malic enzyme, supernatant	0.682	0.000
H3134B01		Lmcd1	LIM and cysteine-rich domains 1	0.605	0.000
H3036E09		Fanca	Fanconi anemia, complementation group A	0.573	0.000
H3060D07		-		2.062	0.019
H3092E09	MGI:95295	Egr1	Early growth response 1	1.842	0.000
H3034E11	MGI:107536	Lgals6	lectin, galactose binding, soluble 6	1.538	0.017
H3126B05		-		1.413	0.007
H3096H11	MGI:1915816	Drd1ip	Dopamine receptor D1 interacting protein	1.338	0.000
H3085B06		A430106J12Rik	RIKEN cDNA A430106J12 gene	1.331	0.002
H3078E06	MGI:2145735	AU020094	Expressed sequence AU020094	1.250	0.007
H3115H02	MGI:1924217	2700049A03Rik	RIKEN cDNA 2700049A03 gene	1.224	0.008
H3076B11		Mier3	Mesoderm induction early response 1, fanily member 3	1.193	0.003
H3051F11		-		1.146	0.000
H3147D03		6430517E21Rik	RIKEN cDNA 6430517E21 gene	1.143	0.000
H3068C09		-		1.103	0.002
H3076A10		Tnfrsf19	Tumor necrosis factor receptor superfamily, member 19	1.096	0.002
H3067H01		Osbpl3	Oxysterol binding protein-like 3	1.054	0.003
H3068C06	MGI:1855699	Vti1a	Vesicle transport through interaction with t-SNAREs homolog 1A (yeast)	1.053	0.004
H3011E04		Dnm2	Dynamin 2	1.036	0.003
H3043F05	MGI:1931874	Dnajb1	DnaJ (Hsp40) homolog, subfamily B, member 1	1.025	0.000
H3003C04	MGI:2140116	Sgip1	SH3-domain GRB2-like (endophilin) interacting protein 1	0.999	0.001
H3151B04	MGI:1933289	Gtf2a2	General transcription factor II A, 2	0.998	0.002

H3044C02	**MGI:3029414**	**Brwd3**	Bromodomain and WD repeat domain containing 3	0.986	0.002
H3035F05		**Cd22**	B lymphocyte cell adhesion molecule (Cd22) mRNA, allele Cd22-c	0.976	0.000
H3058F03		-		0.960	0.002
H3058C09	**MGI:96646**	**Jun**	Jun oncogene	0.950	0.000
H3065B04		-		0.931	0.001
H3153H04	**MGI:2442653**	**C030048B08Rik**	RIKEN cDNA C030048B08 gene	0.915	0.000
H3073A02	**MGI:88529**	**Cs**	Citrate synthase	0.902	0.000
H3054D05	**MGI:1913917**	**Hspbap1**	Hspb associated protein 1	0.899	0.000
H3009D05	**MGI:96777**	**Lgals1**	lectin, galactose binding, soluble 1	0.897	0.000
H3059F09	**MGI:108384**	**Btg2**	B-cell translocation gene 2, anti-proliferative	0.883	0.001
H3066A09	**MGI:1913781**	**2310061C15Rik**	RIKEN cDNA 2310061C15 gene	0.881	0.000
H3072A01	**MGI:1344406**	**Ptpn21**	Protein tyrosine phosphatase, non-receptor type 21	0.870	0.001
H3051A10	**MGI:1919669**	**2700049P18Rik**	RIKEN cDNA 2700049P18 gene	0.866	0.000
H3066E07		**Gm1473**		0.865	0.000
H3015D04	**MGI:2156528**	**Elovl6**	ELOVL family member 6, elongation of long chain fatty acids (yeast)	0.865	0.001
H3016H05	**MGI:106253**	**D19Bwg1357e**	DNA segment, Chr 19, Brigham & Women's Genetics 1357 expressed	0.861	0.000
H3056D04	**MGI:97763**	**Prlr**	Prolactin receptor	0.857	0.004
H3035A05		-		0.844	0.002
H3153H02		**Rerg**	RAS-like, estrogen-regulated, growth-inhibitor	0.842	0.000
H3009A09	**MGI:108392**	**Slc20a1**	Solute carrier family 20, member 1	0.840	0.000
H3016C02	**MGI:2444113**	**5330401P04Rik**	RIKEN cDNA 5330401P04 gene	0.838	0.000
H3084A03	**MGI:1924968**	**6030443O07Rik**	RIKEN cDNA 6030443O07 gene	0.838	0.000
H3044D11		**C4bp**	Complement component 4 binding protein	0.830	0.000
H3086E12	**MGI:2140844**	**Nsun5**	NOL1	0.828	0.000
H3060B03		-		0.821	0.004
H3024G05	**MGI:1915147**	**Rnmt**	RNA (guanine-7-) methyltransferase	0.816	0.000
H3081D07		-		0.814	0.000
H3030H06	**MGI:109345**	**Sh2bp1**	SH2 domain binding protein 1 (tetratricopeptide repeat containing)	0.809	0.000
H3065B02	**MGI:1916754**	-	Transcribed locus	0.806	0.000
H3103H04	**MGI:2138232**	**Glt25d2**	Glycosyltransferase 25 domain containing 2	0.806	0.001
H3063F01	**MGI:1353460**	**Prpf39**	PRP39 pre-mRNA processing factor 39 homolog (yeast)	0.804	0.000
H3064B08		-	Similar to Flj37300-A-prov protein	0.804	0.000

H3017A01	**MGI:2144276**	**Zfp692**	zinc finger protein 692	0.803	0.000
H3008H11		**1700020I14Rik**	RIKEN cDNA 1700020I14 gene	0.798	0.002
H3059E01		-		0.797	0.002
H3061H01		**Hephl1**	PREDICTED: similar to hephaestin [Mus musculus], mRNA sequence	0.791	0.000
H3070A11	**MGI:2444365**	**Rapgef5**	Rap guanine nucleotide exchange factor (GEF) 5	0.789	0.000
H3033C03		-		0.787	0.000
H3051A11	**MGI:2145525**	**Asb13**	Ankyrin repeat and SOCS box-containing protein 13	0.786	0.000
H3074B02	**MGI:1923385**	-		0.785	0.001
H3074B11	**MGI:2145731**	**Xpo4**	Exportin 4	0.784	0.000
H3106D06		-		0.783	0.000
H3110G07	**MGI:1914947**	**Gadd45gip1**	growth arrest and DNA-damage-inducible, gamma interacting protein 1	0.777	0.000
H3049H12	**MGI:1858202**	**Rab11a**	RAB11a, member RAS oncogene family	0.772	0.001
H3071G01	**MGI:1932101**	**Ankrd17**	DNA segment, Chr 5, Wayne State University 152, expressed	0.769	0.001
H3075G12	**MGI:109533**	**Abcb7**	ATP-binding cassette, sub-family B (MDR/TAP), member 7	0.768	0.000
H3099B02	**MGI:1917489**	**Gtf3c5**	General transcription factor IIIC, polypeptide 5	0.765	0.002
H3049A02	**MGI:102499**	-		0.763	0.000
H3029E08		**Zfp367**	Zinc finger protein 367	0.761	0.000
H3068C03		**RP23-221M24.3**	Similar to hypothetical protein MGC42415	0.761	0.000
H3068E07	**MGI:2144842**	**Cbll1**	Casitas B-lineage lymphoma-like 1	0.755	0.000
H3093A03		-		0.753	0.000
H3132A11		-		0.748	0.000
H3070B05		**Evi5**	Ecotropic viral integration site 5	0.747	0.000
H3034B07	**MGI:109249**	**Adamts1**	A disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif 1	0.743	0.000
H3044C01		-	Similar to Es1 protein	0.742	0.001
H3069H04	**MGI:2149960**	**Rasa2**	RAS p21 protein activator 2	0.739	0.000
H3041B12	**MGI:2144114**	**AI316828**	expressed sequence AI316828	0.739	0.000
H3009H05	**MGI:109338**	**Rap1ga1**	RIKEN cDNA 1810058N05 gene	0.736	0.000
H3001G04		-		0.736	0.002
H3024G04	**MGI:102504**	-		0.734	0.000
H3009A07	**MGI:1915984**	**D130020L05Rik**		0.731	0.000
H3087C03	**MGI:1289260**	**Kcnmb2**	Potassium large conductance calcium-activated channel, subfamily M, beta member 2	0.725	0.000

H3055B01		-		0.725	0.000
H3013E06	**MGI:1916890**	**2310040C09Rik**	RIKEN cDNA 2310040C09 gene	0.722	0.000
H3155A04	**MGI:1334444**	**Zfpm2**	Zinc finger protein, multitype 2	0.720	0.000
H3084F10	**MGI:1891693**	**Mtmr7**	myotubularin related protein 7	0.717	0.001
H3072G07		-		0.717	0.000
H3106B07	**MGI:1100526**	**Nr2e1**	Nuclear receptor subfamily 2, group E, member 1	0.715	0.000
H3047E07		-		0.714	0.001
H3144F06	**MGI:87920**	**Adfp**	Adipose differentiation related protein	0.712	0.000
H3148A08		-		0.709	0.000
H3158D08	**MGI:1917028**	**1600021P15Rik**	hypothetical protein A430031N04	0.706	0.000
H3077E05	**MGI:1860764**	**Pde3a**	Phosphodiesterase 3A, cGMP inhibited	0.698	0.000
H3034E12		-		0.696	0.000
H3157A03	**MGI:1926020**	**2700007P21Rik**	RIKEN cDNA 2700007P21 gene	0.694	0.000
H3031C03		**Clca6**	chloride channel calcium activated 6	0.691	0.000
H3047E04		-		0.688	0.000
H3077H03	**MGI:102890**	**Ccng1**	Cyclin G1	0.687	0.000
H3048D01		-	Hypothetical LOC236262	0.687	0.000
H3045G01		-		0.685	0.000
H3060E01		-		0.684	0.000
H3068B06		-		0.683	0.000
H3001A09		-		0.681	0.000
H3090H05		**1110003E01Rik**	RIKEN cDNA 1110003E01 gene	0.680	0.000
H3010C10		-		0.679	0.000
H3089D12		-		0.679	0.001
H3080H12	**MGI:1270841**	**Cry1**	Cryptochrome 1 (photolyase-like)	0.678	0.000
H3130H04		**4632419I22Rik**		0.678	0.000
H3070A01		-		0.677	0.000
H3038H05	**MGI:1923848**	**Gtf2f1**	General transcription factor IIF, polypeptide 1	0.674	0.000
H3058G02	**MGI:1097166**	**Tcl1**	T-cell lymphoma breakpoint 1	0.673	0.000
H3113A02	**MGI:1917438**	**Eif2c2**	eukaryotic translation initiation factor 2C, 2	0.673	0.000
H3140A12	**MGI:1347487**	**Foxm1**	Forkhead box M1	0.672	0.000
H3005G09	**MGI:108088**	**Gab1**	Growth factor receptor bound protein 2-associated protein 1	0.669	0.000

H3079A07	**MGI:2156528**	**Elovl6**	ELOVL family member 6, elongation of long chain fatty acids (yeast)	0.669	0.000
H3045B05	**MGI:2139494**	**Pbx3**	RIKEN cDNA B930068K11 gene	0.668	0.000
H3122H06	**MGI:1929899**	**Sqrdl**	Sulfide quinone reductase-like (yeast)	0.667	0.002
H3098F02		-		0.666	0.000
H3112C05		-		0.665	0.000
H3115F11		**Nid2**	Nidogen 2	0.664	0.000
H3035C08	**MGI:2144624**	**Map2k3**	Mitogen activated protein kinase kinase 3	0.664	0.000
H3107C04	**MGI:2145403**	**AW011956**	Expressed sequence AW011956	0.660	0.000
H3079D12	**MGI:1333782**	**Pld3**	Phospholipase D3	0.658	0.000
H3043B01	**MGI:2444798**	**D530031C13Rik**	PREDICTED: Mus musculus RIKEN cDNA D530031C13 gene (D530031C13Rik), mRNA	0.658	0.000
H3074C10		**Ap1m2**	Adaptor protein complex AP-1, mu 2 subunit	0.657	0.000
H3026E09		**Ncor1**	nuclear receptor co-repressor 1	0.654	0.000
H3074D05	**MGI:1343460**	**Gnpat**	Glyceronephosphate O-acyltransferase	0.653	0.000
H3066A04		-		0.653	0.000
H3052A05		**1200007D18Rik**	RIKEN cDNA 1200007D18 gene	0.653	0.001
H3109F06		**1810013D10Rik**	RIKEN cDNA 1810013D10 gene	0.652	0.002
H3041C01		-		0.651	0.000
H3027A09		**Ets1**	E26 avian leukemia oncogene 1, 5' domain	0.648	0.000
H3035A08	**MGI:1922858**	**Chmp4b**	chromatin modifying protein 4B	0.648	0.000
H3067B08	**MGI:97583**	**Pik3r1**	Phosphatidylinositol 3-kinase, regulatory subunit, polypeptide 1 (p85 alpha)	0.647	0.004
H3084E02	**MGI:1920093**	-		0.646	0.000
H3078E02	**MGI:2681523**	**Tnpo1**	Transportin 1	0.645	0.000
H3091G03	**MGI:1277977**	**Ubr1**	3 complex, subunit 2	0.644	0.000
H3120B05	**MGI:88054**	**Apoc2**	apolipoprotein C-II	0.643	0.000
H3075A04	**MGI:2655198**	**Padi6**	Peptidyl arginine deiminase, type VI	0.643	0.000
H3033C02	**MGI:101783**	**Plk4**	Polo-like kinase 4 (Drosophila)	0.642	0.000
H3051E08	**MGI:2444364**	**Supt3h**	suppressor of Ty 3 homolog (S. cerevisiae)	0.640	0.000
H3102H12	**MGI:1316740**	**Morc1**	microrchidia 1	0.640	0.001
H3046C07		**C80148**		0.639	0.000
H3088A04		**Fndc3b**	fibronectin type III domain containing 3B	0.638	0.000
H3155F05		**Mkl2**	MKL/myocardin-like 2	0.637	0.007

H3068G09	**MGI:109242**	**Spin**	Spindlin	0.635	0.000
H3105E02	**MGI:1915723**	**Mobkl1a**	MOB1, Mps One Binder kinase activator-like 1A (yeast)	0.635	0.000
H3005G04		-		0.633	0.000
H3124C02		-		0.633	0.000
H3036C01	**MGI:1289159**	**Jarid2**	Jumonji, AT rich interactive domain 2	0.630	0.000
H3108C03	**MGI:1328317**	**Trex1**	Three prime repair exonuclease 1	0.627	0.000
H3072C07	**MGI:1927469**	**AU024549**		0.627	0.000
H3046C01	**MGI:2141315**	**C80140**	Expressed sequence C80140	0.627	0.000
H3044E02	**MGI:98181**	**Rrm2**	Ribonucleotide reductase M2	0.626	0.000
H3077E01	**MGI:3574110**	**Xlr5d**	X-linked lymphocyte-regulated 5D	0.626	0.000
H3005C06	**MGI:1929722**	**Anapc5**	Anaphase-promoting complex subunit 5	0.624	0.000
H3070F11		**C130086A10**	Hypothetical protein C130086A10	0.621	0.000
H3094E01		-		0.620	0.000
H3025A06	**MGI:1916974**	**Rnaseh2a**	Ribonuclease H2, large subunit	0.618	0.000
H3090E09	**MGI:2141554**	-		0.618	0.000
H3030H04		-		0.614	0.000
H3067G10	**MGI:1929492**	**Arl6ip2**	ADP-ribosylation factor-like 6 interacting protein 2	0.610	0.000
H3079D08	**MGI:1914634**	**Bag4**	BCL2-associated athanogene 4	0.607	0.002
H3089G06		-		0.607	0.000
H3053E09		-		0.606	0.000
H3067G01		-		0.604	0.000
H3107A04		-		0.602	0.000
H3019B02		**Nfat5**	Nuclear factor of activated T-cells 5	0.601	0.000
H3014B05	**MGI:2141074**	-		0.600	0.000
H3015C03	**MGI:107738**	**Dynclli2**	Dynein, cytoplasmic, light intermediate polypeptide 2	0.596	0.000
H3003A04	**MGI:2140151**	**Nol6**	Nucleolar protein family 6 (RNA-associated)	0.595	0.000
H3061A07		**Srpk2**	Serine/arginine-rich protein specific kinase 2	0.595	0.000
H3072D01		-		0.594	0.000
H3036B12	**MGI:1098779**	**Cdk2ap2**	CDK2-associated protein 2	0.593	0.000
H3030A07	**MGI:1339984**	**Pak2**	P21 (CDKN1A)-activated kinase 2	0.589	0.000
H3054D09	**MGI:2143434**	-		0.585	0.000
H3132D11		-		0.584	0.000

H3151E02	**MGI:102492**	-		0.583	0.000
H3113C11	**MGI:1929655**	**1200020A08Rik**	RIKEN cDNA 1200020A08 gene	0.576	0.000
H3019D10	**MGI:891995**	**Mapre1**	Microtubule-associated protein, RP/EB family, member 1	0.576	0.000
H3026B11	**MGI:103297**	**Atp7b**	ATPase, Cu++ transporting, beta polypeptide	0.576	0.000
H3020A05	**MGI:1919712**	**2600005C20Rik**	RIKEN cDNA 2600005C20 gene	0.575	0.000
H3060C03	**MGI:1347008**	**Plod3**	Procollagen-lysine, 2-oxoglutarate 5-dioxygenase 3	0.573	0.000
H3112F07	**MGI:2447834**	**2610024E20Rik**	RIKEN cDNA 2610024E20 gene	0.572	0.000
H3062H04	**MGI:1095419**	**Utx**	Ubiquitously transcribed tetratricopeptide repeat gene, X chromosome	0.572	0.000
H3076E06	**MGI:98911**	**Upk1a**	Uroplakin 1A	0.571	0.000
H3076D04	**MGI:1891158**	**Tbx19**	T-box 19	0.570	0.000

MGI: Mouse genome informatics

Appendix II: Differentially down-regulated genes in HCV transgenic mouse livers:

Clone ID	MGI ID	Symbol	Description	With Dominancy Correction	
				fold-change	p-value
H3135D11	MGI:98810	Tpm2	Tropomyosin 2, beta	-0.831	0.000
H3002H06	MGI:1351332	Azi2	5-azacytidine induced gene 2	-0.654	0.000
H3148G12	MGI:2142808	Cog4	Component of oligomeric golgi complex 4	-0.811	0.000
H3112B11	MGI:1924058	Rpl18a	Ribosomal protein L18A	-0.782	0.000
H3070C08	MGI:96233	Hsd3b1	Hydroxysteroid dehydrogenase-1, delta<5>-3-beta	-0.778	0.000
H3144H05	MGI:1918974	Aox3	Aldehyde oxidase 3	-0.731	0.000
H3084H09	MGI:1353578	Pttg1	Pituitary tumor-transforming 1	-0.713	0.000
H3071C03	MGI:3505790	Fcho2	2-4-dienoyl-Coenzyme A reductase 2, peroxisomal	-0.697	0.000
H3031E10	MGI:87968	Ahcy	S-adenosylhomocysteine hydrolase	-2.119	0.021
H3082D08	MGI:1341200	Pwp2h	PWP2 (periodic tryptophan protein) homolog, yeast	-1.652	0.016
H3134F05	MGI:95860	Gstm1	glutathione S-transferase, mu 1	-1.436	0.004
H3113F09	MGI:96236	Hsd3b4	Hydroxysteroid dehydrogenase-4, delta<5>-3-beta	-1.413	0.000
H3007E02	MGI:95865	Gstp1	glutathione S-transferase, pi 1	-1.409	0.000
H3044C12	MGI:97167	Msn	Moesin	-1.186	0.007
H3001A02	MGI:99466	Sct	Secretin	-1.142	0.005
H3005E06		-		-1.110	0.000
H3078G12	MGI:98266	Sord	sorbitol dehydrogenase 1	-1.074	0.000
H3154E01	MGI:109292	Rad50	RAD50 homolog (S. cerevisiae)	-1.031	0.003
H3148A01		Sdc2	RIKEN cDNA 4833414L08 gene	-1.026	0.000
H3079F01	MGI:107592	Hmgcs1	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1	-1.012	0.000
H3149F11	MGI:1340051	Gpr56	G protein-coupled receptor 56	-0.962	0.000
H3122C10	MGI:1333818	Rps7	ribosomal protein S7	-0.948	0.002
H3079C09		-		-0.919	0.003
H3001E09		-		-0.919	0.003
H3028C06	MGI:99832	Sec13l1	SEC13-like 1 (S. cerevisiae)	-0.917	0.037
H3144C06	MGI:105943	Rpl10	ribosomal protein 10	-0.907	0.000
H3059B01	MGI:96233	Hsd3b1	Hydroxysteroid dehydrogenase-1, delta<5>-3-beta	-0.906	0.000
H3125H12	MGI:98159	Rps6	ribosomal protein S6	-0.895	0.000
H3059B02	MGI:1918979	Rgs12	Regulator of G-protein signaling 12	-0.894	0.000
H3111H02		Cyp4v3	cytochrome P450, family 4, subfamily v, polypeptide 3	-0.888	0.001

H3111H08		Kcnip1	Kv channel-interacting protein 1	-0.886	0.000
H3053F11	MGI:99678	Actn3	Actinin alpha 3	-0.886	0.000
H3144D01		-		-0.875	0.000
H3155E03	MGI:2385325	Rpl21	ribosomal protein L21	-0.873	0.000
H3069E03	MGI:2146046	Adamts12	A disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif, 12	-0.870	0.000
H3154A01	MGI:1913872	5730410I19Rik	RIKEN cDNA 5730410I19 gene	-0.863	0.000
H3153B02		-		-0.860	0.000
H3117C09	MGI:98073	Rpl7	Ribosomal protein L7	-0.857	0.000
H3096D08		-		-0.854	0.001
H3082B10	MGI:1914693	Map1lc3b	Microtubule-associated protein 1 light chain 3 beta	-0.854	0.000
H3124F09	MGI:1927099	Rplp1	ribosomal protein, large, P1	-0.853	0.000
H3011E01	MGI:1350927	Rpl8	Ribosomal protein L8	-0.845	0.000
H3036C09		Eef1a1	eukaryotic translation elongation factor 1 alpha 1	-0.842	0.000
H3003F06	MGI:95753	Glud1	Glutamate dehydrogenase 1	-0.837	0.000
H3062B12		Nfia	nuclear factor I	-0.833	0.000
H3087C02	MGI:1919110	Wdr16	RIKEN cDNA 1700019F09 gene	-0.817	0.000
H3130H12	MGI:1351605	Rpl3	Ribosomal protein L3	-0.817	0.000
H3078C05	MGI:1914778	Nudt7	Nudix (nucleoside diphosphate linked moiety X)-type motif 7	-0.815	0.000
H3113H11		-		-0.815	0.000
H3061E04	MGI:105925	Cdo1	Cysteine dioxygenase 1, cytosolic	-0.815	0.000
H3115D02	MGI:105381	Rpsa	ribosomal protein SA	-0.814	0.000
H3135B02		4732460K03Rik	PEST-containing nuclear protein	-0.814	0.000
H3155D05		D230040E23	PREDICTED: Mus musculus similar to hypothetical protein FLJ38736 (LOC546144), mRNA	-0.811	0.003
H3142A10	MGI:1095733	Pros1	Protein S (alpha)	-0.811	0.000
H3046C10	MGI:2442264	Idi1	Isopentenyl-diphosphate delta isomerase	-0.806	0.000
H3112F09	MGI:1351605	Rpl3	Ribosomal protein L3	-0.805	0.000
H3115D04	MGI:105381	Rpsa	Laminin receptor 1 (ribosomal protein SA), mRNA (cDNA clone MGC:47040 IMAGE:4500748)	-0.803	0.000
H3043A10	MGI:88049	Apoa1	Apolipoprotein A-I	-0.797	0.000
H3154E10	MGI:88031	Anxa7	Annexin A7	-0.794	0.000
H3130B01	MGI:1915141	Rpl4	Ribosomal protein L4	-0.794	0.000
H3092H11		-		-0.791	0.000
H3011F03	MGI:1351605	Rpl3	Ribosomal protein L3	-0.781	0.001
H3015C01	MGI:1350933	Bcap31	B-cell receptor-associated protein 31	-0.776	0.000
H3144C11	MGI:2139530	Rabepk	Rab9 effector protein with kelch motifs	-0.773	0.000
H3151E07		Klhl7	Kelch-like 7 (Drosophila)	-0.771	0.000

H3028F03	**MGI:88564**	**Ctsl**	Cathepsin L	-0.769	0.000
H3119H04	**MGI:1921455**	**Utp14b**	Acyl-CoA synthetase long-chain family member 3	-0.769	0.000
H3129B12		**Eef2**	Eukaryotic translation elongation factor 2	-0.768	0.001
H3082H07		-		-0.764	0.000
H3147G03	**MGI:2151064**	**Strn3**	striatin, calmodulin binding protein 3	-0.760	0.000
H3123C07	**MGI:1096881**	**Eef1a1**	eukaryotic translation elongation factor 1 alpha 1	-0.759	0.000
H3122B04		-		-0.758	0.000
H3028F02	**MGI:1927347**	**Smarce1**	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily e, member 1	-0.756	0.000
H3129C02		**Eef2**	Eukaryotic translation elongation factor 2	-0.753	0.000
H3083G01		**9030611N15Rik**	RIKEN cDNA 9030611N15 gene	-0.752	0.000
H3020D07	**MGI:1096881**	**Eef1a1**	eukaryotic translation elongation factor 1 alpha 1	-0.751	0.001
H3118A10		-		-0.746	0.000
H3014E07	**MGI:1913536**	**Sec11l3**	Sec11-like 3 (S. cerevisiae)	-0.746	0.000
H3139G09	**MGI:87881**	**Prkcb1**	protein kinase C, beta 1	-0.744	0.000
H3119F02	**MGI:1202305**	**Vps72**	Transcription factor-like 1, mRNA (cDNA clone MGC:6868 IMAGE:2651158)	-0.743	0.000
H3051A09	**MGI:1917274**	**Mcm10**	Minichromosome maintenance deficient 10 (S. cerevisiae)	-0.742	0.000
H3028F04	**MGI:88564**	**Ctsl**	Cathepsin L	-0.739	0.000
H3109E05		**Pnma2**	Paraneoplastic antigen MA2	-0.739	0.000
H3147E01	**MGI:1916851**	**Dab2ip**	Disabled homolog 2 (Drosophila) interacting protein	-0.738	0.000
H3083E04	**MGI:1914370**	**Ccdc39**	DNA segment, Chr 3, ERATO Doi 789, expressed	-0.734	0.000
H3070E05	**MGI:2441684**	-		-0.733	0.000
H3115D03	**MGI:105381**	**Rpsa**	ribosomal protein SA	-0.732	0.000
H3115G05	**MGI:1913739**	**Rpl35**	ribosomal protein L35	-0.730	0.000
H3007F02		-		-0.730	0.000
H3030C01	**MGI:1916457**	**Sfrs11**	splicing factor, arginine/serine-rich 11	-0.729	0.000
H3132F05	**MGI:1355295**	**Nsbp1**	Nucleosome binding protein 1	-0.727	0.000
H3046F11	**MGI:1915141**	-		-0.722	0.000
H3149G08	**MGI:1927204**	**Gosr2**	Golgi SNAP receptor complex member 2	-0.719	0.000
H3026E11	**MGI:1918634**	**5430440L12Rik**	RIKEN cDNA 5430440L12 gene	-0.719	0.000
H3095A09		-		-0.715	0.000
H3144A12	**MGI:87968**	**Ahcy**	S-adenosylhomocysteine hydrolase	-0.714	0.000
H3138G03	**MGI:108077**	**Sdfr1**	Stromal cell derived factor receptor 1	-0.711	0.000
H3115G03	**MGI:1914780**	**Uqcrb**	Fc receptor-like mucin-like 1 (Fcrlm1), mRNA	-0.711	0.000
H3115D01	**MGI:105381**	**Rpsa**	ribosomal protein SA	-0.711	0.000
H3071H02		-		-0.706	0.001

H3143H04	**MGI:1928387**	**B4galt4**	UDP-Gal:betaGlcNAc beta 1,4-galactosyltransferase, polypeptide 4	-0.705	0.000
H3118C05	**MGI:102854**	**Rpl5**	Ribosomal protein L5	-0.701	0.000
H3130A12	**MGI:1915141**	**Rpl4**	Ribosomal protein L4	-0.699	0.000
H3129A03	**MGI:2675859**	**BC037674**	CDNA sequence BC037674	-0.698	0.000
H3024E03	**MGI:1919363**	**Adck1**	aarF domain containing kinase 1	-0.698	0.000
H3151F03		-	Inactive X specific transcripts	-0.696	0.000
H3062H11	**MGI:99511**	**Ptpn11**	Protein tyrosine phosphatase, non-receptor type 11	-0.696	0.000
H3142C10	**MGI:96569**	**Inha**	Inhibin alpha	-0.696	0.000
H3122C01	**MGI:894659**	**Cog5**		-0.695	0.000
H3057A02	**MGI:2141783**	**C87102**		-0.693	0.000
H3012C08	**MGI:1921664**	**Polr3c**	Polymerase (RNA) III (DNA directed) polypeptide C	-0.693	0.000
H3092C07		-	Similar to 60S ribosomal protein L7	-0.689	0.000
H3063B10	**MGI:1924248**	**1700124P09Rik**	RIKEN cDNA 1700124P09 gene	-0.689	0.001
H3006C12	**MGI:109345**	**Sh2bp1**	SH2 domain binding protein 1 (tetratricopeptide repeat containing)	-0.689	0.000
H3120G11	**MGI:1336208**	**Kcnab3**	Potassium voltage-gated channel, shaker-related subfamily, beta member 3	-0.688	0.000
H3131B04	**MGI:98254**	**Scp2**	Sterol carrier protein 2, liver	-0.685	0.000
H3005F03	**MGI:98865**	**Ttr**	Transthyretin	-0.685	0.000
H3113B06	**MGI:96157**	**Hmgb2**	High mobility group box 2	-0.685	0.000
H3108G12	**MGI:1914347**	**Rps10**	Ribosomal protein S10	-0.684	0.000
H3153C02	**MGI:1923990**	**C920006C10Rik**	RIKEN cDNA C920006C10 gene	-0.682	0.000
H3126F08	**MGI:2146851**	**Tfb1m**	Transcription factor B1, mitochondrial	-0.676	0.001
H3094B10	**MGI:1201609**	**Hiat1**	Hippocampus abundant gene transcript 1	-0.676	0.000
H3143E04		-		-0.669	0.000
H3138D12		**Eefla1**	Eukaryotic translation elongation factor 1 alpha 1	-0.669	0.000
H3101E09		-		-0.667	0.000
H3156H07	**MGI:2448523**	**Npepl1**	Aminopeptidase-like 1	-0.667	0.001
H3113H01	**MGI:104890**	-		-0.666	0.000
H3091E01		-		-0.665	0.000
H3046D11		**Elavl1**	ELAV (embryonic lethal, abnormal vision, Drosophila)-like 1 (Hu antigen R)	-0.665	0.000
H3103G06	**MGI:1914436**	-		-0.663	0.000
H3107B12	**MGI:1919266**	**1600002H07Rik**	RIKEN cDNA 1600002H07 gene	-0.663	0.000
H3159B02	**MGI:2136405**	**Glce**	Glucuronyl C5-epimerase	-0.662	0.000
H3159F08		**Rab8a**	RAB8A, member RAS oncogene family	-0.662	0.000
H3004H09	**MGI:107906**	**Rsbn1**	rosbin, round spermatid basic protein 1	-0.662	0.000
H3112F11	**MGI:1351605**	**Rpl3**	Ribosomal protein L3	-0.661	0.000

H3047D02	**MGI:87991**	**Alb1**	Albumin 1	-0.659	0.000
H3033G02	**MGI:1339972**	**Bhmt**	betaine-homocysteine methyltransferase	-0.656	0.000
H3009C10	**MGI:894668**	**Serpinb9b**	Serine (or cysteine) proteinase inhibitor, clade B, member 9b	-0.653	0.000
H3108F06	**MGI:104583**	**Eps15**	epidermal growth factor receptor pathway substrate 15	-0.653	0.000
H3157A11		-		-0.650	0.000
H3148C09	**MGI:1889377**	**AA792894**	EST AA792894	-0.649	0.000
H3026E07	**MGI:2137677**	**Sfxn1**	Sideroflexin 1	-0.646	0.000
H3148D02	**MGI:1917127**	**Nt5m**	5',3'-nucleotidase, mitochondrial	-0.645	0.000
H3051G05		-		-0.645	0.000
H3119H07	**MGI:2444777**	**Brrn1**	Barren homolog (Drosophila)	-0.644	0.000
H3109E03	**MGI:95921**	**H2-DMb1**	histocompatibility 2, class II, locus Mb1	-0.641	0.000
H3143C05	**MGI:1918128**	**4921511K06Rik**	RIKEN cDNA 4921511K06 gene	-0.641	0.000
H3146F03	**MGI:1921753**	**Bbx**	Bobby sox homolog (Drosophila)	-0.637	0.000
H3129A12		-		-0.636	0.000
H3066H02	**MGI:1861453**	**Actl6a**	Actin-like 6A	-0.632	0.000
H3128D12		**Nsfl1c**	NSFL1 (p97) cofactor (p47)	-0.632	0.001
H3158C01		-	Transcribed locus, weakly similar to NP_083693.1 RIKEN cDNA 9030605E16 gene [Mus musculus]	-0.631	0.000
H3028A05	**MGI:97749**	**Ppia**	Peptidylprolyl isomerase A	-0.630	0.000
H3025D09	**MGI:109175**	**Dab2**	Disabled homolog 2 (Drosophila)	-0.628	0.000
H3131A05	**MGI:1261437**	**Atp5c1**	ATP synthase, H+ transporting, mitochondrial F1 complex, gamma polypeptide 1	-0.625	0.000
H3095A07		-		-0.624	0.000
H3118H01		-		-0.623	0.000
H3111A03		**Nek10**		-0.621	0.000
H3131C11	**MGI:1919431**	**Nsun4**	RIKEN cDNA 2810405F18 gene	-0.621	0.000
H3139B08	**MGI:1914280**	**Fancl**	Fanconi anemia, complementation group L	-0.620	0.000
H3036C07	**MGI:105943**	**Rpl10**	ribosomal protein 10	-0.615	0.000
H3135E03	**MGI:1889797**	-		-0.614	0.000
H3035D02	**MGI:1347014**	**Psmb3**	Proteasome (prosome, macropain) subunit, beta type 3	-0.613	0.000
H3007C01	**MGI:1914273**	**2010315L10Rik**	RIKEN cDNA 2010315L10 gene	-0.612	0.000
H3126A06	**MGI:1096881**	**Eef1a1**	eukaryotic translation elongation factor 1 alpha 1	-0.608	0.000
H3022A07	**MGI:1313286**	**Eif2b3**	Eukaryotic translation initiation factor 2B, subunit 3	-0.608	0.000
H3084C05	**MGI:1914713**	**1200014M14Rik**	RIKEN cDNA 1200014M14 gene	-0.608	0.000
H3111C02	**MGI:2385053**	**BC011248**	CDNA sequence BC011248	-0.608	0.000
H3122H03	**MGI:107757**	**Gfer**	Growth factor, erv1 (S. cerevisiae)-like (augmenter of liver regeneration)	-0.607	0.000
H3059A04	**MGI:1924301**	**4632413K17Rik**	RIKEN cDNA 4632413K17 gene	-0.607	0.000

H3126A07		-		-0.605	0.000
H3100D04	**MGI:101816**	**Msh2**	MutS homolog 2 (E. coli)	-0.604	0.000
H3034B08	**MGI:1917374**	**Trim47**	Tripartite motif protein 47	-0.604	0.000
H3020E03	**MGI:2145443**	**Utp15**	Expressed sequence AW544865, mRNA (cDNA clone MGC:28378 IMAGE:4021387)	-0.602	0.000
H3141E04	**MGI:3042273**	**Nsfl1c**	NSFL1 (p97) cofactor (p47)	-0.601	0.000
H3143C08	**MGI:894835**	**Rbbp6**	Retinoblastoma binding protein 6	-0.600	0.000
H3093A10	**MGI:1353651**	**Gnl3**	guanine nucleotide binding protein-like 3 (nucleolar)	-0.600	0.000
H3062G06	**MGI:1929601**	**Ndfip1**	Nedd4 family interacting protein 1	-0.599	0.000
H3146G01	**MGI:101934**	**Tfdp1**	Transcription factor Dp 1	-0.597	0.000
H3015E11	**MGI:1860611**	**Pdlim1**	PDZ and LIM domain 1 (elfin)	-0.597	0.000
H3075C08	**MGI:1342287**	**Klf4**	Kruppel-like factor 4 (gut)	-0.594	0.000
H3138G10	**MGI:1921605**	**Smchd1**	SMC hinge domain containing 1	-0.592	0.000
H3144E01	**MGI:101785**	**Crtac1**	cartilage acidic protein 1	-0.590	0.000
H3008C07	**MGI:1928486**	**Tdo2**	Tryptophan 2,3-dioxygenase	-0.587	0.000
H3026C05	**MGI:2146015**	**AI451006**	Expressed sequence AI451006	-0.586	0.000
H3051E05	**MGI:2444286**	**Zzef1**	Zinc finger, ZZ-type with EF hand domain 1	-0.583	0.000
H3148C01	**MGI:1924038**	**2410127E18Rik**	RIKEN cDNA 2410127E18 gene	-0.582	0.000
H3051H09		-		-0.580	0.000
H3111D10	**MGI:2148202**	**Ces3**	Carboxylesterase 3	-0.580	0.000
H3133G05	**MGI:1096881**	**Eef1a1**	eukaryotic translation elongation factor 1 alpha 1	-0.580	0.026
H3089B09	**MGI:1914192**	**Ddx18**	DEAD (Asp-Glu-Ala-Asp) box polypeptide 18	-0.579	0.000
H3150H05	**MGI:106016**	**5730405G21Rik**	8 days embryo whole body cDNA, clone:5730405G21 product:silica-induced gene 41	-0.576	0.000
H3132C12		-		-0.575	0.000
H3087B04	**MGI:1913667**	**Mat2b**	Methionine adenosyltransferase II, beta	-0.574	0.000
H3082C01	**MGI:891967**	**Serpina1e**	Serine (or cysteine) proteinase inhibitor, clade A, member 1e	-0.573	0.000
H3118B01		-		-0.571	0.000
H3106F10	**MGI:2146998**	**AU041480**		-0.571	0.000
H3156H09		**Ext1**	Exostoses (multiple) 1	-0.571	0.000

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Doctor of Philosophy 2001-2006
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WORK EXPERIENCE:

Ph. D candidate 2001-2006
Department of Biochemistry, Microbiology & Immunology.
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Lab demonstrator 1995-1999
Faculty of Pharmacy
Department of Microbiology and Immunology
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Pharmacist 1994-2000
Working in private pharmacy
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LAB WORK EXPERIENCE:

- More than 5 years research experience in working in Microbiology and Immunology research.
- Expertise in molecular biology techniques e.g. DNA, RNA extraction, cloning, PCR, real-time RT-PCR and sequencing.
- Thorough knowledge in protein purification, SDS-PAGE and western blotting.
- Expertise in different types of ELISA, proliferation assays, Intracellular staining and ELIspot assay.
- Thorough knowledge in conducting Flow Cytometry and cDNA microarray,
- Extensive experience on working with different types of established cell lines, and primary cell cultures.
- Knowledge of handling and maintaining mice for immunization, and pathological tests.

SUPERVISION AND TRAINING OF STUDENTS:

Supervision and laboratory training in different techniques such as PCR, ELISA, intracellular staining, flow cytometry, manipulation, mice-handling and injection.

- From La Cité Collégiale, Biotechnologie students internship

Marie Claude Renaud	January – April 2004
Danica Albert	January – April 2002
Annie Jawed	January - April 2002

- Honour student:

Nicole Scherling	September 2004-April 2005

PUBLICATIONS:

- Characterization of liver histopathology in a transgenic mouse model expressing genotype 1a hepatitis C virus core and envelope proteins 1 and 2. **Naas T**, Ghorbani M, Alvarez-Maya I, Lapner M, Kothary R, De Repentigny Y, Gomes S, Babiuk L, Giulivi A, Soare C, Azizi A, Diaz-Mitoma F. J Gen Virol. 2005 Aug; 86(Pt 8): 2185-96.

- A combined nucleocapsid vaccine induces vigorous SARS-CD8+ T-cell immune responses. Azizi A, Aucoin S, Ghorbani M, Soare C, **Naas T**, Tadesse H, Frost R, Diaz-Mitoma F. Genet Vaccines Ther. 2005 Aug 22; 3(1): 7.

- Comparison of Antibody and Cell-Mediated Immune Responses after Intramuscular Hepatitis C Immunizations of BALB/C mice: Predominance of IgG1 against Hepatitis C by a Combination of DNA Vector, Core E1/E2 Protein and Montanide. Ghorbani M, Azizi A, **Nass T,** Soare C, Aucoin S, Giulivi A, Anderson DE, Diaz-Mitoma F. Viral Immunology. 2005;18(4):637-48.

- Immunogenicity of single and combined HIV-1/HCV candidate vaccines in HLA-A2.1 mice. Azizi A, Ghorbani M, Mojibian M, Soare C, **Naas T**, Diaz-Mitoma F. Current HIV Research. Accepted

IN PROCESS MANUSCRIPTS:

- Identification of novel hepatic gene expression alterations caused by HCV structural proteins in a transgenic mouse model using microarray analysis. **Naas T**, Ghorbani M, Alvarez-Maya I, Soare C, Lapner M, Kryworuchko M, Smith B, Famili F, Kothary R, De Repentigny Y , Diaz-Mitoma, F.

- Adoptive transfer of splenocytes to study cell-mediated immune responses in hepatitis C infection using HCV Transgenic mice. **Naas T**, Ghorbani M, Soare C, Scherling N, Muller R, Diaz-Mitoma, F.

ABSTRACTS:

- Characterization of Liver tumors and differential gene expression in hepatitis C transgenic mice. **Naas T**, Diaz-Mitoma F. 14th SPORE Investigators' Workshop. Baltimore, August 9, 2006.

- Adoptive transfer of anti-HCV splenocyts in an HCV transgenic mouse model. **Naas T**, Ghorbani M, Soare C, Scherling N, Azizi A, Diaz-Mitoma F. 12th International Symposium on Hepatitis C and Related Viruses. Montréal, Canada, October 2-6, 2005.

- Liver histopathology in a transgenic mouse model expressing genotype 1a hepatitis C virus core and envelope proteins 1 and 2. **Naas T**, Ghorbani M, Diaz-Mitoma F. 10th Annual BioNorth. November 17-19, 2003, Ottawa Congress Centre, Ottawa , Ontario

PRESENTATIONS:

- Characterization of a transgenic mouse model expressing genotype 1a hepatitis C virus structural proteins (core and Envelope 1 & 2). **Naas T**, Diaz-Mitoma F. Biochemistry, Microbiology & Immunology department, University of Ottawa. Research seminar, July 2006.

- Liver histopathology in a transgenic mouse model expressing hepatitis C virus structural proteins (core and Envelope 1 & 2). **Naas T**, Diaz-Mitoma F. Biochemistry, Microbiology & Immunology department, University of Ottawa. Student seminar series, February 2003.

- Hepatitis C virus structural proteins (core and Envelope 1 & 2) are expressed in the liver and salivary glands in a transgenic mouse model.

Naas T, Diaz-Mitoma F. Biochemistry, Microbiology & Immunology department Poster Day. April 2002. University of Ottawa.

OTHER INFORMATION:

Fluent in English (spoken and written).

(References and copies of certificates will be provided upon request)