UNIVERSITY OF NAPOLI FEDERICO II

Dipartimento di Biologia e Patologia Cellulare e Molecolare
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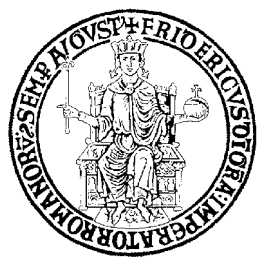

Doctorate School in Molecular Medicine

Doctorate Program in Genetics and Molecular Medicine
XXII Cycle

"GENERATION OF A MOUSE MODEL FOR THE *IN VIVO* STUDY OF HCV-HOST INTERACTION"

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Napoli 2010

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ABSTRACT

The natural species tropism of hepatitis C virus (HCV) is limited to humans and higher primates. Due to the limited availability of chimpanzees, the establishment of a small-animal model for the study of HCV infection has a high priority. The recent identification of the human cellular receptors that are essential for HCV entry together with the advances in mouse genome engineering provides the bases for creating an inbred mouse model of HCV infection and propagation.

In our study we designed a gene targeting strategy to produce novel humanized mouse strains expressing human-specific HCV entry receptors. Human genes for HCV essential entry factors were introduced in the mouse genome by homologous recombination in murine embryonic stem cells. To achieve the native expression patterns of their murine orthologues, we adopted a knock-in approach replacing the murine genes with human coding sequences. Three novel humanized mouse strains were generated, expressing respectively human CD81, scavenger receptor class B member 1 and occludin. Molecular analyses of mice genomic DNA and messenger RNA confirmed the presence and germ line propagation of the human genes, and their transcription.

The native regulated expression of human-specific HCV entry receptors can overcome the major block to HCV replication in murine cells. To verify the virus entry into the cells of our humanized mice, we successfully optimized isolation and culture of murine primary hepatocytes and HCV pseudotype particles infection assay. Genetic humanization of mice for species-specific HCV receptors could allow HCV glycoproteins mediated cell entry and could be an important tool for *in vivo* studying of virus-host interactions.

1. BACKGROUND

1.1 HEPATITIS C

1.1.1 Biology of hepatitis C virus (HCV)

The hepatitis C virus (HCV) is a small, enveloped RNA virus that causes liver fibrosis, cirrhosis and hepatocellular carcinoma. The liver is its primary target organ and the hepatocyte is its primary target cell. More than 170 million people (nearly 3% of worldwide population) are currently infected with HCV. A notable feature of HCV infection is its tendency to become chronic: ~70% of acute infections become persistent, and chronic cases are often associated with serious liver disease (Hoofnagle 2002, Alter and Seeff 2000).

The molecular characterization of HCV became possible approximately twenty years ago with the cloning of the viral genome, which led to its classification in a separate genus, *Hepacivirus*, of the Flaviviridae family (Choo et al. 1989).

HCV circulates in various forms in the serum of an infected host, including virions bound to lipoproteins, which appear to represent the infectious fraction, virions bound to immunoglobulins and free virions (Bradley et al. 1991, Thomssen et al. 1993). HCV virions have not yet been visualized conclusively by means of electron microscopy, and therefore information on their tridimensional structure is lacking. By analogy with the known structures of closely related flaviviruses and alphaviruses, HCV is thought to adopt a classical icosahedral scaffold in which its two envelope glycoproteins, E1 and E2, are anchored to the host cell-derived double-layer lipid envelope (Ferlenghi et al. 2001, Khun et al. 2002). Underneath the membrane is the nucleocapsid that is likely composed of multiple copies of the core protein, forming an internal icosahedral viral coat that encapsidates the genomic RNA (Penin et al. 2004).

The HCV genome consists of a positive single-stranded RNA of 9.6 kilobases. It encodes a single open reading frame (ORF) that is flanked by 5' and 3' untranslated regions (UTRs), including control elements required for translation and replication (Simmonds et al. 2005) (**Fig 1**).

The 5' UTR contains an internal ribosome entry site (IRES) that facilitates the translation of the capless HCV RNA. The 5' UTR also contains essential replication signals for the negative-strand RNA, which serves as the replicative intermediate (Friebe et al. 2001). The 3' UTR has a tripartite structure and is essential for HCV replication. In addition to directing the synthesis of the negative-strand RNA, the 3' UTR can also increase HCV IRES-mediated translation in the proper genomic context (Yi and Lemon 2003).

The UTRs flank an uninterrupted ORF encoding a single polyprotein of approximately 3000 amino acids, which is co-translationally and post-translationally processed by cellular and viral proteases to form three structural

protein (core, E1 and E2), an ion channel protein (p7) and six other non-structural proteins (NS) (Penin et al. 2004).

The structural proteins are located at the N-terminus and are released from the polyprotein by host signal peptidases that cleave signal peptides located between core/E1, E1/E2, E2/p7 and p7/NS2 (Penin et al. 2004). The cleavage of the signal sequence between core/E1 yields to the immature form of the core protein, which is further processed by another host peptidase into a mature protein of approximately 21 kDa. This maturation process promotes the transport of the core from the endoplasmic reticulum (ER) membrane to the surface of the lipid droplets, the site of HCV particle assembly. The core's N-terminus is highly basic and has been implicated in RNA binding and homo-oligomerization, both important properties of a nucleocapsid protein (Targett-Adams et al. 2008). E1 and E2 are envelope glycoproteins on the viral surface that are responsible for receptor binding and HCV entry into target cells. The C-terminal transmembrane domains of these proteins direct them to the ER membrane, the presumed site where HCV capsid particles pick up their envelopes before leaving the cell. E1 (192 amino acids) and E2 (363 amino acids) are both heavily glycosylated and form a non-covalent heterodimer (Brazzoli et al. 2005). Although the correct folding of these two proteins appears to depend on each other, E2 is the major ligand that binds to CD81 and SR-BI (scavenger receptor class B type I), two of the several HCV receptors identified so far (Pileri et al. 1998, Scarselli et al. 2002).

The p7 protein is a hydrophobic peptide of 63 amino acids. Both N- and C-termini of p7 face the ER lumen, indicating that it contains two TM domains. HCV p7 oligomerizes on the ER membrane and forms hydrophobic pores with ion channel activity. Although dispensable for RNA replication, p7 is essential for the production of infectious viruses both *in vivo* and *in vitro*, indicating a potential role of this protein in viral assembly and release (Griffin et al. 2003).

Down-stream of the structural protein, NS proteins are located in the order: NS2, NS3, NS4A, NS4B, NS5A, NS5B. They coordinate the intracellular processes of viral life cycle. Proteolytic processing within the NS region occurs through the action of two viral enzymes: the NS2 cysteine protease, which cleaves at the NS2/3 junction, and the NS3-4A serine protease, cleaving at all downstream sites (Bartenschlager et al. 1994).

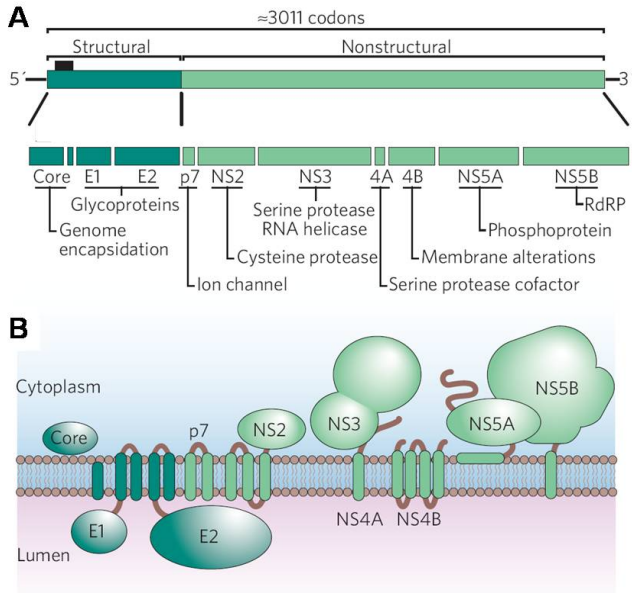


Fig 1 - HCV genes and gene products (Lindenbach and Rice 2005). Panel A: The structure of the viral genome, including the long ORF encoding structural and nonstructural genes, and 5′ and 3′ UTRs. RdRP, RNA-dependent RNA polymerase. Panel B: The topology of HCV proteins with respect to a cellular membrane.

Enveloped HCV particles interact with specific surface receptors and are internalized. Fusion of the viral and cellular membranes, presumably triggered by the low pH of the endocytic compartment, leads to the release of the single-stranded RNA genome into the cytoplasm of a newly infected cell. This genome serves multiple roles within the virus life cycle: firstly as a messenger RNA (mRNA) for translation of the viral proteins; subsequently as a template for RNA replication; and finally as a nascent genome packaged within new virus particles. Virions, presumably, form by budding into the ER and leave the cell through the secretory pathway (Brett et al. 2005) (**Fig 2**).

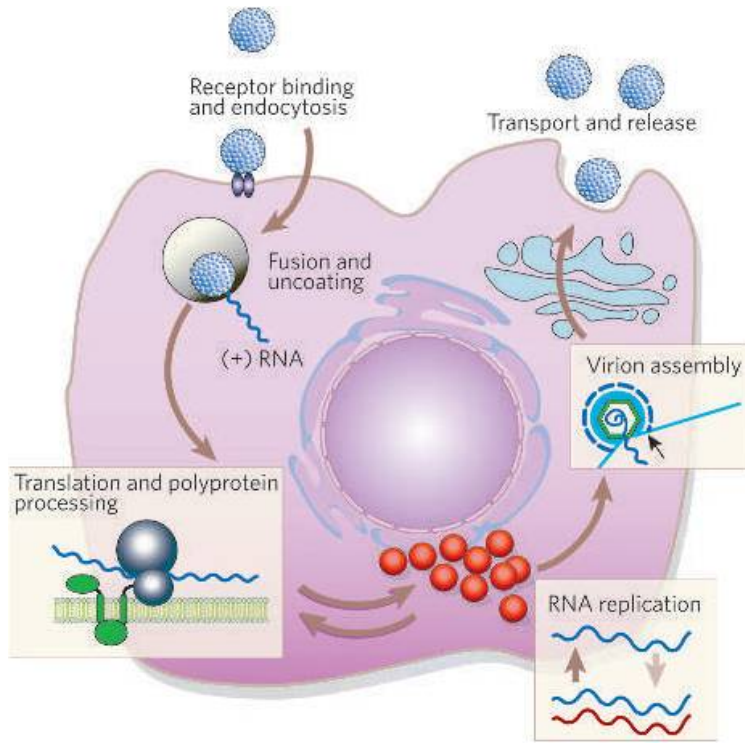


Fig 2 - HCV life cycle (Lindenbach and Rice 2005). After entry into the cell and uncoating, the HCV genome plays three main roles: translation, replication and packaging into nascent virions.

The first step of HCV infection is the attachment of the virus to the host cell surface, where attachment molecules such as glycosaminoglycans and low-density lipoprotein receptor (LDL-R) may mediate this initial interaction. Subsequently, specific binding between viral envelope glycoproteins and entry receptors induces a receptor-mediated endocytosis and the internalization of HCV particles through the cell plasma membrane (Koutsoudakis et al. 2006, Agnello et al.1999).

HCV RNA does not contain a structure similar to the 5' cap required for eukaryotic translation; instead it exploits an IRES-based cap-independent approach for protein synthesis. HCV IRES, located within the 5' UTR, can induce conformational changes in the 40S subunit of the ribosome that allow the formation of the active 80S complex in the absence of a 5' cap or ATP-dependent scanning (Wang et al. 1993).

Like in all positive-strand RNA viruses, HCV replication occurs on intracellular membranes. In fact HCV forms a replication complex (RC) on the ER membrane that includes the viral genome, NS proteins and cellular cofactor (Quinkert et al. 2005).

HCV assembly appears to occur on lipid droplets, and the core protein clearly coats the surface of this organelle. Although the details are still lacking,

a generalized model of HCV assembly has been proposed. This model suggests an important role of the very low-density lipoproteins (VLDL) pathway and NS proteins in the virus particles assembly. A reasonable proposal is that NS proteins are divided into at least two pools in the infected cells; one participates in the replication process and is incorporated into the RC, whereas the other is involved in particle assembly. In addition, it is likely that the association with lipoproteins and lipids affects the buoyant density of the virions (Quinkert et al. 2005).

1.1.2 *In vitro* model systems to study HCV infection

Discerning the mechanisms of HCV cell infection has been difficult for a long time due to the absence of an appropriate animal model and an efficient *in vitro* cell culture system supporting the complete HCV life cycle and enabling the production of infectious virus particles. For unknown reasons, serum-derived HCV poorly replicates in primary human hepatocytes and hepatoma cells *in vitro*. Thus, several surrogate models have been used in studies of virus entry before the development of an *in vitro* cell culture system, allowing the reproduction of all steps of the HCV replication cycle, including cell entry (Wakita et al 2005).

In the absence of a reliable *in vitro* model for virus multiplication, a truncated, soluble form of recombinant E2 glycoprotein (sE2) was used to search for candidate receptors involved in HCV cell entry. Two major HCV receptors, the human tetraspanin CD81 and the human scavenger receptor, were isolated in screening based on sE2 binding. The limitation of this system was the fact that the viral glycoproteins E1 and E2 form a heterodimer on the viral envelope and thus isolated E2 may behave differently (Flint et al. 1999).

An important breakthrough in getting access to a system that most closely mimics entry of authentic HCV cell entry was the development of HCV pseudotypes. This system is based on the production of retroviral or lentiviral particles that incorporate unmodified HCV glycoproteins into the lipid envelope (Bartosch et al, 2003b, Hsu et al, 2003). The production of HCV pseudotypes is achieved by co-transfection of 293T cells with plasmids encoding three components: full-length E1 and E2 glycoproteins, retroviral or lentiviral core and polymerase proteins, and a proviral genome carrying a marker gene, such as green fluorescent protein or luciferase. These particles are infectious and show a tropism for human liver cells. Moreover, cell entry of HCV pseudotypes is neutralized by antibodies directed against the E2 protein (Bartosch et al, 2003a). The main drawback of this system is that it mimics only the very early steps of infection: from particle binding to the liberation of the capsid.

The development of the first *in vitro* model reproducing the complete viral replication cycle and supporting the production of authentic virus particles that are infectious *in vitro* and *in vivo* was an important milestone in the HCV field. Cell culture-produced HCV (HCVcc) model is based on a particular genotype 2a virus strain, JFH-1, cloned from the serum of a patient with

fulminant hepatitis C. Human hepatoma Huh-7 clones transfected with the JFH-1 genome efficiently replicate the virus and secrete infectious particles (Lindenbach et al, 2005, Wakita et al, 2005). Although this HCV *in vitro* replication model mimics a natural HCV infection, it has some important limitations: it is restricted to two particular cell lines (Huh-7 and LH86), which have abnormal lipoprotein metabolism, and essentially to the JFH-1 strain (Burlone and Budkowska 2009).

1.1.3 Cellular receptors for HCV entry and host specificity

Virus entry is defined as the steps from particle binding to the host cell up to the release of the viral genome to its replication site within the target cell. This process relies on specific interactions between virus components, mainly envelope proteins and several cellular factors. HCV entry is a slow and complex multistep process involving the presence of several entry factors. Initial host cell attachment may involve glycosaminoglycans and the LDL-R, after which the particle appears to interact sequentially with four entry receptors: the tetraspanin CD81, the scavenger receptor class B member 1 (SR-BI) and the tight-junction proteins claudin-1 (CLDN-1) and occludin (OCLN) (Tang and Grisé 2009).

CD81, a member of the tetraspanin protein family, is probably the best-characterized receptor molecule for HCV entry. It was first identified as a soluble E2-binding protein (Pileri et al. 1998), and its critical role in HCV entry has been confirmed in all model systems (Helle and Dubuisson, 2008). The E2-binding site on CD81 has been mapped to a large extracellular loop of 89 amino acids. Ectopically expressed CD81 is necessary, but not sufficient, to allow entry of HCV into non-permissive cells, indicating the presence of additional entry factors (Zhang et al. 2002).

Accumulating evidence supports a critical role for SR-BI in the HCV entry process: both anti-SR-BI antibodies and RNA interference directed at SR-BI mRNA could inhibit infection of HCV pseudoparticles and HCVcc (Dubuisson et al. 2003, Zeisel et al. 2007). Like that of CD81, the expression level of SR-BI in Huh-7-derived cells lines can regulate cell permissiveness or HCV infectivity (Zeisel et al. 2007). Because SR-BI interacts with both high-density lipoproteins and soluble E2, it remains unclear whether the interaction between SRBI and HCV is direct or mediated by HCV-associated lipoproteins (Grove et al. 2007).

Getting evidence that even the simultaneous expression of CD81 and SR-BI on the cell surface of many cell lines was insufficient to support HCV pseudoparticles entry, Evans and colleagues set out to identify additional entry cofactors using an expression cloning approach. Their effort successfully identified CLDN-1, a tight-junction protein, that revealed to be essential for HCV entry. Interestingly, an interaction between CD81 and CLDN-1 has been observed, suggesting a co-receptor complex formation (Harris et al. 2008, Yang et al. 2008).

CLDN1 has been shown to be essential for HCV infection of human hepatoma cell lines, even though there is still no evidence that CLDN1 binds HCV directly. In addition, human cell lines such as HeLa and HepH (CD81- and SR-BI-positive) remained HCV resistant when overexpressing CLDN1, suggesting that additional factors were required for successful HCV cell entry (Evans et al. 2007). Recent studies have provided evidence that OCLN, another transmembrane component of the tight junctions, plays an important role in HCV cell entry and initiation of a productive HCV infection (Liu et al. 2009). Targeting CLDN1 and OCLN by RNA interference demonstrated that reduction of the expression of both of these molecules inhibited HCV pseudoparticles and HCVcc cell entry (Liu et al., 2009). Furthermore OCLN interacts directly with E2 glycoprotein as demonstrated by co-immunoprecipitation and pulldown assays (Benedicto et al. 2008, Liu et al. 2009).

Of the four entry factors identified so far, CD81 and OCLN determine the species specificity of HCV entry between mouse and human, as mouse CLDN-1 and SR-B1 worked similarly as the human counterparts (Ploss et al. 2009).

Very little is known about how these factors co-ordinate to facilitate the actual viral entry process. A current model predicts a multistep process that includes attachment and receptor binding, post-binding association with tight-junction proteins and then internalization by endocytosis, which is followed by a pH-dependent step that results in the fusion of membranes and the release of viral RNA into the cytoplasm of the host cells (**Fig 3**) (Ploss et al. 2009).

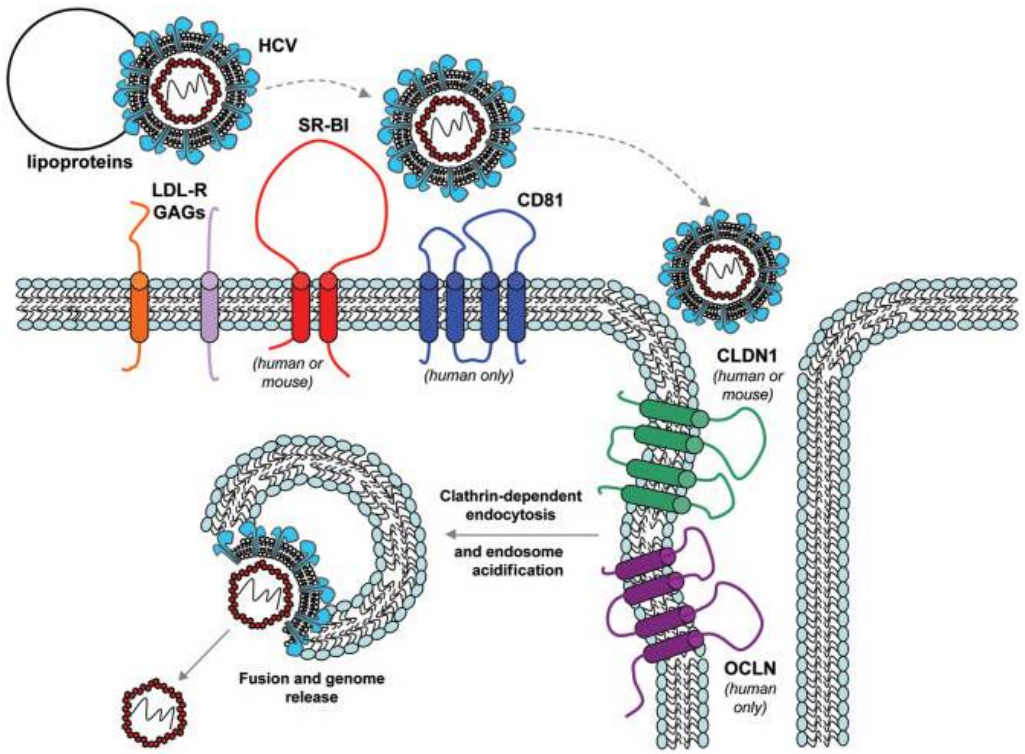


Fig 3 - Current model of HCV entry into host cells (Ploss et al. 2009). In the blood, virions are complexed to lipoproteins. These complexes are thought to interact with a number of hepatocyte cell surface proteins, including LDL-R, glycosaminoglycans, SR-BI, CD81, and, most likely at later stages, the tight junction proteins CLDN1 and OCLN. CD81 and OCLN represent the minimal human-specific entry factors. Although both mouse and human variants of SR-BI and CLDN1 can mediate HCV entry in the cell culture model system, human variants of CD81 and OCLN are mandatory for HCV entry. After receptor engagement, the virion is internalized via clathrin-dependent endocytosis. The early endosome is then acidified, and this leads to fusion of the viral envelope with the endosomal membrane and release of the viral genome into the cytoplasm.

1.1.4 Development of *in vivo* small models for HCV infection studies

The natural species tropism of HCV is limited to humans and chimpanzees. Due to the limited availability of chimpanzees, high costs and ethical aspects, the establishment of a small-animal model for the study of HCV infection has a high priority. Rodents are certainly the most appropriate model for all biological studies. Their short gestation period (around 20 days for mice and rats), their small size and their low cost are particularly advantageous. A mouse model with exogenously introduced HCV susceptibility traits could facilitate *in vivo* studies of HCV infection. Two alternative approaches can be proposed to achieve this: the virus could be adapted to infect non-human cells, or rodent tissues could be humanized (**Fig 4**). The latter might be achieved either by xenotransplantation of human

tissues, or by genetic manipulation to express or ablate key genes (Ploss and Rice 2009).

To produce a virus that efficiently enters mouse cells, HCV glycoproteins could be adapted *in vitro* to use mouse orthologous of human essential entry factors. Although mouse SR-BI and CLDN1 can mediate efficient HCV pseudoparticles uptake, CD81 and OCLN must be of human origin to render mouse cells permissive to HCV infection (Ploss et al. 2009). The HCV glycoproteins have remarkable plasticity, as shown by the continuous escape of the virus from neutralizing antibodies along chronic infection (von Hahnt et al. 2007). Three mutations in E1 and E2 were selected after the serial passage of HCV on human cells that express only mouse CD81 and these changes enhanced mouse CD81-dependent uptake to levels comparable with infection using the human orthologue CD81 (Bitzegeio and Pietschmann, 2008). A similar approach could be envisioned for the adaptation of HCV to entry through mouse OCLN. However the adaptive mutations required for the use of mouse CD81 might not be compatible with the changes needed to allow the engagement of mouse OCLN.

Adapting the murine environment to support the replication of HCV is an alternative approach that has already met with success. Chimeric mice that harbor HCV-permissive tissue can be obtained by transplanting human hepatocytes into mouse recipients with liver injury and severe immunodeficiency (Meuleman and Leroux-Roels 2008). The most commonly used recipient strain is a transgenic mouse overexpressing urokinase-type plasminogen activator. This enzyme overexpression is hepatotoxic and results in homozygous mice with severe liver damage (Heckel et al. 1990), which can be rescued by transplanting non-transgenic (human) hepatocytes. These chimeric-liver mice are susceptible to human hepatotropic pathogens, including HCV. The inoculation of HCVcc or sera from HCV-positive patients into these mice leads to a rapid increase in viraemia (Lindenbach et al. 2006). Unfortunately, chimeric-liver mice can be produced only in small numbers, and their use is limited by logistical constraints and substantial variability. Pathogenesis and immunity studies are also limited in liver chimeric mice, as the animals lack a functional immune system.

An inbred mouse model with inheritable susceptibility to HCV would overcome the technical difficulties of the xenotransplantation model. After the discovery of CD81 as an essential HCV entry factor (Pileri et al. 1998), transgenic mice expressing the human protein in a wide variety of tissues were produced (Masciopinto et al. 2002). However, human CD81-transgenic mice were resistant to HCV infection. This led to the conclusion that the expression of human CD81 alone is insufficient to confer susceptibility to HCV infection in mice. Enthusiasm for creating an inbred mouse model for HCV has recently been rekindled by the identification of OCLN as the second human factor that is essential to overcome the cross-species barrier at the level of entry (Ploss et al. 2009). However, to accurately reproduce the complex process of HCV entry *in vivo*, it will be important to achieve native expression patterns of the

human HCV entry factor orthologues. Advances in mouse genetics, including bacterial artificial chromosome transgenics and knock-in approaches, will undoubtedly be crucial in achieving this. Such a model would allow HCV-glycoprotein-mediated entry in an inbred mouse strain, and would be an invaluable tool for analyzing HCV entry *in vivo* and for preclinical testing of new intervention strategies.

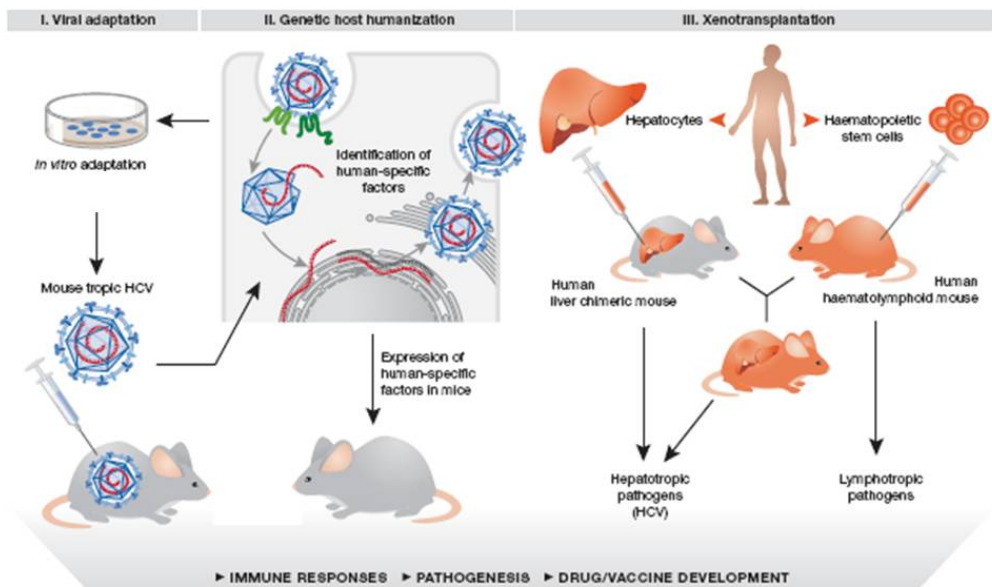


Fig 4 - Strategies to create mouse models for HCV (Ploss and Rice 2009). Viral adaptation (I), genetic host humanization (II) and humanization by xenotransplantation (III) are the proposed approaches to create mouse models for HCV infection.

1.2 GENETICALLY ENGINEERED MICE AS MODEL SYSTEM FOR BIOMEDICAL RESEARCH

Experimental models are required to understand human biology in both its normal and pathological aspects. Clinical studies certainly have many intrinsic limits: inducing pathology is unacceptable, patients tissue samples are not always available, the embryonic life is not very accessible. *In vitro* and cell culture systems are a very important tool, but they do not allow us to study physiological systems in their entirety.

The mouse is an excellent experimental model for defining human biology because of its anatomic, physiologic, and genetic similarity to humans. The recent genomic sequencing efforts suggest that we share 99% of our genes with the mouse. Furthermore the mouse is small, has a short gestation period (around 20 days), can be inbred and maintained cost-effectively. The mouse is also a popular model because its genome can be readily manipulated by molecular biology techniques. For example, mouse geneticists can eliminate or overexpress genes in the whole animal or in a specific tissue, introduce large regions of DNA into the genome, and engineer whole chromosomes. Furthermore, inbred strains of mice also provide the opportunity to study a disease trait in a defined genetic background. In the last few years, a number of significant technological advances have dramatically increased our ability to create mouse models of human diseases. These technological advances have been greatly aided thanks to the of mouse genome sequencing and the subsequent mouse genomic resources that have been developed (Palgen 2003, van der Weiden et al. 2002).

Viral or mammalian DNA injected into mouse oocytes could be incorporated into the genome. Since the first report of transgenic mice generated by injecting DNA into the pronucleus of one-cell mouse embryos, this technique has been immensely useful in creating model organisms for research purposes (Gordon et al. 1980). Normally, the transgenic construct consists of a selected enhancer and/or promoter, which may also direct gene expression to a specific tissue or developmental stage, linked to the sequence to be expressed. Using this approach, one may directly test in the mouse the role of selected gene products, dominant negative mutants, or specifically designed proteins. In theory, once integrated into the murine genome, the injected DNA can manifest its function. However, as the insertion occurs randomly, positional variegation effects may be considered, and both the function of endogenous genes might be affected by the insertion of a transgene as well as the expression of the transgene itself may also be severely compromised by surrounding elements.

In 1981, two laboratories reported the isolation of cell lines with the properties of pluripotent embryonic cells from the culture of early mouse embryos (Martin 1981, Evans and Kaufman 1981). These cells, called embryonic stem (ES) cells, after being injected into a young embryo were capable of colonizing all its tissues including the germ line, thus giving birth to

chimeras. The ES cell genotype could then be transmitted to future generations. It was demonstrated that these cells could be genetically modified *in vitro*, introducing of a transgene and that the corresponding transgenic mice could be obtained (Gossler et al. 1986, Robertson et al. 1986). Thus a totally new method for obtaining transgenic mice was born, considerably widening the possibility of ‘‘classical’’ transgenesis by microinjection of DNA in the zygote. Indeed, it became possible for the investigator to use procedures selecting rare genetic modifications in the ES cells and to obtain the corresponding mutant mice.

Important research advances have been made using mice as a model for the study of various biological systems. However, mice are not humans, and there is a growing need for animal models to carry out *in vivo* studies of human cells, tissues and organs. Humanized mice have been developed and are now an important research tool for the *in vivo* study of human cells and tissues. Humanized mice are defined as immunodeficient mice engrafted with haematopoietic cells or tissues, or mice that transgenically express human genes. The development of humanized mice is an opportunity to advance in our understanding of human biological processes, providing new *in vivo* models to study human haematopoiesis, immunity system, infectious diseases and cancer biology (Shultz et al. 2007). For example, humanized mice by transgenic approach are produced for immunological research. A functional human immune system was established by the expression of human genes, such as HLA molecules or immunoglobulins, in immunocompetent mice. HLA transgenic mice are used to identify antigens presented to T cells by HLA molecules, whereas human immunoglobulin-transgenic mice are used mainly to generate human monoclonal antibodies, such as the epidermal growth factor receptor (EGFR)-specific antibody which have being developed for therapeutic applications (Bleeker et al. 2004).

1.2.1 Manipulating mouse genome by gene targeting

Gene targeting is a process by which desired changes are introduced into the nucleotide sequence of a chosen gene (Capecchi 1989). A crucial advantage of gene targeting approaches for introducing genome modifications into mice is that the genetic locus to modify can be chosen. Furthermore, the genetic modification can be precisely designed to address specific biological questions. Such modifications could include the creation of null mutations, the introduction of reporter genes to follow gene expression and manipulation to restrict the effects of a mutation to any desired organs or to any chosen temporal period during the mouse life (Capecchi 2005).

Mammalian cells have the enzymatic machinery to appropriately recombine homologous DNA exactly with their counterparts on the chromosome, even if this is relatively rare compared with the random

integration of the same DNA (Smithies et al. 1985, Wong and Capecchi 1986). Gene targeting is normally carried out in murine ES cells as these cells have high rates of homologous recombination and are able to contribute to the germline when re-introduced into mouse embryos at the morula or blastocyst stage of development (van der Weyden et al. 2002, Bradley 1991, Bradley 1993).

A gene targeting vector will usually consist of a core region containing the desired genetic modification together with a positive marker that allows the selection of ES clones that have incorporated the exogenous DNA into their genome (conferring resistance to antibiotics). This core region is flanked by two homology arms which are absolute identical to the genomic regions immediately upstream and downstream of the target site. These arms are required for homologous recombination to take place, thus introducing the required modification into the genome. Homologous recombination in ES cells occurs much less frequently than random integration of DNA. To overcome this limit, improved strategies are needed to enrich in recombined ES cell clones and distinguish them from those where random integrations have occurred. These enrichment strategies usually take advantage of positive and negative selection markers. The most commonly used positive marker for the selection of vector incorporating ES clones is the neomycin resistance cassette. To discriminate homologous recombination from random integrations of the targeting vector, negative selection is used. A negative marker, such as the herpes simplex virus type 1 thymidine kinase gene or the diphtheria toxin α-chain coding region, is placed outside the homology region of the targeting vector. ES cell clones correctly recombined will not contain this marker, while most randomly integrated targeting constructs still retain the negative selection marker, thus permitting acounter selection against randomly integrated clones.

The targeting construct is electroporated into murine ES cells. Positive and negative selections can be used to enrich for ES cell lines that contain the desired modified gene. The surviving ES cell colonies are then isolated and examined for the presence of the targeted allele (to ensure that the desired recombination event has occurred) by PCR amplification and Southern blot analysis. A correctly targeted ES clone is then injected into a recipient pre-implantation mouse embryo, a blastocyst. These embryos are then surgically transferred to a recipient pseudopregnant foster mother to allow the embryos to come to term. The resulting pups are then examined for their degree of chimerism (percentage of the genetic contribution by the injected ES clone). Male mice showing a high percentage of chimerism are then mated with wild type mice to check for germ line transmission of the targeted allele in the offspring (**Fig 5**).

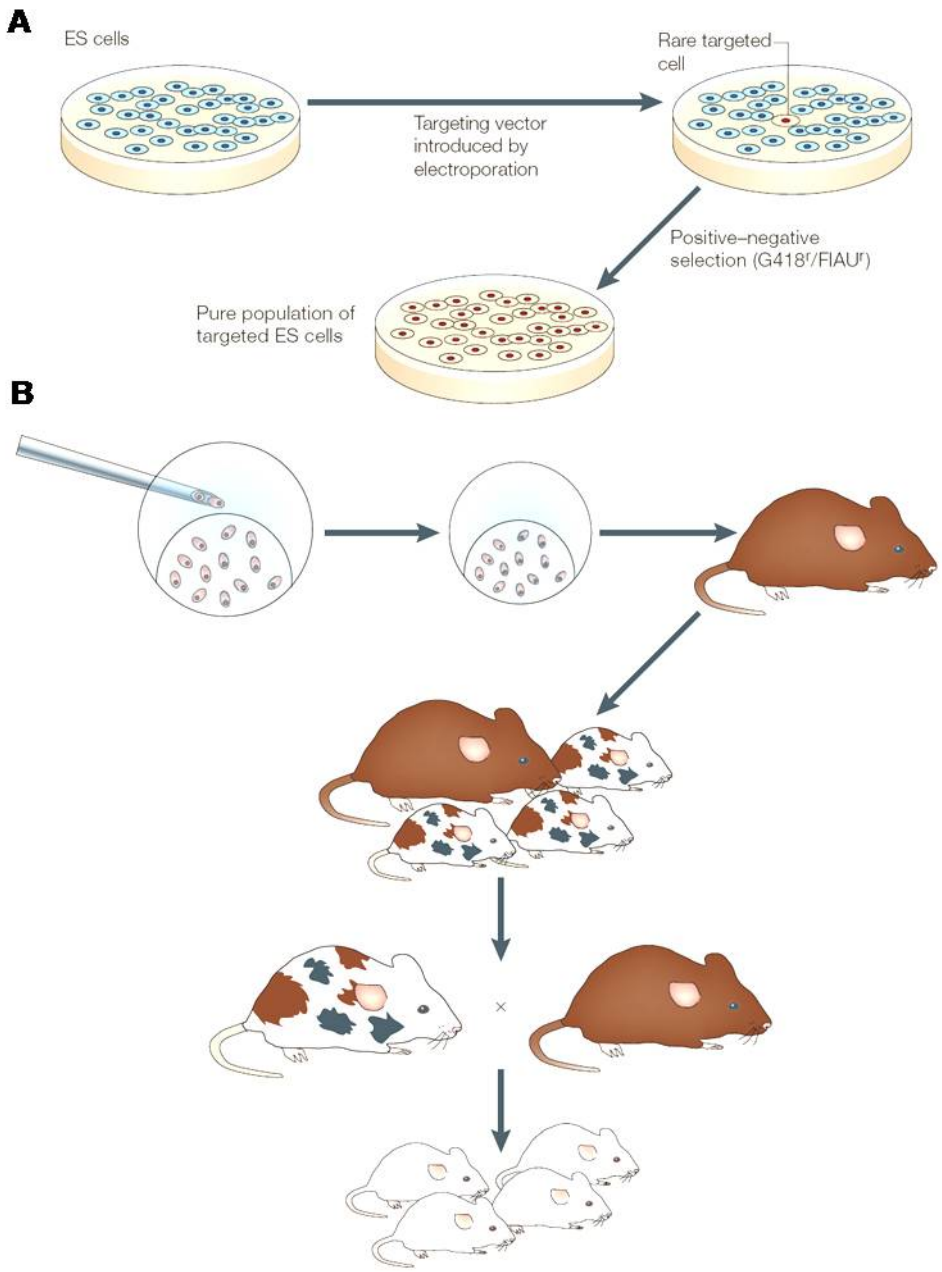


Fig 5 – Gene targeting in mouse (Capecchi 2005). Panel A: Isolation of ES cell clones that contain the desired targeted genomic modification. Panel B: Selected ES cells are used to generate chimeric mice that are able to transmit the genomic modification to their offspring. This is accomplished by injecting targeted ES cells into a recipient blastocyst. These embryos are then transferred to a recipient pseudopregnant foster mother to allow the embryos to come to term. The extent of the contribution of ES cells to the formation of the chimeric mouse can be evaluated by visual assessment of coat-colour chimerism. ES cell contribution to the germ line can be evaluated by observing the coat colour of the progeny that is derived by mating the chimeric mouse with black mice.

Gene targeting is most commonly used to create mice bearing null mutations in any cloned gene (knockout mice) (**Fig 6**). A knockout experimental strategy consists of disrupting a chosen gene in the mouse by replacing the coding exons of the gene with a selectable marker gene. Gene targeting can also be used to introduce a gene of interest in a specific locus of the murine genome (knock-in mice). Following homologous recombination with the chosen target locus, the modified allele expresses the inserted gene in replacement of the endogenous one. To study a specific mutation in a gene, knock-in approaches were developed to exchange a mutated DNA sequence for the endogenous one without gene disruption. Some knock-in strategies rely on site-specific DNA recombination system using the Cre recombinase from bacteriophage P1, or the Flp recombinase from *S. cerevisiae*. Both Cre and Flp cleave DNA at a distinct target sequence and ligate it to the cleaved DNA of a second identical site, to generate a contiguous strand. Including Cre or Flip recombination sites (respectively loxP or FRT) in the targeting vector, it is possible to replace a gene sequence with a sequence of the investigator's choice and to delete unnecessary sequences. Furthermore the genes for Cre or Flip recombinases can be insert into targeted loci in a way that brings their expression under the control of the endogenous gene promoter, thus allowing tissue-specific or temporal specific expression of the enzymes and hence recombination of loxP or FRT sites that flank the gene of interest.

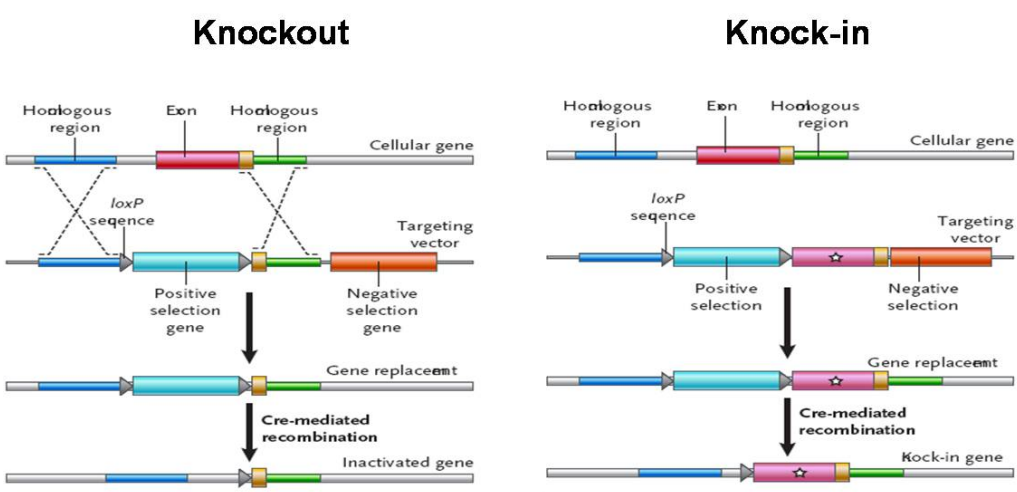


Fig 6 – Knockout and knock-in strategies. Targeting vectors contain positive and negative selection genes and two arms of homology with the genomic regions upstream and downstream the target site. The positive selection gene is flanked by two loxP sites and it can be removed by Cre-mediated recombination. Knockout strategy allows the deletion of targeted exon, whereas an exogenous gene is introduced in the targeted locus by knock-in approach.

1.2.2 *Recombineering* to construct targeting vectors

A limitation of gene targeting to modify the murine genome is that it requires the production of complicated molecular constructs. Traditional genetic engineering technology uses restriction endonucleases and DNA ligase to cut and rejoin DNA fragments. Traditional cloning methods break down when large DNA molecules have to be manipulated. Indeed, even rare restriction sites occur frequently in DNA sequences containing hundreds of kilobases, such as bacterial artificial chromosomes (BACs) or P1 artificial chromosomes (PACs), making the finding of unique sites almost impossible. Furthermore, the *in vitro* manipulation of large linear DNAs of this length is extremely difficult.

The development of phage-based homologous recombination systems in the last years (Murphy 1998, Muyrers et al. 1999, Yu et al. 2000) has greatly simplified the generation of targeting constructs, making it possible to engineer large segments of genomic DNA, such as those carried on BACs or PACs that replicate at low-copy number in *E. coli*. Using phage recombination to carry out genetic engineering has been called "recombinogenic engineering" or "recombineering" (Muyrers et al. 2001). Recombineering offers exciting new opportunities for creating inbred mouse models by gene targeting in ES cells.

To perform recombineering, a bacterial strain expressing a bacteriophage recombination system is required. The phage enzymes can be expressed from either their own promoter or from a heterologous regulated promoter. Expressing the genes from their endogenous phage promoter confers the advantage of tight regulation and coordinate expression, which results in higher recombination frequencies. This is an important advantage, since in many cases high recombination frequencies will be essential to obtain a desired recombinant. After induction of the recombination functions, the modifying DNA, either a double-stranded PCR product or a synthetic single-stranded oligonucleotide, is introduced into the phage enzymes expressing strain by electroporation. Recombinants are obtained either by selection or screening of the population of cells surviving electroporation.

In λ-mediated recombineering target DNA molecules are precisely altered by homologous recombination in strains of *E.coli* expressing phage-derived protein pairs, either RecE/RecT from the Rac prophage, or Redα/Redβ from λ phage (Muyrers et al. 1999). These protein pairs are functionally and operationally equivalent. RecE and Redα are 5′→3′ exonucleases, and RecT and Redβ are DNA annealing proteins. A functional interaction between RecE and RecT, or between Redα and Redβ is also required in order to catalyse the homologous recombination reaction (**Fig 7**).

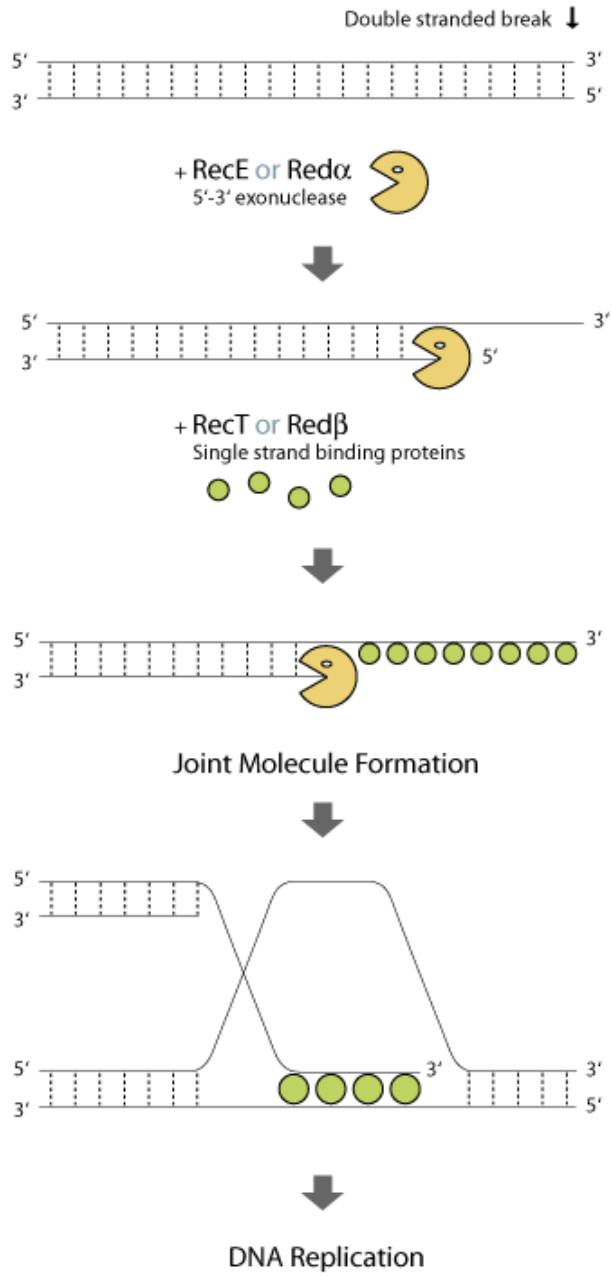


Fig 7 - Mechanism of Red/ET Recombination. Bacteriophage λ contains a homologous recombination system termed Red, which requires two proteins: Red-α (RecE from Rac prophage) and Red-β (RecT from Rac prophage). Red-α/RecE are 5'→3' exonucleases that act on linear double-strand DNA. Red-β/RecT bind to the 3' single-strand DNA overhangs created by Red-α/RecE and stimulate annealing to a complementary strand, promoting strand invasion and exchange.

Recombination occurs through homology regions, which are stretches of DNA shared by the two molecules that recombine. Since the sequence of the homology regions can be chosen freely, any position on a target molecule can be specifically altered. Homologous recombination allows the exchange of genetic information between two DNA molecules in a precise, specific and faithful manner, qualities that are optimal for DNA engineering regardless of its size.

Red/ET homologous recombination can efficiently modify chromosomal or plasmidic DNA *in vivo* by recombination with linear DNA electroporated into a cell. The most important aspects of recombineering are that only short homology segments are required to direct the recombination. These homologous recombination systems enable large fragments of genomic DNA to be modified or subcloned in a way that was unimaginable only a few years ago. The high frequency of recombination offered by these plasmid and phage systems allows BAC DNA to be manipulated without drug selection. These recombination systems allow to construct complicated targeting vectors and generate knock-in and knockout mice (Angrand et al. 1999, Lee 2001).

2. AIMS OF THE STUDY

The limited species tropism of HCV to human and higher primates causes the lack of suitable animal models to study hepatitis C. Chimpanzees are the only established *in vivo* experimental system, but their use is limited by restricted availability, expense and ethical aspects. The recent identification of the minimal human factors that are essential for viral uptake together with the advances in mouse genome engineering has laid the foundations for creating an inbred mouse model for HCV.

The aim of our study is the development of a novel mouse strain humanized for the expression of human HCV receptors. Initially we directed our attention to the two first entry cellular factors identified, CD81 and SR-BI. These human transmembrane receptors are involved in HCV entry and their differences than the murine orthologues contribute to render the mouse cells not permissive to the HCV infection (Bartosch B et al. 2003b). We designed a gene targeting strategy to produce humanized mice introducing human CD81 and SR-BI genes in the murine genome, to achieve native expression patterns of their orthologues.

Human occludin is the final essential HCV entry factor recently identified by Ploss and colleagues (2009). Their results provide strong evidence that the expression of human CD81 and occludin in the context of mouse claudin-1 and SR-BI can be sufficient to overcome the mouse resistance to HCV entry. Therefore we added occludin to the chosen human receptors for producing novel knock-in mouse strains.

Our humanized mouse models could allow HCV glycoproteins mediated entry and could be an important tool for *in vivo* studying of virus-host interactions.

3. MATERIALS AND METHODS

3.1 Targeting vectors

Targeting vectors to insert human CD81, SR-BI and OCLN genes in the murine genome were constructed using both traditional genetic engineering and Red/ET recombineering technologies. For each gene, human coding sequence (hCDS) was inserted in a modified pL451 plasmid (NCI- Frederick), that contains SV40 polyadenilation signal (pA) followed by neomycin resistance cassette (Neo^R, constituted by FRT - PGK promoter - EM7 promoter - neomycin resistance gene – bGH pA - FRT) (pL451-pA); the hCDS was specifically digested and ligated upstream SV40 pA. The human gene cassette (hBOX, constituted by CDS – SV40 pA – Neo^R) was amplified by high-fidelity PCR (AccuPrime *Pfx* – Invitrogen) using specific primers designed with 50 bps homology arms to their 5'. These arms mediated homologous recombination in Red/ET expressing *E. Coli* strain that allowed the insertion of hBOX in a specific site of the BAC including murine orthologous gene. The region of the recombined BAC constituted by the hBOX flanked by two asymmetric murine genomic arms was subcloned in pBluescript II KS+ (pBS, Agilent Technologies) by Red/ET recombineering: pBS was amplified by PCR using primers designed with short segments homologous to the upstream and downstream ends of interesting BAC region, and introduced in recombined BAC containing bacteria by electroporation. Red/ET recombination led to the retrieving of the BAC region of interest in pBS and the targeting vector was obtained (**Fig 8**). Plasmids and primers used to construct the three targeting vectors are indicated in table 1 (**Tab 1**). All PCR primers used in our study were designed using Vector NTI software (Invitrogen). Every cloning step including PCR amplification was verified for nucleotide mismatches by direct sequencing analysis of plasmids (Stazione Zoologica A. Dohrn, Napoli).

3.2 Gene targeting and generation of humanized knock-in mice

The targeting vectors were linearized with Eco RV (CD81 and OCLN vectors) and with Pvu I (SR-BI vector), and introduced in murine ES cells by electroporation (MicroPorator MP-100 Digital Bio).

The ES cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 15% FBS (HyClone), 2 mM L-Glutamine (Gibco), 1X NEAA (Gibco), 50 μg/ml Penicillin/Streptomycin (Gibco), 0.1 mM β-mercaptoethanol (Sigma), 1000 U7ml LIF (Chemicon), 1 mM Sodium Pyruvate and 400 μg/ml, Geneticin (G418, Gibco).

ES cells that survived G418 selection were screened for correct targeting by PCR (**Tab 2**) and Southern blotting as described in section 4. The correctly targeted ES cell clones were electroporated with a FLPe-expressing plasmid (pCAGGS-FLPe) to delete the Neo^R by recombination between the FRT sites.

Correctly targeted ES clones, with Neo^R or with Neo^R removed, were micro-injected into C57BL/6 blastocysts to generate chimeric mice. Chimeric male mice were bred with C57BL/6 females and tested for germ line transmission. The germ line transmitted F1 mice were further inter-crossed to generate mice used in this study. Mice with Neo^R were further crossed with transgenic mice expressing FLPe recombinase protein in both somatic and germ cells under the control of human ACTB promoter (Rodriguez et al. 2000).

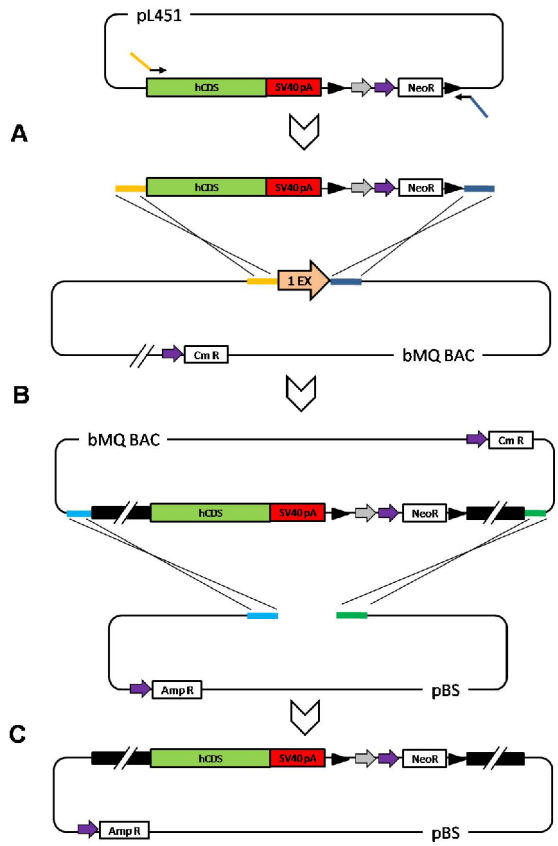


Fig 8 – Targeting vector construction by recombineering strategy. Panel A: hCDS (green bar) was inserted by traditional cloning in pL451-pA plasmid, upstream SV40pA (red bar) and neomycin cassette (closed triangles indicate FRT sites, grey arrow PGK promoter, purple arrow prokaryotic EM7 promoter). hBOX was amplified by PCR with primers with 5' short homology arms (yellow and blue bars). Panel B: hCDS PCR product was introduced in Red/ET bacteria containing a specific bMQ BAC, and inserted in place of the murine exon sequence in the BAC by homologous recombination between short arms. Neomycin resistant bacteria colonies were selected and screened for correct recombination. Panel C: Recombined BAC region including hBOX flanked by two asymmetric homology arms (black bars) was subcloned in pBS: primers with 5' short homology arms (light blue and green bars) were used to amplify pBS, and the PCR product was introduced in Red/ET bacteria containing recombinant BAC. Red/ET homologous recombination mediated the incorporation of BAC region in pBS. Only the plasmid correctly recombined conferred ampicillin resistance to the bacteria.

GENE	cDNA plasmid	bMQ BAC	PCR PRIMERS		
			step	*name*	*sequence 5'-3'*
CD81	clone # 839D11134D (RZPD)	clone # 164G9 (geneservice)	cloning in pL451-pA	C-XhoI-1	CTCGAG-CAGACCACCAACCTCCTGTATCTGGA
				C-HindIII-2	AAGCTT-TGGGTCCTAGTACACGGAGCTGTTCC
			cloning in in bMQ BAC (recombineering)	C-50bps-3	GGCTGGAGGCGTGATCCTAGGTGTAGCTCTGTGGTTGCGTCATGATCCA-CAGACCACCAACCTCCTGTATCTGG
				C-50bps-4	TGGGTGCCGGTTTGTTTCCCAGTTCCAGGTACAGCAGGCTGGTGGTCTGT-GGCCGCTCTAGAACTAGTGGATCCA
			subcloning in pBS (recombineering)	C-50bps-5	GCCTGGGAGGGAGGCGCTGCCAGTATCAGAAGCTGGGGAAAGGCAGCA CA-TACGACTCACTATAGGGCGAATTGG
				C-50bps-6	CTGCCACACTCCACAGTATGTGTTTGTCACACTCCACAAGCCCTTGCAAC-GGTGATGGTTCACGTAGTGGGCCA
SR-BI	clone # 8327505 (geneservice)	clone # 186k05 (geneservice)	cloning in pL451-pA	S-SalI-1	GTCGAC-ACATGGGCTGCTCCGCCAAA
				S-HindIII-2	AAGCTT-TCACGGTGTCCTCAGGACCCTA
			cloning in in bMQ BAC (recombineering)	S-50bps-3	CCGTCTCCTTCAGGTCCTGAGCCCCGAGAGCCCCTTCCGCGCACGCGGAC-ATGGGCTGCTCCGCCAAAGC
				S-50bps-4	CCCTGCGTCCCGATCCCCAACCCCCATCTCCCCAAGACACTTCACTCAC-GGCGGCCGCTCTAGAACTAGTGGA
			subcloning in pBS (recombineering)	S-50bps-5	GTCCGCGTGCGCGGAAGGGGCTCTCGGGGCTCAGGACCTGAAGGAGAC GG-GGTGATGGTTCACGTAGTGGGCCA
				S-50bps-6	TCCTCACAGCCCAGGTTCCTCTGAACCTGCTCCCACTCTGTGCCCCTGAT-GGTGATGGTTCACGTAGTGGGCCA
OCLN	clone # 5179203 (geneservice)	clone # Q385N11 (geneservice)	cloning in pL451-pA	O-XhoI-1	CTCGAG-CTGACCATTGACAATCAGCCATGTC
				O-HindIII-2	AAGCTT-CAAACAACTTGGCATCAGCCTTCT
			cloning in in bMQ BAC (recombineering)	O-50bps-3	GCCTGGACATTTTGCTCATCATAAAGATTAGGTGACCAGTGACATCAGCC-ATGTCATCCAGGCCTCTTGAAAGT
				O-50bps-4	GGGAAGGGTCTAGGACTCTGCTGATGAGCAACAGTCCTACCCTAGCTTAA -GCCAAGCTCGAAATTAACCCTCACT
			subcloning in pBS (recombineering)	O-50bps-5	AGATCCTACTATAGCATCAGTCTTTTGAGCTCCATAATTACAGGCACATG-TACGACTCACTATAGGGCGAATTGG
				O-50bps-6	CTTCATCTCATGCTGAAATACAAACATGATCAATTTAAACCCAAAGGAGG-GGTGATGGTTCACGTAGTGGGCCA

Tab 1 – Plasmids and primers used to construct CD81, SR-BI and OCLN targeting vectors.

Targeted ES cells screening - PCR PRIMERS		
gene	*name*	*sequence 5'-3'*
CD81	C-7	TGGCTCAGACAACTGCTTGGCA
	C-8	GCCCACATGACCAACAGCTATGTG
SR-BI	S-5'-7	TTATTGAGTGTGGGGTTACCATGG
	S-5'-8	GCGCACGTTCTTAAGGACCT
	S-3'-7	AAGGTGCCACTCCCACTGTCCTTT
	S-3'-8	TTCCAAACAGAGCGCACCTGTG
OCLN	O-5'-7	TGCGCCATGGTGACTGGTTT
	O-5'-8	CGGCAATGAAACAAAAGGCAGC
	O-3'-7	AAGGTGCCACTCCCACTGTCCTTT
	O-3'-8	GCCTCATACGTGATAAGCAAGCTTTC

Tab 2 – PCR primers used to screen CD81, SR-BI and OCLN targeted ES cell clones. Genomic DNA was isolated from 96-well plates ES clones (section 3.3), and PCR analysis was performed using Expand Long Template PCR System (Roche), according with the manufacturer's procedure.

3.3 Southern blotting

To analyse targeted ES cell clones by Southern blotting, genomic DNA was isolated from Es cell clones in 6-well plates by cell lysis (10 mM Tris pH 7.5, 10 mM EDTA pH 8, 10 mM NaCl, 0,5 % sarcosyl with 1.0 mg/ml Proteinase K; 60°C 14 h) and ethanol precipitation (75 µM NaCl, EtOH 98%).

ES cell clones genomic DNA was digested as described in section 4, and separated by 0.7% agarose gel electrophoresis. The probes were labelled with digoxigenin using PCR DIG Probe Synthesis Kit (Roche). Southern blotting was carried out with Hyond-N^+ nylon membrane (Amersham), using alkaline blotting protocol recommended by the supplier. Hybridization and detection were performed in accordance with the DIG system protocols (Roche).

3.4 PCR mice genotyping

Mice and 10.5 embryos described in our study were genotyped by PCR analysis of genomic DNA isolated respectively from tail biopsies and yolk sacs. Tail biopsies and yolk sacs were lysed (50 mM Tris pH 7.5, 100 mM EDTA pH 8, 100 mM NaCl, 1% SDS, 1.0 mg/ml Proteinase K; 55 °C 14 h) and DNA was precipitated (2 M NaCl, 50% Isopropanol). PCR genotyping was designed as described in section 4, using primers indicated in table 3 (**Tab 3**). To genotype preimplantation embryos, each embryo was collected in PCR tube in dry ice. They were heat shock lysed transferring the tubes from dry ice to room temperature. Nested PCR was performed on the whole embryo to determine its genotype.

Mice genotyping - PCR PRIMERS		
gene	*name*	*sequence 5'-3'*
CD81	C-9	GTTACAACCAAGAGGAGCTGG
	C-10	GTGAAGAACGTCCCCAGCAG
	C-11	GAGGTTCACTAGAAATGCCCA
SR-BI	S-9	TAGACCAGCAACCACCAGGA
	S-10	GATGTAGTCGCTCTCCGAGC
	S-11	TGGTGAGGAGGTAGCTCCAC
OCLN	O-9	AAAGATAACTTGAGTGCCAGGG
	O-10	GCCACTTCCTCCATAAGGGT
	O-11	GCAACAGACCCCAGTAGAAAAG
	O-9'	CAAGATCTCATGCTAACCTCGA
	O-10'	GGCAATGAAACAAAAGGCAG
	O-11'	TGTTTCACCGTGTCTGGTTTAT

Tab 3 – PCR primers used to genotype CD81, SR-BI and OCLN humanized knock-in mice. (O9', O10', O11' are used to perform the first reactions in nested PCR to genotype preimplantation embryos).

3.5 Reverse Transcription (RT)-PCR and real time PCR

Different mouse tissues (kidney, liver, lung, tail) was disrupted and homogenized by TissueLyser (Qiagen) and total RNA was isolated by Trizol reagent extraction (Invitrogen). First strand cDNA synthesis was perfomed using M-MuLV Reverse Transcriptase RNase H^- and oligo dT primers (Finnzymes), according with the manufacturer's procedures.
RT-PCR and real time PCR primers were designed to amplify specific regions of wild type and humanized transcripts (**Tab 4**), as described in section 4. RNA samples without the addition of reverse transcriptase were also included as negative PCR controls.

RT-PCR was performed using high-fidelity Taq polimerase (Phusion, Finnzymes) and purified PCR products (Qiaex II PCR purification Kit, Qiagen) were analysed by direct sequencing (Stazione Zoologica A Dohrn, Napoli).

The amplification efficiency of primer pairs used for real time PCR was preliminary calculated by cDNA standard dilutions reactions. Real time PCR was performed using Power SYBR Green PCR reagents and ABI Prism 7900 Real Time PCR System (Applied Biosystems). Threshold cycle values (Ct) were determined and analyzed by Applied Biosystems software. To calculate mRNA levels of specific genes Ct values were corrected for primers efficiency and normalized for the housekeeping GAPDH, according to the Pfaffl's model (Pfaffl 2001).

RT-PCR and real time PCR PRIMERS				
gene	*analysis*	*genotype*	*name*	*sequence 5'-3'*
CD81	RT-PCR	*wild type*	C-12	CTTCGTCTTCAATTTCGTCTTCTGGC
			C -13	CTACAAAGCCTCTGGGCAAG
		humanized	C-14	CTTCGTCTTCAATTTCGTCTTCTGGC
			C-15	TAGTAACGGCCGCCAGTGTG
SR-BI	RT-PCR	*wild type*	S-12	AGATGTGGGCACCCTTCATGACACC
			S-13	AAATAAGGACCCTGAGGGGCCTGCC
		humanized	S-14	TTCATGACTCCTGAGTCCTCGC
			S-15	TGAGTTTGGACAAACCACAAC
OCLN	RT-PCR	*wild type*	O-12	TTTCCTTAGGCGACAGCGGT
			O-13	TGGTGGGGAACGTGGCCGAT
		humanized	O-14	TTTCCTTAGGCGACAGCGGT
			O-15	GAACCCAGTTTCCTTATGTG
	real time PCR	*wild type*	O-16	CTGTGATGTGTGTTGAGCTTTG
			O-17	AAACCCTGTCTGGAAAACCA
		humanized	O-18	AAACTTTCACACCCCAGACG
			O-19	GAACCCAGTTTCCTTATGTG
GAPDH	real time PCR	*wild type*	G-1	CATGGCCTTCCGTGTTCCTA
			G-2	CCTGCTTCACCACCTTCTTGAT

Tab 4 – RT-PCR and real time PCR primers.

3.6 Western blotting

Mouse protein extract for electrophoresis was generated lysing 1 mg of liver by TissueLyser (Qiagen) in lysis buffer (20 mM TrisHCl pH 8, 150 mM NaCl, 2 mM EDTA, 1% TRITON X 100, 1 mM DTT, 1mM PMSF, 1 mM Na_3VO_4, 1mM NaF, 2 mM AEBSF, 130 μM Bestatin, 14 μM E-64, 1 μM Leupeptin, 0.3 μM Aprotinin). Samples were run on 4-12% NuPAGE polyacrylamide gradient gels (Invitrogen) and blotted onto Immobilon-P PVDF membrane (Millipore) at 8V/cm 1 hour in 25 mM Tris-HCl:150 mM glycine buffer, pH 8.3 containing 10% methanol. Blots were blocked in PBS: 0.3% Tween 20: 5% non-fat milk, before incubating with rabbit polyclonal anti-human occludin antibody (Invitrogen #71-1500) and mouse monoclonal anti-β-actin (Sigma #A2228) 1 μg/ml in blocking buffer for 1 hour. Antibody binding was detected with HRP-labelled secondary antibody (Amersham) diluted in blocking buffer. Blots were developed using ECL chemiluminescence (Pierce).

3.7 Murine primary hepatocytes isolation

4 month old mice were anesthetized with Avertin 2.5 (Sigma) and the abdomen opened using Wagner scissors 10.5 cm in order to expose the bowel. The hepatic *vena porta* was isolated and cleaned from the surrounding fat tissue and then clumped with a stitch at the caudal end. A small incision made with 2.5 mm spring scissors allowed the insertion of a cateter, then secured to the vein with a second stitch. Perfusion was performed with a 50-60 ml of a pre-perfusion solution (0.9% NaCl, 0.05% KCl, 0.2% HEPES, 0.08 mg/ml EGTA) and subsequently with a 50-60 ml of a digestion solution (0,6% NaCl, 0.05% KCl, 1.2% HEPES, 0.07% $CaCl_2$ and Collagenase type IV from Sigma 0.32 mg/ml) at a flow rate of 5 ml/min. Digestion was stopped when the liver changed both colour and texture. After removing the gall bladder, the liver was minced with a razor blade and homogenized with a pipette device and subsequently filtered through a 70 μm strainer. The homogeneous suspension was then washed (600 rpm, 4'), resuspended in 3 ml of William's Complete Medium E (Gibco) and then loaded on a 37.5 % Percoll cushion to isolate only the viable cells from cell debris and other undesired digestion products (1050 rpm, 3'). Viable isolated hepatocytes were counted in a Burker chamber and then seeded in gelatine coated 6 wells plates at the desired concentration.

Total RNA was extracted from 48 hours cultured primary cells by Trizol reagent protocol (Invitrogen) and it was retro-transcribed using QuantiTect Reverse Transcription kit (Qiagen), according with the manufacturer's procedures. RT-PCR was performed to amplify two hepatic markers, albumin (5'CATACGATGAGCATGCCAAAT 3'; 5'GCACACAACTTATCTCCAAAAAGA 3') and α-fetoprotein (5'ACCTTTACCCAGTTTGTTCCG 3'; 5'ACAAATTTCATCCAGAAACACAGATA 3').

3.8 HCV pseudotypes infection assay

Primary hepatocytes isolated from our mice were plated on gelatine 0.2% (Sigma) coated 6-wells at $80x10^3$ cells/well and cultured in William's Supplemented Medium (William's Medium E, 4% inactivated FBS, 1% pen/strep, 1% Glutamax, EGF 50 ng/mL, Insulin 1 ug/mL, Transferrin 10 µg/mL, Hydrocortisone 1.3 ug/mL) 48 hours before infection. Hep3B and Hepa1.6 cell lines were seeded on 6-wells at $120x10^3$ cells/well and cultured on DMEM (10% FBS, 1% pen/strep, 1% Glutamax) 24 hours before infection.

293T cells were transfected using Calcium Phosphate with 12.5 µg of pCMV-E1E2 (from Granier C, Viral Envelopes and Retrovirus Engineering) and 12.5 µg of pNL4-3.Luc.R^-E^- (NIH AIDS Reaserch and Reference Reagent Program) and 12 hours after transfection fresh culture media was added. 48 hours and 72 hours after transfection viral surnatants from 5 transfections were collected. They were pooled and filtered through a 0,22 µm syringe driven filter units. To help viral attachment on cell surface, polybrene 4 ng/µl was added and then the viral surnatant was dispensed 3 ml/well on primary hepatocytes, Hep3B and Hepa1.6 cells. To further improve infection yeld the plates were then spinoculated at 1200 g for 90' at 37°C. 24 hours after the last round of infection, cells were collected and lysed; luciferase assay (Promega) was performed. Bradford assay (Biorad) allowed to normalize the luciferase values for the total protein amount of each sample.

4. RESULTS AND DISCUSSION

4.1 Generation of humanized CD81 knock-in mouse

The tetraspanin CD81 has been proposed as a first putative cellular receptor involved in hepatitis C virus (HCV) entry into the host cell. A soluble form of HCV glycoprotein E2 (sE2) binds to human cells and was used to identify CD81 as an HCV receptor molecule (Pileri et al. 1998). The large extracellular loop of human CD81 binds to sE2 and this binding appears to be species-specific, as sE2 does not bind mouse CD81 (Flint et al. 2006). Non-permissive human hepatoma cell lines, which do not express CD81, become susceptible to HCV pseudoparticle infection upon ectotopic expression of CD81 after transduction (Bartosch et al. 2003b, Cormier et al. 2004b, Lavillette et al. 2005, Zhang et al. 2004). The binding of sE2 to the liver tissue was confirmed in transgenic mice expressing human CD81. However the expression of human CD81 alone in transgenic mice is not sufficient to confer susceptibility to HCV infection (Masciopinto et al. 2002). There are at least two possible reasons for this failure: other human receptors are required, and the exogenous genes have to be regulated according to the native expression patterns of their orthologues. The knock-in approach allows to insert an exogenous gene in a desired specific site of the murine genome, therefore it is possible to substitute the murine genes with their human orthologues for the putative HCV receptors. We chose this strategy to produce novel mouse strains expressing human HCV receptors, including CD81.

CD81 human and murine genes are highly homologous: they are both constituted by 8 exons and they share the 87% of the coding sequence and the 92% of the amino acid sequence. We manipulated the murine genome so that CD81 gene was translated in the human protein. Using a gene targeting approach, we created a CD81 chimeric gene by inserting in the murine gene the human coding sequence (CDS) beginning at the position of the first codon which differs between the two species (AA43) (**Fig 9**).

We designed and constructed a molecular vector to modify CD81 murine gene by homologous recombination in mouse embryonic stem (ES) cells (**Fig 10**). This targeting vector was constituted by two asymmetric regions of genomic sequence, the homology arms, obtained from a 129S7/SvEv mouse bMQ BAC comprising the genomic locus of CD81. These homology arms flanked the partial CDS of human CD81, from the nucleotide 127 to the stop codon, followed by SV40 polyadenilation signal. The neomycin resistance gene, under the control of PGK promoter, was included by two FRT sites (Neo^R cassette) and inserted downstream the human CDS. The targeting vector was linearized and introduced into the murine R1/129/Sv ES cell line (Nagy et al. 1993) by electroporation. The recipient clones were then screened for correct targeting by PCR analysis (**Fig 11**). On 288 G418 selected clones we found 8 correctly recombined clones by PCR analysis. These clones were further analyzed by Southern blotting to verify the correct recombination and the absence of unspecific integrations of the exogenous DNA into their genome

(**Fig 12**). The correct targeting was confirmed by Southern blotting for every 8 clones, showing no random integration.

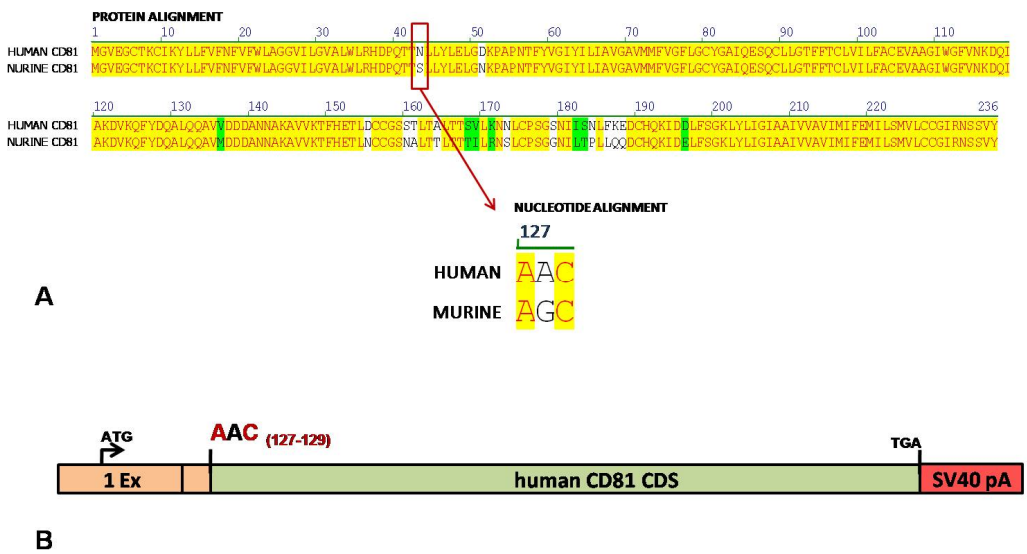


Fig 9 – CD81 human and murine proteins alignment and chimeric transcript. Panel A: the protein alignment between human and murine CD81 sequences is represented: yellow indicates identical amino acids, green similar amino acids, white different "non-similar" amino acids. The red box indicates the first amino acid substitution between human and murine sequences (N43S), corresponding to the missense nucleotide substitution A128G, as indicated by the arrow. Panel B: to humanize the mouse for CD81 protein, its human coding sequence (CDS) was inserted in the second exon of the murine orthologous gene (pink bar), exactly from the first missense substitution (A128G) to the stop codon (green bar), followed by SV40 polyadenilation signal (red bar).

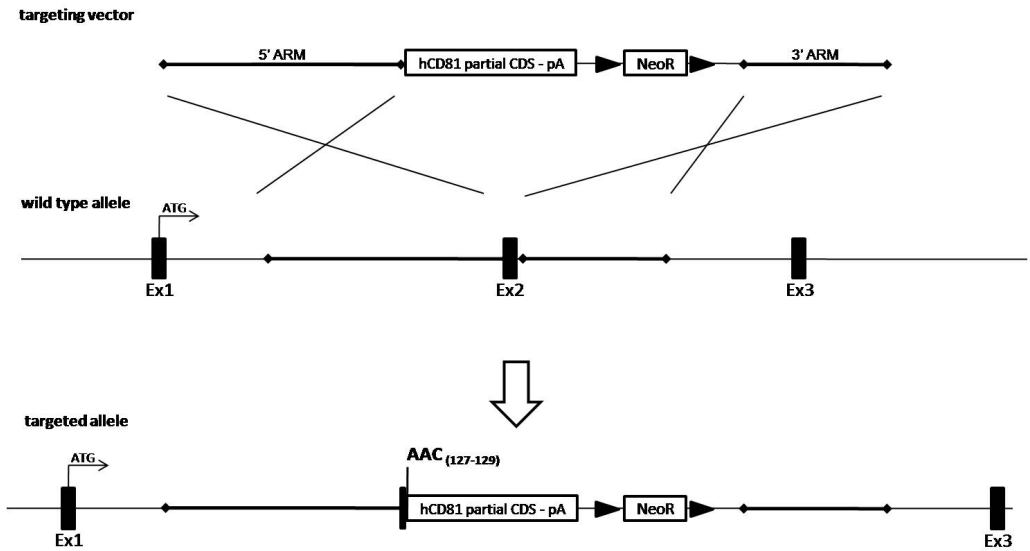


Fig 10 – Gene targeting strategy to humanize CD81 murine gene. A targeting vector to insert the human CDS of CD81 in the murine locus was constructed: two asymmetric homology regions (5' arm of 5 kb, and 3' arm of 3 kb) flank partial human CD81 CDS (from the nucleotide 127 to the stop codon) followed by SV40 polyadenilation signal (pA) and the neomycin resistance gene (NeoR) included by two FRT sites (closed triangles). These exogenous elements were inserted by homologous recombination in the murine locus, replacing the second exon, so that the human sequence was in frame with the murine one.

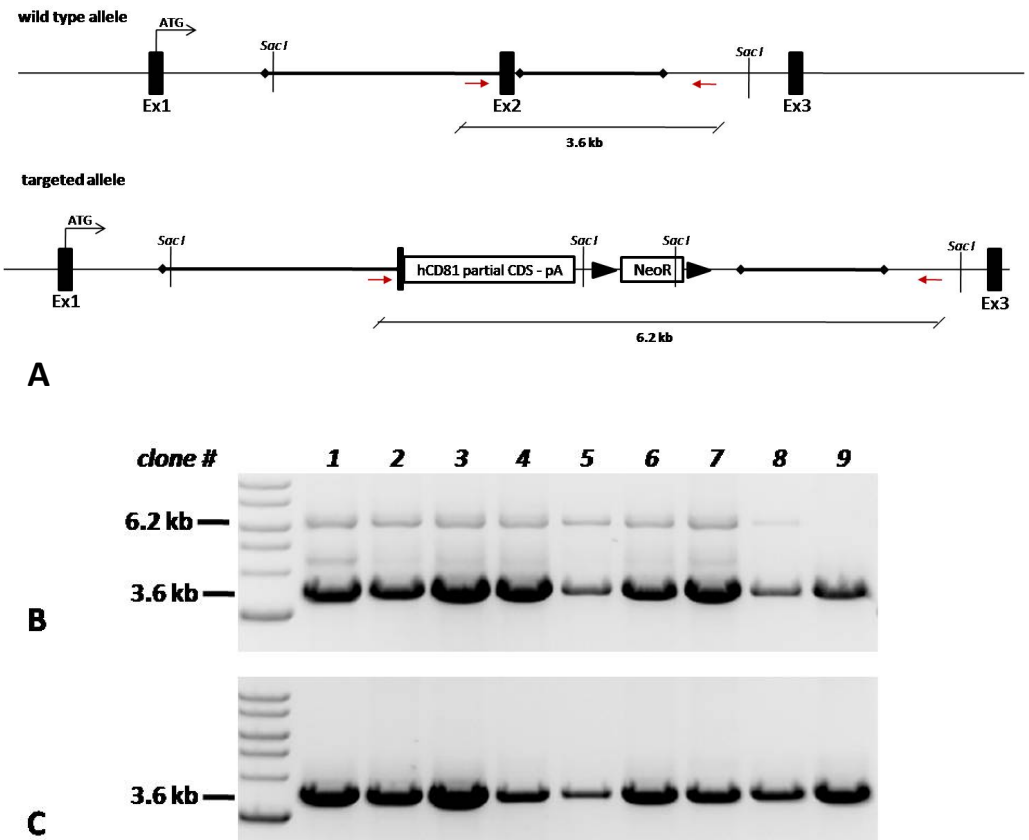
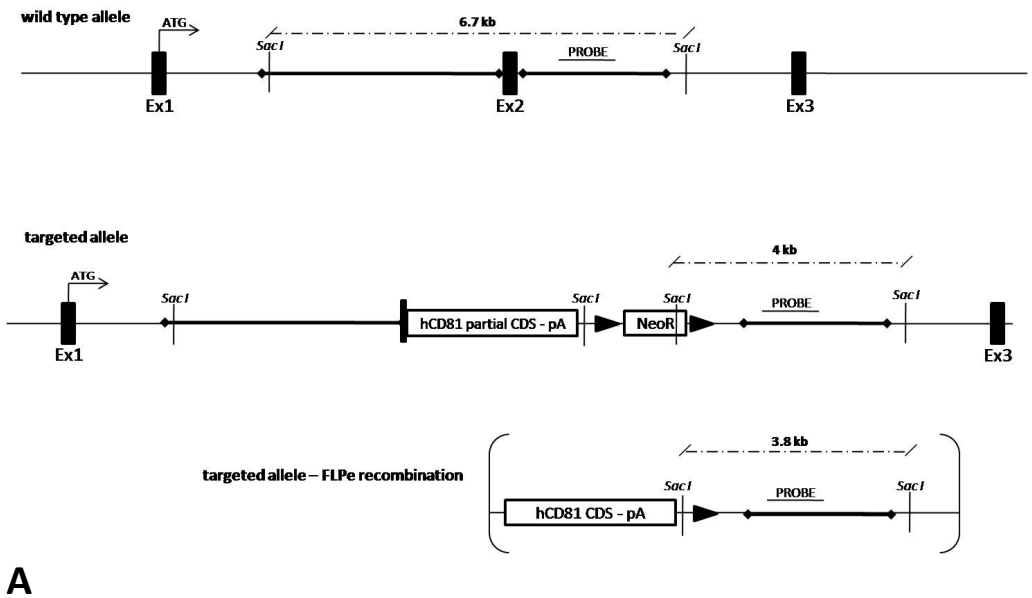


Fig 11 – CD81 targeted murine ES cell screening by PCR analysis. G418 resistant cell clones were screened for the correct targeting by PCR analysis. Panel A: CD81 wild type and recombinant loci are represented; the sense primer (red rightwards arrow) anneals on the genomic sequence within the 5' homology arm, the antisense primer (red leftwards arrow) anneals on the genomic sequence downstream the 3' arm. They amplify a 3.6 kb product on the wild type allele and a 6.2 kb product on the targeted allele. Panels B and C: PCR results are reported for nine clones. The clones 1-8 showed both the wild type and the recombinant products, so they were correctly targeted. The clone 9 had only the wild type product and it did not correctly recombine (B). ES clones genomic DNA was further digested with Sac I before PCR: Sac I cut outside the wild type amplified region and inside the recombinant amplified region, as shown in panel A. After this digestion the targeted PCR product disappeared, demonstrating its specificity (C).



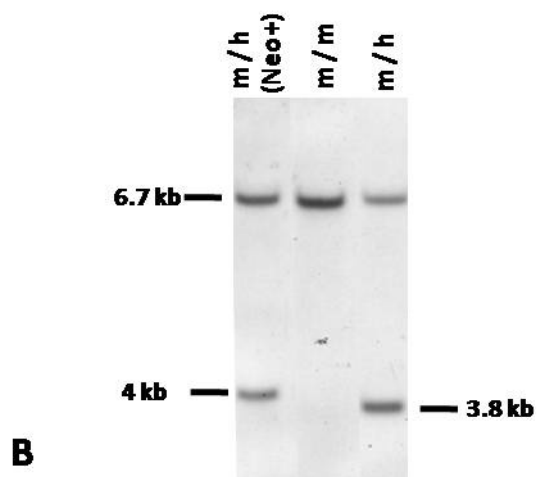


Fig 12 – Southern blot analysis of CD81 humanized ES cells. To verify the correct recombination and the absence of unspecific integrations, targeted ES clones were analyzed by Southern blotting. Panel A: CD81 wild type and recombinant loci are represented; oligonucleotide probe annealed on the 3' homology arm genomic sequence and after Sac I digestion it detected a wild type restriction product of 6.7 kb and a recombinant restriction product of 4 kb (or 3.8 kb when neomycin cassette was removed). Panel B: Southern blotting results are reported for a correctly targeted clone, before and after the FLPe treatment, m/h (Neo+) and m/h respectively. A wild type clone, m/m, was reported too.

One targeted ES clone was microinjected in C57BL/6 blastocyst to generate chimeric mice. These chimeras were mated with C57BL/6 wild type mice and their offspring derived entirely from 129/Sv injected cells, as indicated from their coat color (agouti). The genomic modification produced in the embryonic cells was successfully transmitted to the germ line.

The mice were genotyped for the specific knock-in allele performing a three primers PCR on genomic DNA extracted from their tails biopsies (**Fig 13**).

The produced knock-in mice still contained the Neo^R cassette, therefore they were later bred with transgenic mice expressing FLPe recombinase protein in both somatic and germ cells under the control of human ACTB promoter (Rodriguez et al. 2000) to remove the Neo^R cassette.

More than 60 mice were produced from the mate of the chimera with wild type mice and with FLPe transgenic mice, and about the half presented heterozygous genotype for CD81 locus, CD81 murine / CD81 human (CD81 m/h), according to the Mendelian ratio. Heterozygous CD81 m/h mice were viable, with normal growth and exhibited no overt gross phenotypes.

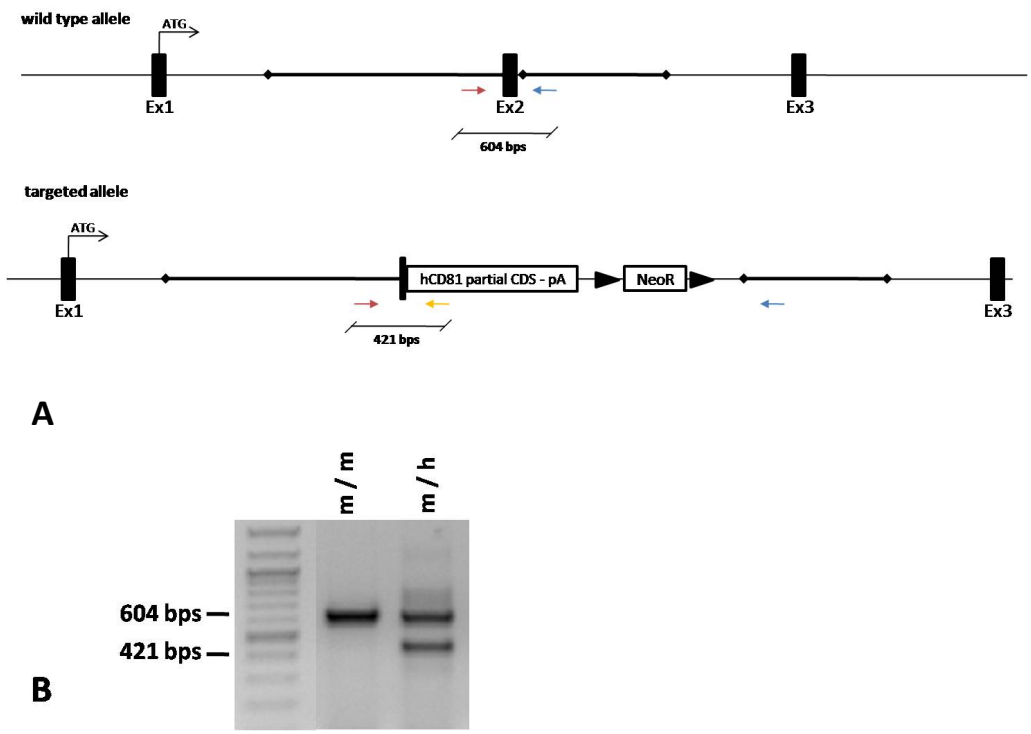
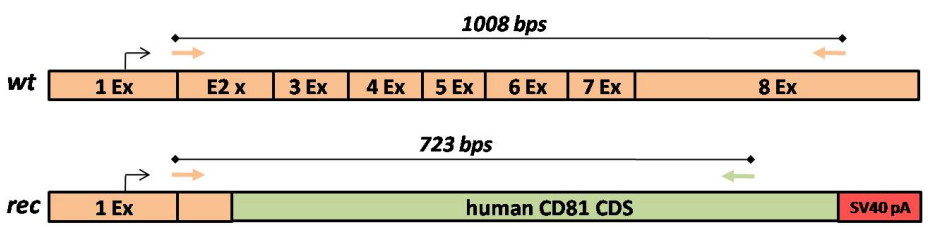


Fig 13 – Humanized CD81 knock-in mice PCR genotyping. Genomic DNA was extracted from mice tails to determine their genotype by PCR. Panel A: CD81 wild type and recombinant loci are represented; the sense primer (red arrow) amplifies a 604 bps product with one antisense primer (blue arrow) on the wild type allele, and a 421 bps product with the other antisense primer (yellow arrow) on the targeted allele. Panel B: Genotyping results are reported for a wild type mouse (m/m) and a heterozygous mouse (m/h).

4.2 CD81 expression in humanized CD81 knock-in mouse

The nucleotide sequence coding the human form of CD81 was introduced in the genome so that the second exon of the murine orthologous gene was deleted (**Fig 10**). The transcription of the exogenous sequence is regulated by the promoter of CD81 murine gene; SV40 polyadenylation signal was inserted downstream the human CDS to allow transcription termination and to stabilize the mRNA (Proudfoot et al. 1991). The targeted allele transcription leads to the production of CD81 chimeric transcript instead of the murine endogenous one. In CD81 heterozygous mice both the wild type and humanized transcripts are produced; when homozygous mice will be generated only the humanized gene will be transcribed.

To verify if the gene manipulation corresponded to the expected phenotype, we performed RT-PCR analysis of CD81 transcripts (**Fig 14**). Total RNA was extracted from different CD81 expressing tissues (lung, liver, kidney). Heterozygous CD81 m/h and wild type CD81 m/m total RNA samples were collected and treated to completely remove genomic DNA contamination. They were retro-transcribed and then used as template for specific PCR. We designed two reactions to discriminate humanized transcript from the murine wild type one. Both recombinant and wild type products were found in heterozygous samples, demonstrating that the exogenous gene for human CD81 was expressed. We also determined the sequence of the amplified products and their specificity was confirmed (**Fig 7**). As figure 15 shows, CD81 was expressed as both wild type and human transcripts in heterozygous mouse liver. These data suggest that a novel humanized CD81 murine line was permanently established.



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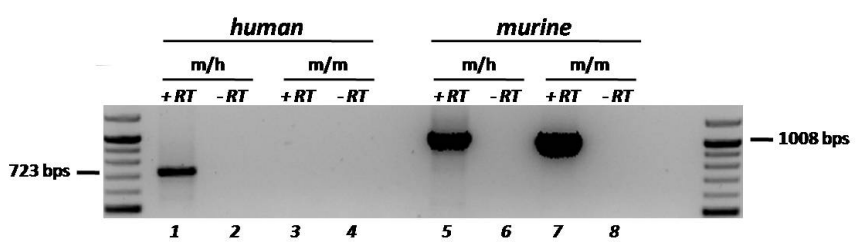


B

Fig 14 – RT-PCR analysis of CD81 transcripts.

Fig 14 – RT-PCR analysis of CD81 transcripts. Panel A: The structure of wild type and recombinant CD81 transcripts is schematically shown. Pink bars represent murine exons, green bar represents the inserted human CDS, red bar represents SV40 polyadenylation signal. PCR was performed using a sense primer annealing on the murine sequence shared by both wild type and recombinant transcripts (pink rightwards arrows) and two different anti-sense primers: one annealing only on the wild type transcript (pink leftwards arrow), the other annealing only on the human sequence (green leftwards arrow). Panel B: PCR results on liver cDNA were reported. Liver was dissected from wt mice (m/m) and from CD81 heterozygous mice (m/h). RNA control samples, in which no retrotranscriptase was added (-RT), were also included (lanes 2, 4, 6, 8). Amplification using the sense primer paired with the antisense primer annealing only on the human sequence produced the specific 723 bps fragment for m/h and no one for m/m (lanes 1, 3), while the reaction with the antisense primer annealing only on the wild type transcript produced the specific 1008 bps fragment for both m/h and m/m (lanes 5, 7). No product was observable in –RT controls.

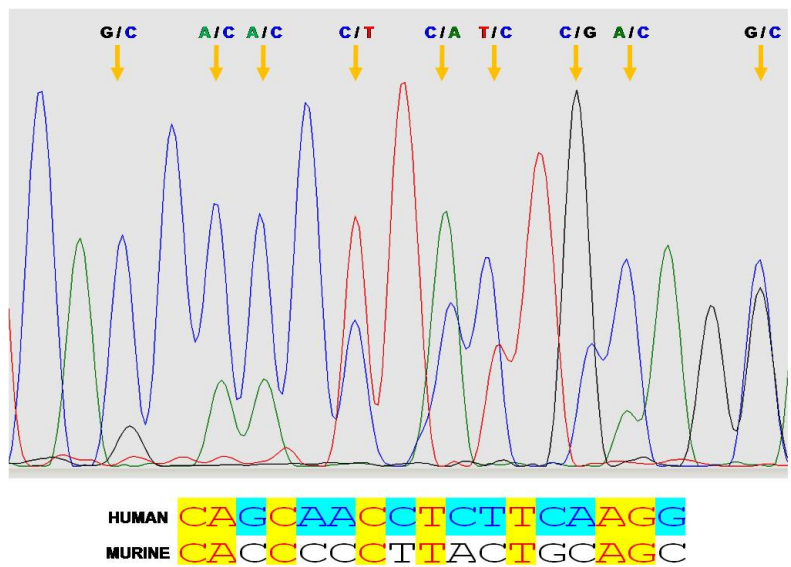


Fig 15 – Sequence analysis of CD81 RT-PCR products. Liver cDNA from heterozygous CD81 m/h mouse was specifically amplified to discriminate humanized transcript from the wild type one. Two different PCR products were obtained and they were further mixed and sequenced. Only a small region of the whole sequence is reported: yellow arrows indicate double picks corresponding to the nucleotide substitutions between human and murine sequences. These results demonstrated the liver expression of both wild type and humanized CD81 transcripts in the heterozygous mouse.

4.3 Generation of humanized SR-BI knock-in mouse

Scavenger receptor class B member 1 (SR-BI) has been proposed to act as a putative HCV entry molecule on the basis of its reactivity with HCV sE2 (Scarselli et al. 2002). SR-BI binding to sE2 appears to be species-specific, as mouse SR-BI does not bind sE2. Antibodies against SR-BI also significantly reduce HCV pseudoparticles infectivity (Bartosch et al. 2003b).

Similar to CD81, SR-BI is a cellular receptor involved in HCV entry and the differences between the murine protein and the human one could contribute to render the mouse cells not permissive to the HCV psuedoparticles infection. We decided to produce a novel mouse strain expressing human SR-BI, as done for CD81. Using a similar gene targeting approach, we inserted SR-BI human CDS in murine genome to replace the orthologous gene.

SR-BI human and murine genes are both constituted by 13 exons and they share the 80% of the coding sequence and the 80% of the amino acid sequence. A chimeric gene was created inserting the complete human CDS of SR-BI in the murine gene replacing the first coding exon, exactly from the start codon (**Fig 16**).

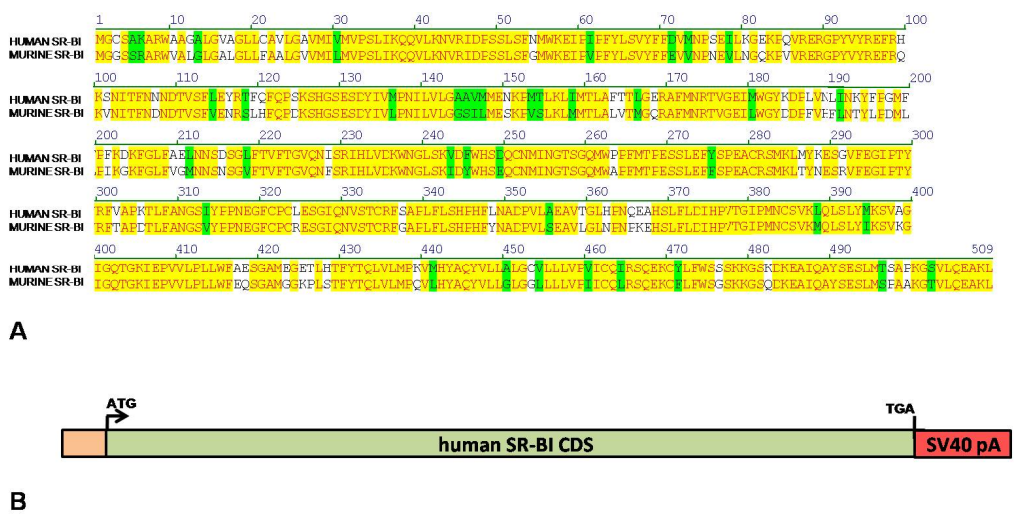


Fig 16 – SR-BI human and murine proteins alignment and chimeric transcript. Panel A: the protein alignment between human and murine SR-BI sequences is represented: yellow indicates identical amino acids, green similar amino acids, white different "non-similar" amino acids. Panel B: to humanize the mouse for SR-BI protein, its human CDS was inserted in the first exon of the murine orthologous gene (pink bar), exactly from the start codon to the stop codon (green bar), followed by SV40 polyadenilation signal (red bar).

We designed and constructed a targeting vector to modify SR-BI murine gene by homologous recombination in mouse ES cells (**Fig 17**). Two asymmetric homology arms, obtained from a 129S7/SvEv mouse bMQ BAC comprising the genomic locus of SR-BI, flanked the SR-BI CDS followed by SV40 polyadenilation signal. The neomycin resistance cassette was also included downstream the human gene. The targeting vector was linearized and electroporated into the murine W4/129S6/SvEv ES cell line (Auerbach W et al. 2000). The recipient clones were then screened for correct targeting by PCR analysis (**Fig 18**). On 96 G418 selected clones we found 2 correctly recombined clones by PCR analysis. To verify the absence of random integrations, targeted clones were further analyzed by Southern blotting (**Fig 19**). The correct targeting was confirmed by Southern blotting for both clones, and no random integration was observed.

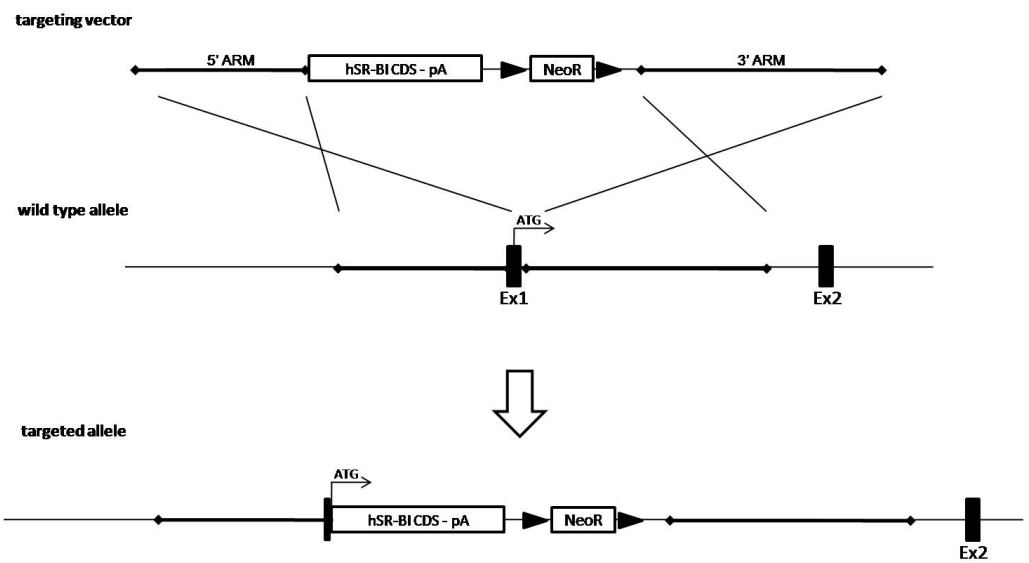
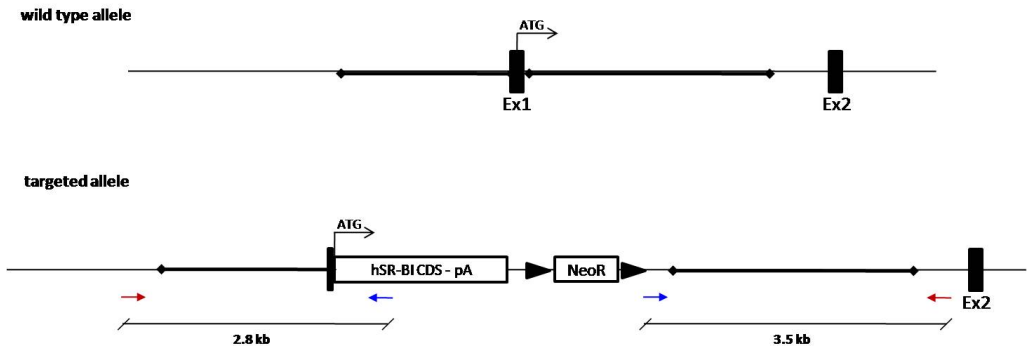


Fig 17 – Gene targeting strategy to humanize SR-BI murine gene. A targeting vector to insert the human CDS of SR-BI in the murine locus was constructed: two asymmetric homology regions (5' arm of 2 kb, and 3' arm of 3 kb) flank human SR-BI complete CDS followed by SV40 polyadenilation signal (pA) and the neomycin resistance gene (NeoR) included by two FRT sites (closed triangles). These exogenous elements were inserted by homologous recombination in the first murine exon, exactly from the start codon.



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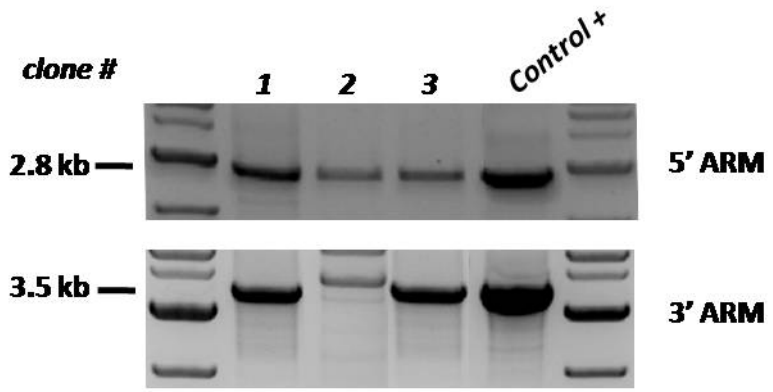


B

Fig 18 – SR-BI targeted murine ES cell screening by PCR analysis. G418 resistant cell clones were screened for the correct targeting by PCR analysis. Panel A: SR-BI wild type and recombinant loci are represented. Two different reactions were designed to amplify a genomic region including the 5' homology arm (2.8 kb) and another region including the 3' homology arm (3.5kb). Both the PCR primers pairs annealed outside the homology arms, one primer of each pair on the wild type genomic sequence (red arrows), the other on the inserted exogenous sequence (blue arrows). Panel B: PCR results are reported for three clones. The specific 5' product was amplified for every three clones, while only clones 1 and 3 presented the specific 3' product. A positive control was also included and it consisted of modified BAC used to construct the targeting vector.

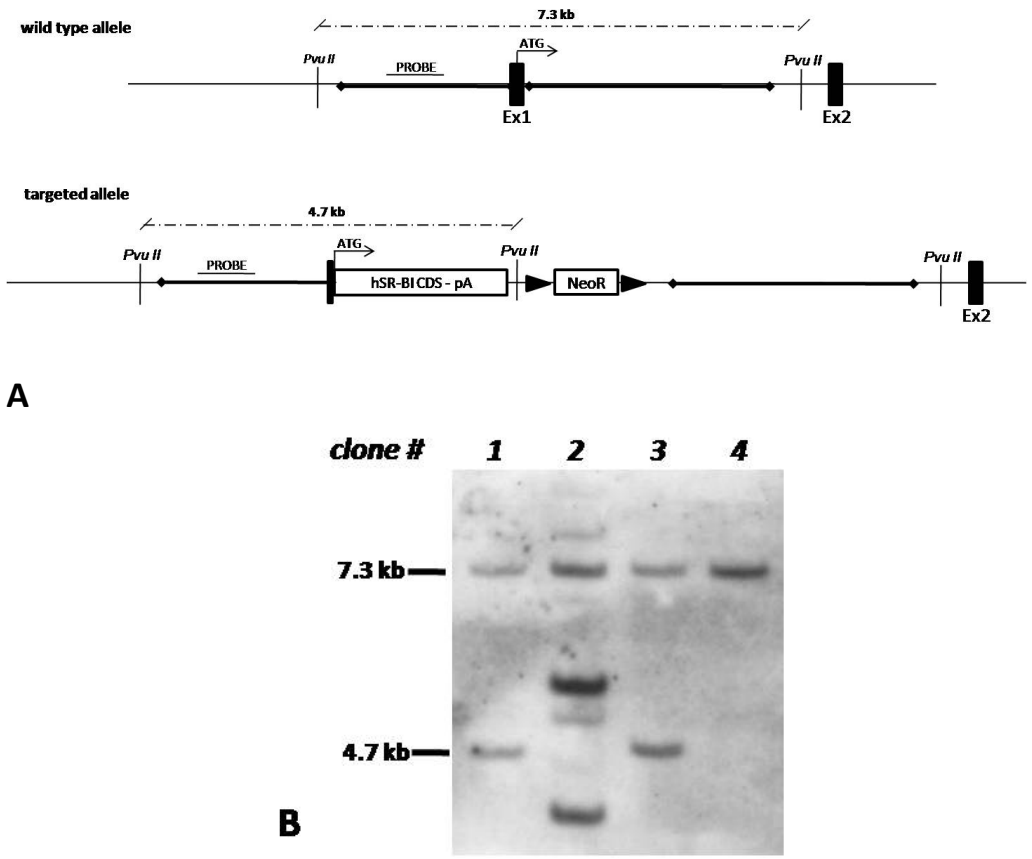


Fig 19 – Southern blot analysis of SR-BI humanized ES cells. To verify the correct recombination and the absence of unspecific integrations, targeted ES clones were analyzed by Southern blotting. Panel A: SR-BI wild type and recombinant loci are represented; oligonucleotide probe annealed on the 5' homology arm genomic sequence and after Pvu II digestion it detected a wild type restriction product of 7.3 kb and a recombinant restriction product of 4.7 kb. Panel B: Southern blotting results are reported for the PCR analyzed clones shown in figure 18 (1-3) and for a wild type clone (4). Clones 1 and 3 presented only the wild type and the specific recombinant products, while clone 2 presented more fragments corresponding to random integrations.

One targeting ES clone was microinjected in a C57BL/6 blastocyst to generate chimeric mice that transmitted the correct modification to the germ line. A three primers PCR performed on genomic DNA extracted from tail allowed to determine the mice genotype for the SR-BI targeted allele (**Fig 20**). The heterozygous knock-in mice, containing the neomycin resistance gene, were interbred with the available FLPe transgenic mice.

21 mice were generated from the mate of the chimera with wild type mice, and about the half presented heterozygous genotype for SR-BI locus (SR-BI m/h), according to the Mendelian ratio. Heterozygous SR-BI m/h mice were viable, with normal growth and exhibited no overt gross phenotypes.

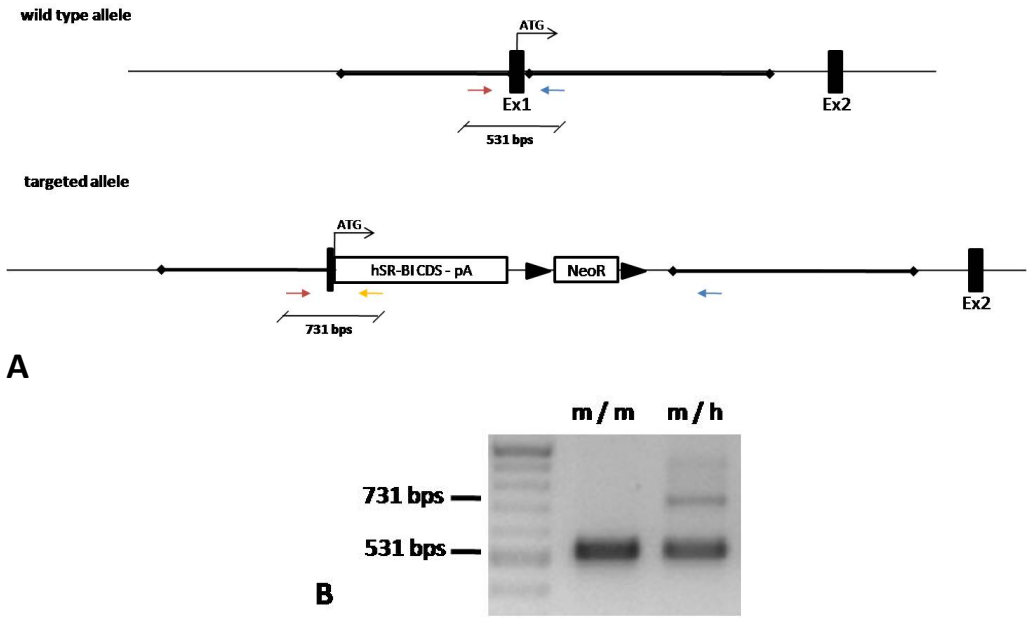


Fig 20 – Humanized SR-BI knock-in mice PCR genotyping. Genomic DNA was extracted from mice tails to determine their genotype by PCR. Panel A: SR-BI wild type and recombinant loci are represented; the sense primer (red arrow) amplifies a 531 bps product with one antisense primer (blue arrow) on the wild type allele, and a 731 bps product with the other antisense primer (yellow arrow) on the targeted allele. Panel B: Genotyping results are reported for a wild type mouse (m/m) and a heterozygous mouse (m/h).

4.4 SR-BI expression in humanized SR-BI knock-in mouse

SR-BI murine locus was modified inserting the complete human CDS, followed by a polyadenilation signal, in the place of the first exon by homologous recombination in ES cells (**Fig 17**). The transcription of this modified gene produces a SR-BI chimeric transcript constituted by murine five prime untranslated region (5' UTR), the human CDS and SV40 polyadenilation signal (**Fig 21 A**). In humanized SR-BI heterozygous mice both the wild type and chimeric transcripts are produced; when homozygous mice will be generated only the humanized gene will be transcribed.

RT-PCR was performed to analyze SR-BI transcripts, as done for humanized CD81 mice (**Fig 21**). Liver was dissected from heterozygous SR-BI m/h and wild type SR-BI m/m mice and total RNA was extracted and treated to completely remove genomic contamination. Two different RT-PCRs were designed to specifically discriminate humanized chimeric transcript and murine wild type transcripts. Both wild type and chimeric products were found in heterozygous samples, demonstrating that the exogenous gene for human SR-BI was expressed. We also determined the sequence of the amplified products and their specificity was confirmed **(Fig 22)**.

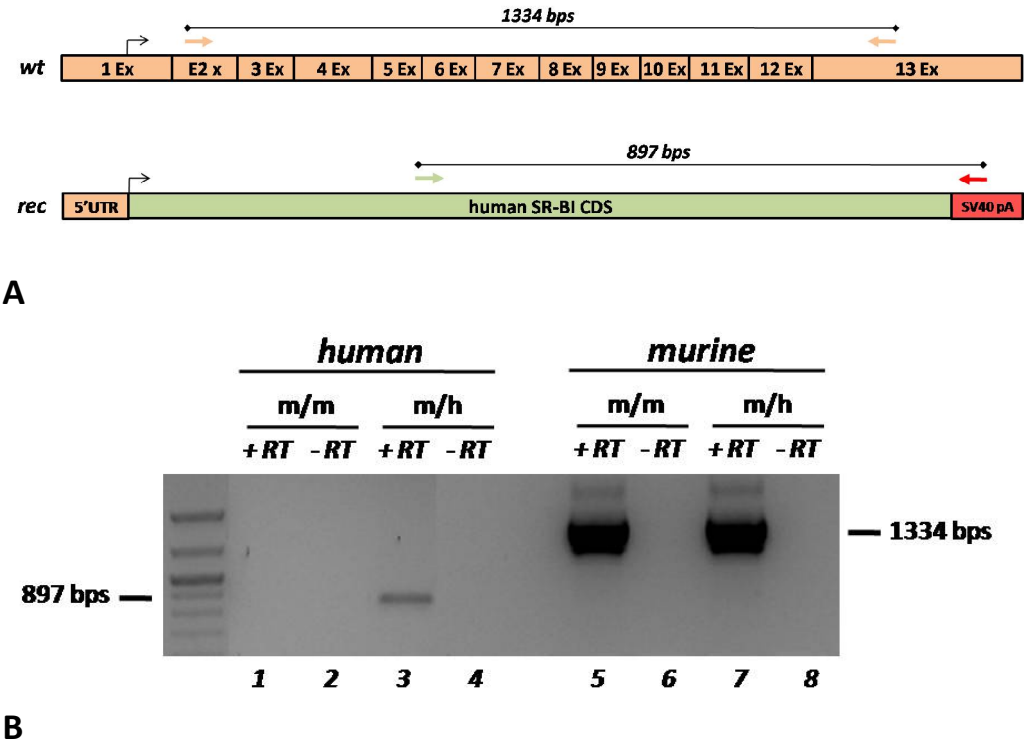


Fig 21 – RT-PCR analysis of SR-BI transcripts. Panel A: The structure of wild type and chimeric SR-BI transcripts is schematically shown. Pink, green and red bars represent murine exons, human CDS and SV40 polyadenylation signal, respectively. RT-PCR performed using primers annealing only on the murine sequence (pink arrows) amplified a wild type product of 1334 bps; amplification using the sense primer annealing on the human sequence (green arrow) and the antisense primer on the polyadenylation signal (red arrow) produced a chimeric fragment of 897 bps. Panel B: PCR on liver cDNA were reported. Liver was dissected from wt mice (m/m) and from SR-BI heterozygous mice (m/h). RNA control samples, in which no retrotranscriptase was added (-RT), were also included (lanes 2, 4, 6, 8). RT-PCR using chimeric transcript specific primers amplified the 897 bps product only for m/h (lanes 1, 3), while the reaction with wild type specific primers amplified the 1334 bps product for both m/h and m/m (lanes 5, 7). No product was observable in –RT controls.

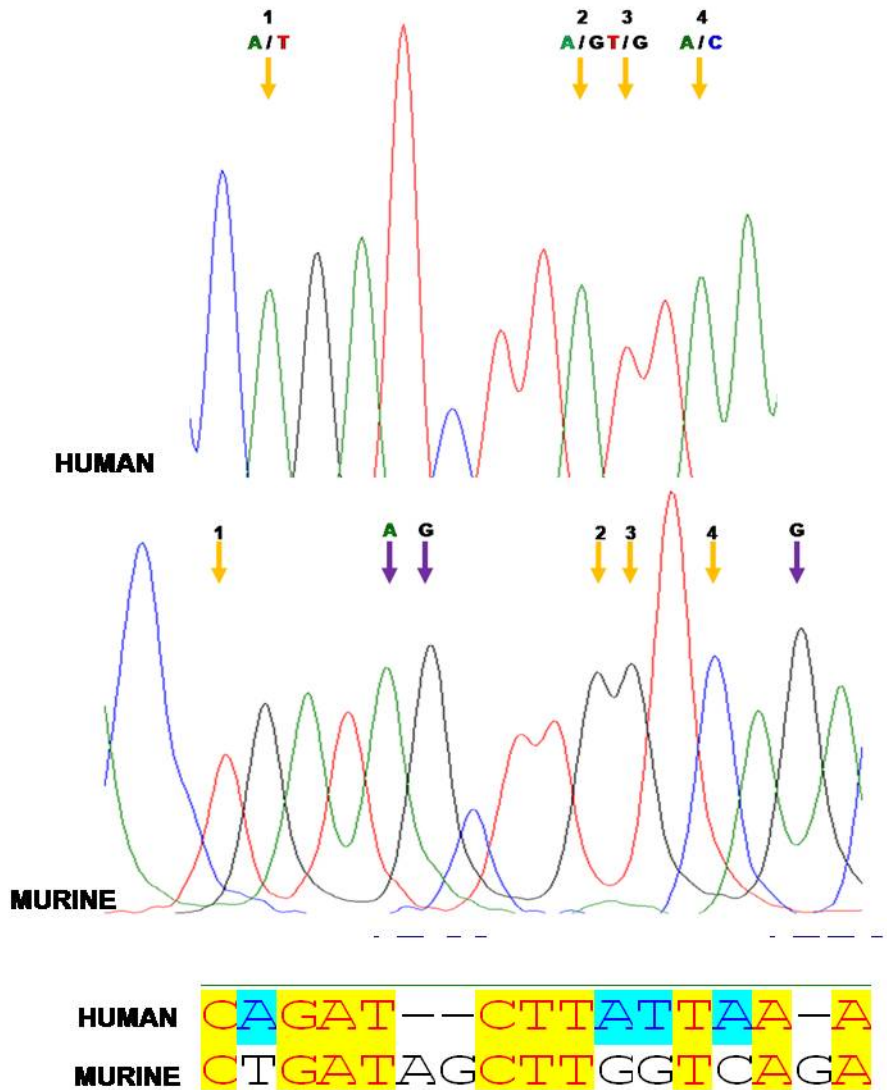


Fig 22 – Sequence analysis of SR-BI RT-PCR products. Liver cDNA from heterozygous SR-BI m/h mouse was specifically amplified to discriminate humanized transcript from the wild type one. Two different PCR products were obtained and sequenced. Only a small region of whole sequence is reported: yellow arrows indicate the nucleotide substitutions (numbered 1-4), purple arrows nucleotide insertions between human and murine sequences. These results demonstrated the liver expression of both wild type and humanized SR-BI transcripts in the heterozygous mouse.

4.5 Generation of humanized occludin knock-in mice

The tight junction protein occludin (OCLN) has been identified as a new HCV entry factor essential for HCV infection together with the already known HCV receptors CD81, SR-BI and CLDN-1 (Ploss et al. 2009, Liu et al. 2009). The experiments by Ploss et al. showed that non-HCV-permissive human and non-human cell lines became susceptible to HCV when all four molecules are expressed. Their results provide strong evidence that OCLN is the final essential entry factor for HCV. Moreover they speculated that the expression of human CD81 and OCLN in the context of mouse CLDN-1 and SR-BI can overcome the major block to HCV replication in murine cells, providing a clear foundation upon which a mouse model for HCV infection can be obtained (Nature 2009;457:797-98,882-86). These conclusions urged us to produce another humanized mouse strain for the last identified essential HCV receptor.

OCLN human and murine genes are both constituted by 9 exons and they share the 86% of the coding sequence and the 89% of the amino acid sequence. A chimeric gene was created inserting the complete human CDS of OCLN in the murine gene replacing the first coding exon, exactly from the start codon (**Fig 23**).

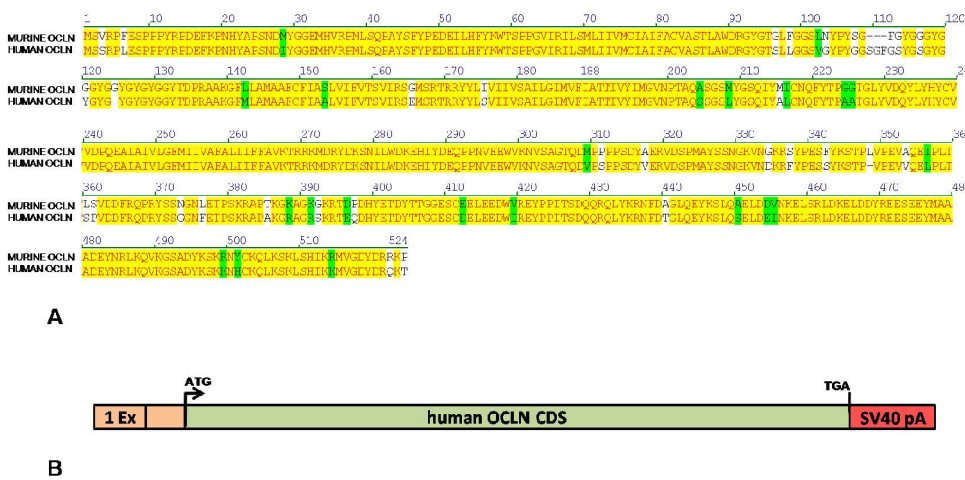


Fig 23 – OCLN human and murine proteins alignment and chimeric transcript. Panel A: the protein alignment between human and murine OCLN sequences is represented: yellow indicates identical amino acids, green similar amino acids, white different "non-similar" amino acids. Panel B: to humanize the mouse for OCLN protein, its human CDS was inserted in the second exon of the murine orthologous gene (pink bar), exactly from the start codon to the stop codon (green bar), followed by SV40 polyadenilation signal (red bar).

A targeting vector to insert the human gene in the place of the murine one by homologous recombination in ES cells was designed and constructed similarly to those described for CD81 and SR-BI (**Fig 24**). The targeting vector was linearized and electroporated into the murine W4/129S6/SvEv ES cell line

(Auerbach et al. 2000). G418 resistant ES cell colonies were screened by PCR analysis (**Fig 25**). On 288 G418 selected clones we found 6 correctly recombined clones by PCR analysis. To verify the absence of unspecific random integrations, targeted clones were further analyzed by Southern blotting (**Fig 26**). The correct targeting was confirmed by Southern blotting for every 6 clones, and no random integration was observed.

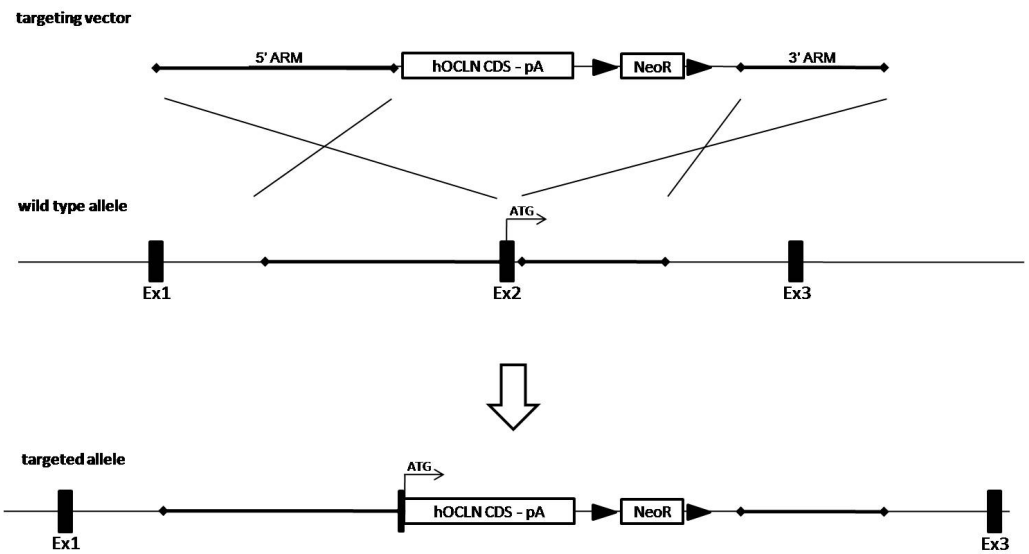
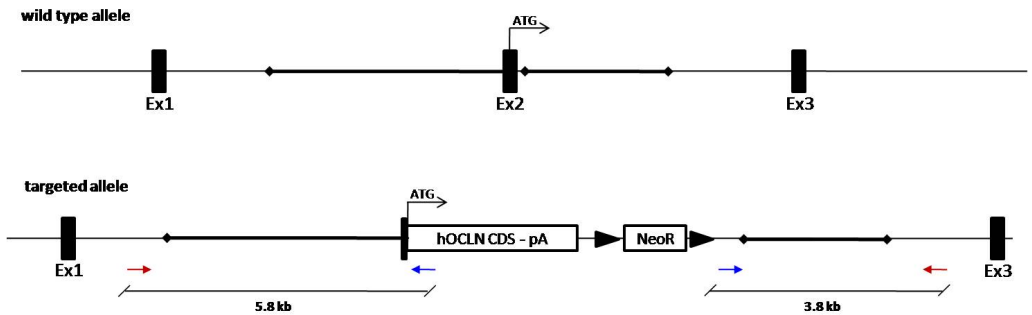


Fig 24 – Gene targeting strategy to insert the human OCLN gene in the murine locus. A targeting vector to insert the human CDS of OCLN in the murine locus was constructed: two asymmetric homology regions (5' arm of 5 kb, and 3' arm of 3.3 kb) flank human OCLN complete CDS followed by SV40 polyadenilation signal (pA) and the neomycin resistance gene (NeoR) included by two FRT sites (closed triangles). These exogenous elements were inserted by homologous recombination in the second murine exon, exactly from the start codon.



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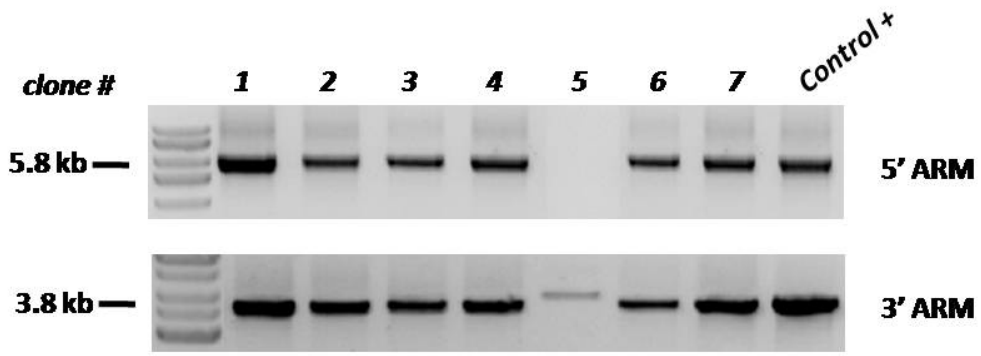

B

Fig 25 – OCLN targeted murine ES cells screening by PCR analysis. G418 resistant cell clones were screened for the correct targeting by PCR analysis. Panel A: OCLN wild type and recombinant loci are represented. Two different reactions were designed to amplify a genomic region including the 5' homology arm (5.8 kb) and another region including the 3' homology arm (3.8kb). Both PCR primers pairs annealed outside the homology arms, one primer of each pair on the wild type genomic sequence (red arrows), the other on the inserted exogenous sequence (blue arrows). Panel B: PCR results are reported for seven clones. The specific products were amplified for six clones, while clone 5 presented only an unspecific product at 3' PCR. A positive control was also included and it consists of modified BAC used to generate the targeting vector.

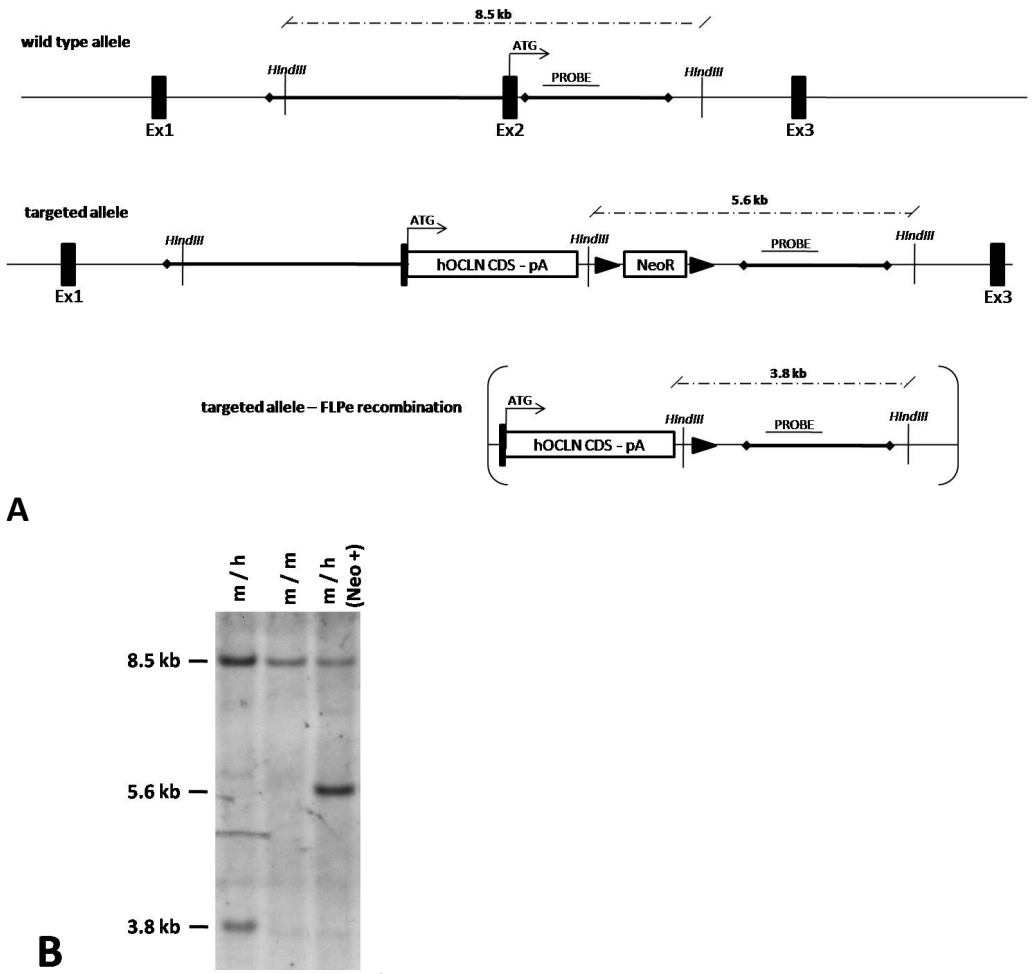

Fig 26 – Southern blot analysis of humanized OCLN ES cells. To verify the correct recombination and the absence of unspecific integrations, targeted ES clones were analyzed by Southern blotting. Panel A: OCLN wild type and recombinant loci are represented; oligonucleotide probe annealed on the 3' homology arm genomic sequence and after Hind III digestion it detected a wild type restriction product of 8.5 kb and recombinant restriction products of 5.6 kb and 3.8 kb, before and after FLPe treatment respectively. Panel B: Southern blotting results are reported for a correctly targeted clone, before and after the FLPe treatment, m/h (Neo+) and m/h respectively. A wild type clone, m/m, was reported too.

Chimeric mice were generated by blastocyst injection of one targeted ES clone containing the Neo^R cassette or with the Neo cassette removed by transient transfection and expression of FLPe recombinase. These chimeras were bred with C57BL/6 wild type mice to generate heterozygous neoOCLN and OCLN mice respectively as demonstrated by PCR genotyping (**Fig 27**).

Mice containing the Neo^R cassette flanked by FRT sites were further bred with the available FLPe transgenic mice, to generate heterozygous mice in which the Neo^R cassette has been removed (herein referred to as OCLN-2 mice).

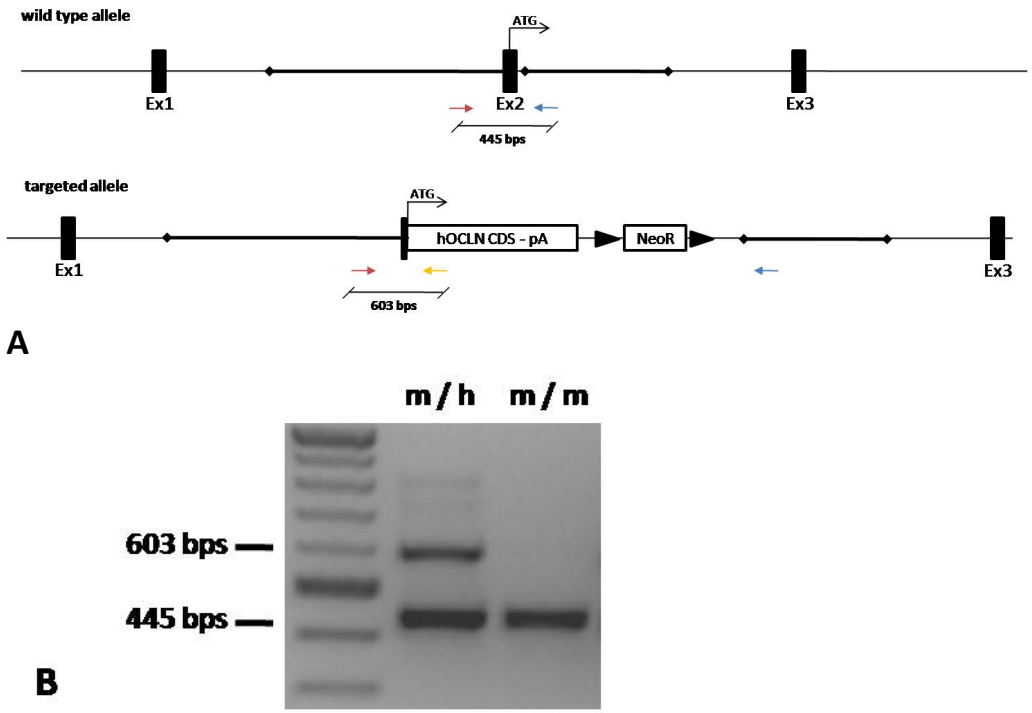

Fig 27 – Humanized OCLN knock-in mice PCR genotyping. Genomic DNA was extracted from mice tails to determine their genotype by PCR. Panel A: OCLN wild type and recombinant loci are represented; the sense primer (red arrow) amplifies a 445 bps product with one antisense primer (blue arrow) on the wild type allele, and a 603 bps product with the other antisense primer (yellow arrow) on the targeted allele. Panel B: Genotyping results are reported for a heterozygous mouse (m/h) and a wild type mouse (m/m).

4.6 OCLN expression in humanized OCLN knock-in mouse

To generate a humanized mouse for the occludin protein, we modified the murine genome introducing the nucleotide sequence coding for the human occludin in the place of the first coding exon of the murine orthologous gene. This genomic modification was transferred in mouse germ line so that a new humanized occludin murine line was permanently established.

To verify if the gene manipulation corresponded to the expected phenotype, we performed RT-PCR analysis of occludin transcripts (**Fig 28**). Total RNA extracted from different tissues of heterozygous OCLN m/h mice was retro-transcribed and then used as template for specific PCRs. We designed two reactions to discriminate humanized transcript from the murine wild type one. Both recombinant and wild type products were found in heterozygous samples, demonstrating that the exogenous gene for human occludin was expressed. We also determined the sequence of these PCR products and their specificity was confirmed (**Fig 29**). As shown in figure 28 shows, OCLN was expressed as both wild type and human transcripts in

heterozygous mouse liver. These data expression demonstrated the effectiveness of the murine genomic modification at the OCLN locus to be successfully converted in the production of a humanized transcript.

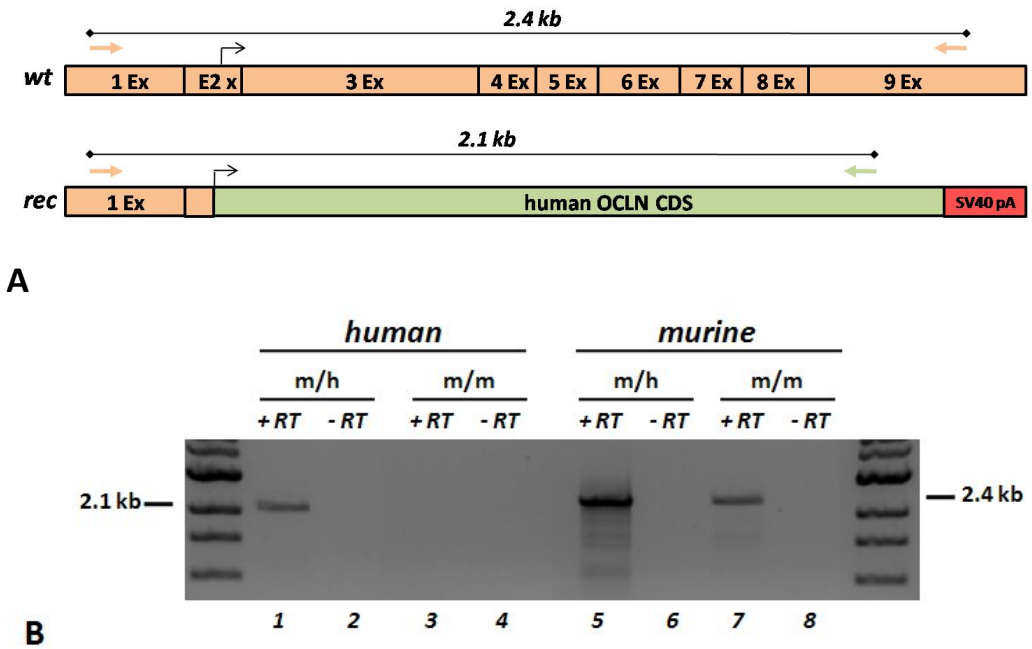

Fig 28 – RT-PCR analysis of OCLN transcripts. Panel A: The structure of wild type and recombinant OCLN transcripts is schematically shown. Pink bars represent murine exons, green bar represents the inserted human CDS, red bar represents SV40 polyadenylation signal. PCR was performed using a sense primer annealing on the murine sequence shared by both wild type and recombinant transcripts (pink rightwards arrows) and two different anti-sense primers: one annealing only on the wild type transcript (pink leftwards arrow), the other annealing only on the human sequence (green leftwards arrow). Panel B: PCR results on liver cDNA were reported. Liver was dissected from wt mice (m/m) and from OCLN heterozygous mice (m/h). RNA control samples, in which no retrotranscriptase was added (-RT), were also included (lanes 2, 4, 6, 8). Amplification using the sense primer paired with the antisense primer annealing only on the human sequence produced the specific 2.1 kb fragment for m/h and no one for m/m (lanes 1, 3), while the reaction with the antisense primer annealing only on the wild type transcript produced the specific 2.4 kb fragment for both m/h and m/m (lanes 5, 7). No product was observable in –RT controls.

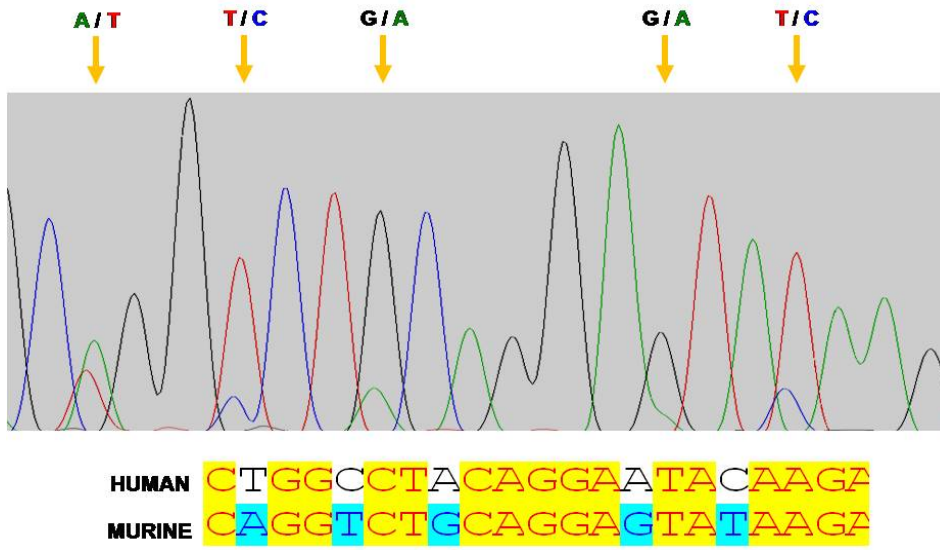

Fig 29 – Sequence analysis of OCLN RT-PCR products. Liver cDNA from heterozygous OCLN m/h mouse was specifically amplified to discriminate humanized transcript from the wild type one. Two different PCR products were obtained and they were further mixed and sequenced. Only a small region of whole sequence is reported: yellow arrows indicate double picks corresponding to the nucleotide substitutions between human and murine sequences. These results demonstrated the liver expression of both wild type and humanized OCLN transcripts in the heterozygous mouse.

4.7 Homozygous OCLN h/h mice are not viable

Heterozygous OCLN m/h mice were viable, with normal growth and exhibited no overt gross phenotypes. To generate homozygous mice, we set several crosses among our heterozygous, as described in Table 1. 291 mice were produced from the different mates. Genotype analysis revealed that about 75% were heterozygous OCLN murine / OCLN human (OCLN m/h) and the others wild type OCLN murine / OCLN murine (OCLN m/m) (**Tab 5**). These data indicate that homozygous insertion of human OCLN gene may lead to prenatal lethality.

neoOCLN m/h x neoOCLN m/h

No.	*m/m*	*m/h*	*h/h*	*χ^2 test*
156	49	107	0	10^{-12}

OCLN m/h x OCLN m/h

No.	*m/m*	*m/h*	*h/h*	*χ^2 test*
34	11	23	0	0.003

OCLN-2 m/h x OCLN-2 m/h

No.	*m/m*	*m/h*	*h/h*	*χ^2 test*
101	21	80	0	10^{-10}

Tab 5 – Humanized OCLN knock-in mice genotypes. In this table is shown the OCLN genotypes of the litters from heterozygous interbreeds, for each of the three OCLN knock-in lines: neomycin cassette including line, neoOCLN m/h, and lines with neomycin cassette removed *in vivo*, OCLN m/h, and *in vitro*, OCLN-2 m/h. These data are statistically significant ($\chi^2 < 0.005$).

4.8 Embryonic lethality for homozygous OCLN h/h knock-in mice

The humanized occludin knock-in mouse that we generated did not show any evident phenotype when carried heterozygous genomic modification. In contrast, when heterozygous mice were interbred no homozygous humanized occludin mouse (OCLN h/h) was produced. The genotype analysis of heterozygous interbreed derived offspring showed Mendelian 1:2 ratio between wild type homozygous (OCLN m/m) and heterozygous (OCLN m/h) littermates (**Tab 5**). This observation indicated that homozygous OCLN h/h condition was lethal during mouse embryonic life. The same results were observed for all the three humanized occludin knock-in lines, demonstrating that this consequence did not correlate either with the presence of neomycin resistance gene or with FLPe recombination events.

To verify murine embryonic lethality for homozygous humanization of occludin locus, we collected thirty embryos at 10.5 dpc (days of gestation) from heterozygous interbreed and analyzed their genotype. Either at this stage no homozygous OCLN h/h was found (**Tab 6**).

neoOCLN m/h x neoOCLN m/h					
Embryos stage	***No.***	***m/m***	***m/h***	***h/h***	***χ^2 test***
E10.5	31	6	25	0	0.0009

Tab 6 – Humanized occludin knock-in embryos genotypes. 10.5 dpc embryos were collected from heterozygous neoOCLN m/h interbreed and they were genotyped. No homozygous OCLN h/h embryos were found at this embryonic stage ($\chi^2 < 0.005$).

Therefore we investigated the genotype in early embryonic stages, collecting preimplantation embryos from superovulated female heterozygous mice crossbred with male heterozygous mice. To determine preimplantation embryos genotype we performed nested PCRs on whole embryos (**Fig 30**). The genotype analysis showed the production of homozygous OCLN h/h at 2-cell (1-2.5 dpc) and morula stages (1-3.5 dpc), whereas no homozygous was found at blastocyst stage (2-5 dpc) (**Tab 7**). These results indicate that the genomic modification introduced in the mouse to humanize occludin gene become lethal in homozygosity; in particular the mortality appears during embryonic development from morula to blastocyst stages.

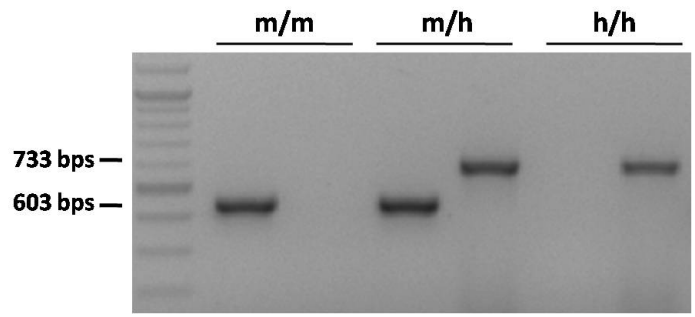

Fig 30 – Humanized occludin knock-in preimplantation embryos PCR genotyping. Nested PCRs were performed on whole preimplantation embryos to amplify a wild type allele product (603 bps) and a recombinant allele product (733 bps). The nested primers are the same used to genotype mice and are indicated in figure 27A. PCR results are shown for all the three genotypes: wild type m/m, heterozygous m/h, and homozygous h/h.

neoOCLN m/h x neoOCLN m/h					
Embryos stage	***No.***	***m/m***	***m/h***	***h/h***	***χ^2 test***
2-cell	35	8	19	8	-
morulae	29	8	16	5	-
blastocysts	22	4	18	0	0,0056

Tab 7 – Humanized occludin knock-in preimplantation embryos genotypes. Preimplantation embryos were collected from superovulated heterozygous OCLN h/m mice interbred with heterozygous OCLN h/m mice, and they were genotyped. Homozygous OCLN h/h embryos were found with Mendelian rate at 2-cell and morula stages, whereas no homozygous blastocyst was produced ($\chi^2 < 0.005$).

Since OCLN null mice have several defects (Saitou et al. 2000), we wondered whether the embryo lethality of our homozygous OCLN h/h could be due to an insufficient expression of human gene. We adopted a real time PCR approach to measure relative expression levels of both humanized and wild type occludin genes in the heterozygous mice, according to Pfaffl model (Pfaffl 2001). Therefore we designed two different oligonucleotide primer pairs annealing on the 3' UTR regions, which represent the least conserved sequence between the two genes, to specifically amplify a wild type allele derived product and the humanized allele derived one. The real time PCR results were calculated regarding amplification efficiency of each primer pairs, and they were normalized for GAPDH reference gene. We analyzed the relative expression levels of human and murine occludin in liver samples extracted from wild type and heterozygous OCLN m/h mice (**Figg 31-32**). The study was conducted on six animals for each genotype, homogeneous for sex and age: wild type OCLN m/m, neoOCLN m/h, OCLN m/h, OCLN-2 m/h. The results indicated a high variability in the expression levels of occludin between animals with the same genotype. However most of the times the levels of human OCLN were comparable to those of the murine transcript in each single heterozygous mouse.

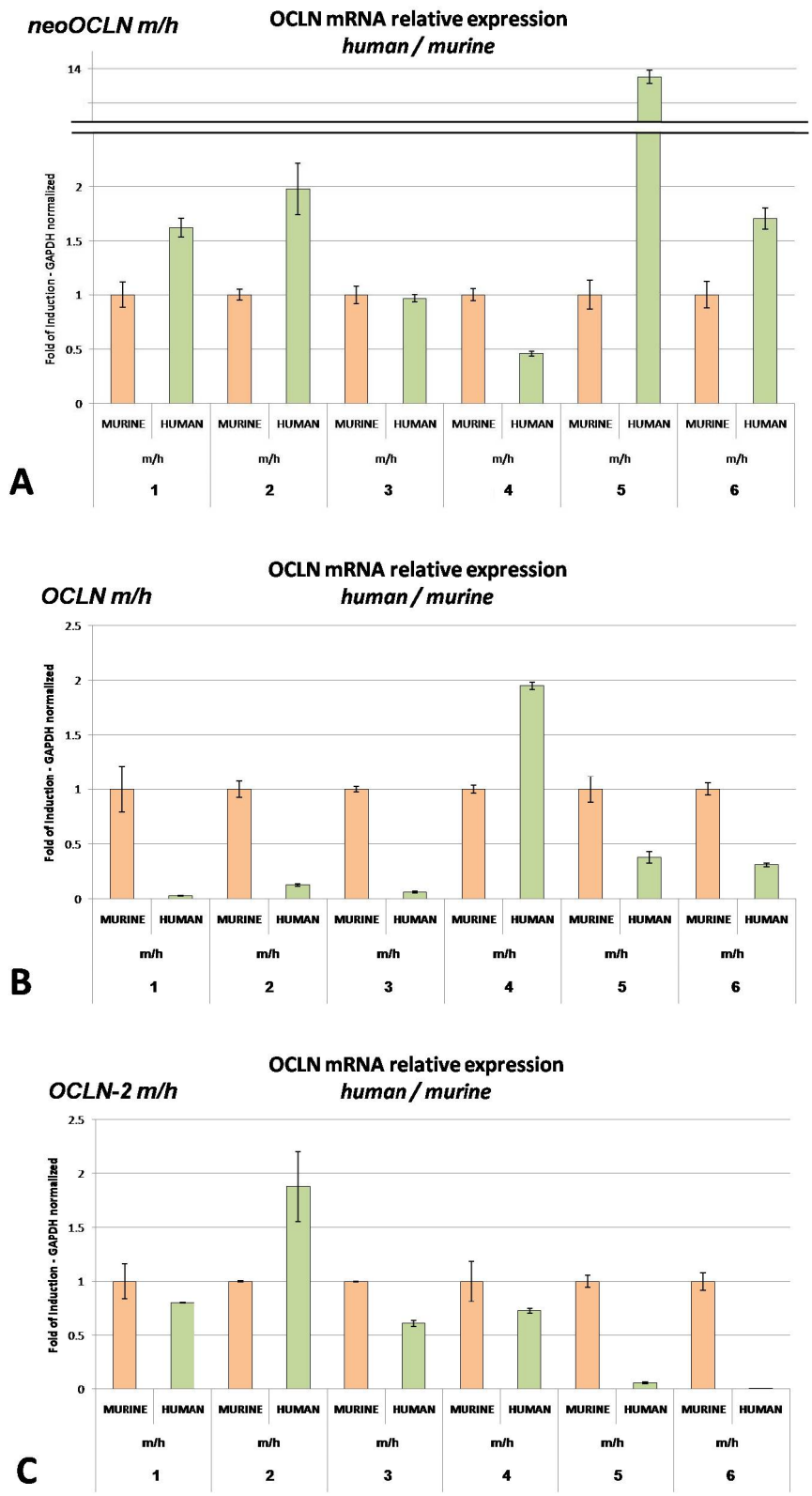

Fig 31 – Determination of OCLN mRNA levels by real time PCR.

Fig 31 – Determination of OCLN mRNA levels by real time PCR. Real time PCR results, normalized for GAPDH, are represented as relative expression of human occludin (green bars) than murine one (pink bars). For each knock-in genotype (A, Neo^R cassette including line; B and C, lines with NeoR cassette removed, *in vivo* and *in vitro* respectively) six animals were analyzed. A high variability in the expression levels of occludin was observed between animals with the same genotype. In 13/18 analyzed heterozygous mice the expression level of human OCLN was comparable than the expression level of murine OCLN.

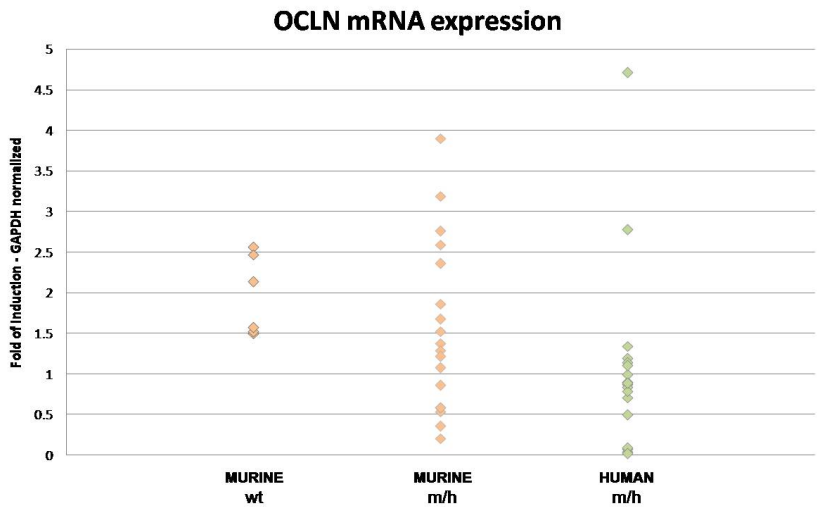

Fig 32 – Determination of OCLN mRNA levels by real time PCR (Absolute values). Real time PCR results, normalized for GAPDH, were reported for murine gene (pink points) and for human gene (green points) in a dispersion graph to compare their levels between wild type and heterozygous m/h mice of every three OCLN murine lines (wt, *N*=6; m/h, *N*=18). No statistically significant variation was observed between distributions.

To analyze the protein expression of OCLN in humanized mice we performed a Western blotting using a polyclonal antibody against the C-terminal 150 amino acid region of human OCLN (**Fig 33**). This antibody reacts specifically with mammalian OCLN, including human and mouse proteins. Indeed it was not possible to discriminate the endogenous protein from the human one in knock-in mice. However we analyzed the expression of OCLN protein in heterozygous m/h mice of each OCLN lines (neoOCLN, OCLN, OCLN-2) to verify possible difference with its expression in wild type mice. Liver was dissected from wild type and heterozygous OCLN m/h mice and total proteins extracts were generated using a lysis protocol preserving transmembrane proteins integrity. A specific band of approximately 60 kDa was detected in every sample, according to the expected molecular weight of OCLN (Furuse M et al. 1993). No quantitative difference was observed between heterozygous and wild type mice. These data indicated that humanized OCLN heterozygous mice did not express lower levels of liver

OCLN protein and no different molecular weight protein was generated in liver.

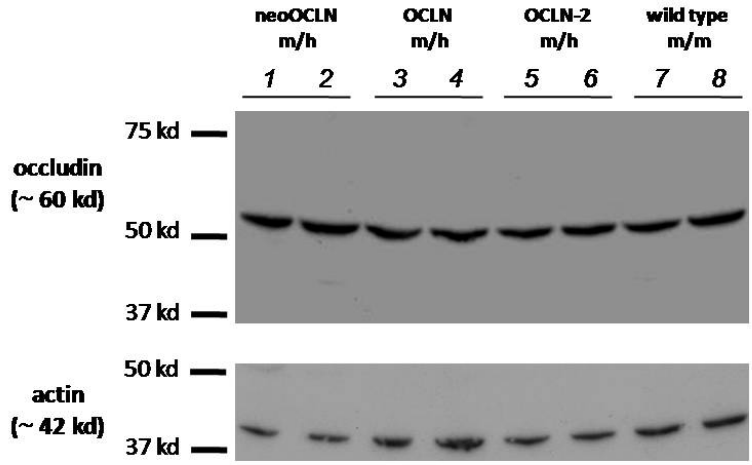

Fig 33 – OCLN Western blot analysis in OCLN knock-in mice. OCLN protein expression was analyzed in humanized mice by Western blotting. Liver extracts were generated from heterozygous OCLN m/h mice containing the Neo^R cassette (lanes 1, 2), or with Neo^R cassette removed *in vivo* (lanes 3, 4) and *in vitro* (lanes 5, 6), and from wild type OCLN m/m mice (lanes 7, 8). Polyclonal anti-OCLN antibody detected a specific band of ~60 kDa for every sample; actin normalization was also performed and no expression levels difference was observed between heterozygous and wild type mice.

4.9 HCV receptors humanized mice as potential model of virus entry and infection

The natural species tropism of HCV is limited to humans and chimpanzees. Indeed the inoculation of HCV-infected sera or of tissue-culture-derived virus into the mouse does not result in detectable infection (Ploss and Rice, unpublished data). This resistance phenotype is probably multifactorial, but is at least partly attributable to a block in HCV entry. The identification of HCV entry essential factors provides the bases to develop an inbred mouse model for HCV. Genetic humanization of a mouse strain for species-specific HCV receptors could overcome the block in entry and could be a useful tool for analyzing HCV infection *in vivo*.

Using knock-in approach, we produced novel inbred humanized mouse strains for three essential HCV receptors, CD81, SR-BI and OCLN. We adopted a gene targeting strategy to insert human genes in the murine genome, to achieve native expression patterns of the human HCV entry factor orthologues. Heterozygous humanized mice are now available for all three HCV receptors and we are waiting for homozygous generation for CD81 and SR-BI; unfortunately humanized OCLN mice showed embryonic lethality for homozygous condition. Humanized mice for single receptors are interbred to

generate mouse strains expressing different combination of human HCV entry factors.

Novel potential interactions between our humanized mice and HCV will be studied. First of all we want to verify the virus entry into the cell of humanized mouse models. Therefore we are optimizing a protocol to isolate and culture primary hepatocytes from the mouse, and we are producing HCV pseudotype particles to perform infectivity assays on the primary hepatocytes.

Murine primary hepatocytes were successfully isolated from CD1 and C57BL/6 mice liver, using a modified Seglen's protocol (Seglen 1976, Gandin et al. 2008). Four-month-old mice were anaesthetized and liver was perfused *in situ* through the portal vein with digestion solution containing a collagenase. After digestion, liver was homogenized and filtered; to isolate only the viable cells, hepatocytes were passed through a Percoll cushion. Viable isolated hepatocytes were then seeded in gelatin coated plates and incubated at 5% CO_2, 37°C (**Fig 34**). To confirm the cellular type specificity of isolated cells we performed RT-PCR analysis to detect hepatic markers. Total RNA was extracted from primary cultured cells and from a frozen liver section as control. RT-PCR for two hepatic markers, albumin and alpha-fetoprotein, was performed and specific products were amplified for both primary cells and liver section samples, indicating the hepatic derivation of isolated cells (**Fig 35**).

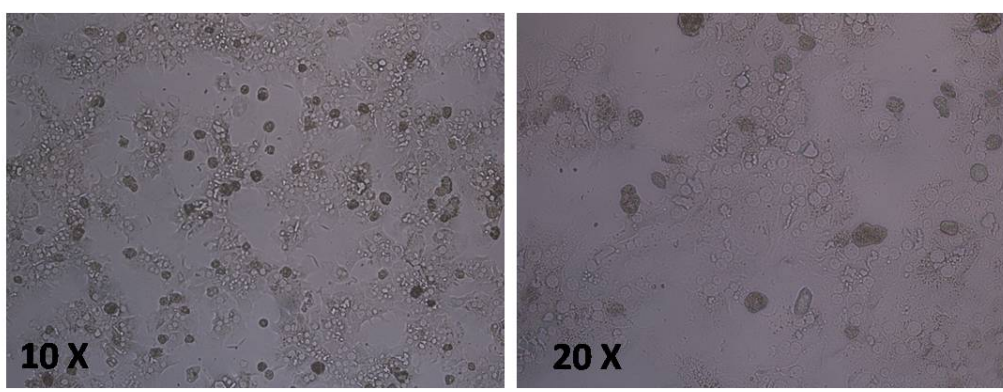

Fig 34 – Primary hepatocytes isolated from mouse liver. CD1 wild type mouse liver was *in vivo* perfused with collagenase digestion solution and manually homogenized. Primary hepatocytes were filtered through 70 µm strainer and passed on 37.5% Percoll cushion. 24 hours primary hepatocytes cultured on gelatin coated plates are shown.

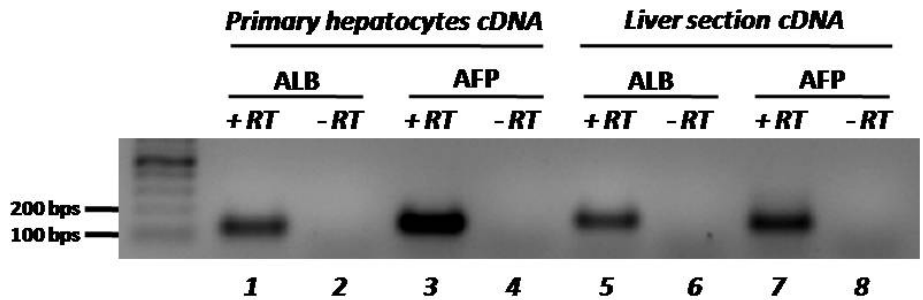

Fig 35 – RT-PCR analysis of isolated murine primary hepatocytes. To verify the cellular type origin of mouse liver isolated cells RT-PCR was performed to detect hepatic markers, albumin (ALB) and alpha-fetoprotein (AFP). Total RNA was extracted from 48 hours cultured primary cells and from liver section and it was retro-transcribed and used as PCR template. ALB and AFP specific primer pairs amplified respectively a 123 bps region on the albumin transcript and a 137 bps region on the alpha-fetoprotein transcript. RT-PCR amplified the specific products for both genes in primary hepatocytes sample (lanes 1, 3) and in liver section control sample (lanes 5, 7). No product was observable in RNA control samples, in which no retrotranscriptase was added (lanes 2, 4, 6, 8).

Luciferase infectivity assays were optimized to test virus entry into human and murine immortalized cell lines and into murine primary hepatocytes. To test HCV entry HIV pseudo-particles were produced as described by McKeating (Journal of Virology 2003;77-7:4070–80). 293T cells were cotransfected with a plasmid encoding an envelope-defective HIV-1 proviral genome expressing a luciferase reporter gene (NL4.3.Luc.R-E-) and a plasmid encoding full-length strain H E1E2 HCV glycoproteins. As control, plasmid encoding vesicular stomatitis virus glycoprotein (VSV-G) was cotransfected to generate pseudotype viruses with known entry characteristics. These pseudotype particles were tested to infect human hepatoma cell line (Hep3B), murine hepatoma cell line (Hepa1.6) and wild type mouse primary hepatocytes (**Fig 36**). As expected, HCV pseudotype particles entered in human Hep3B while murine Hepa1.6 and wild type mouse primary hepatocytes were resistant to the virus entry. Murine hepatic cells were instead permissive to VSV-G pseudoparticles entry.

Isolation and culture of murine primary hepatocytes and HCV pseudotype particles infection assay were successfully optimized, and we will use these model systems to study HCV entry into humanized mice cells.

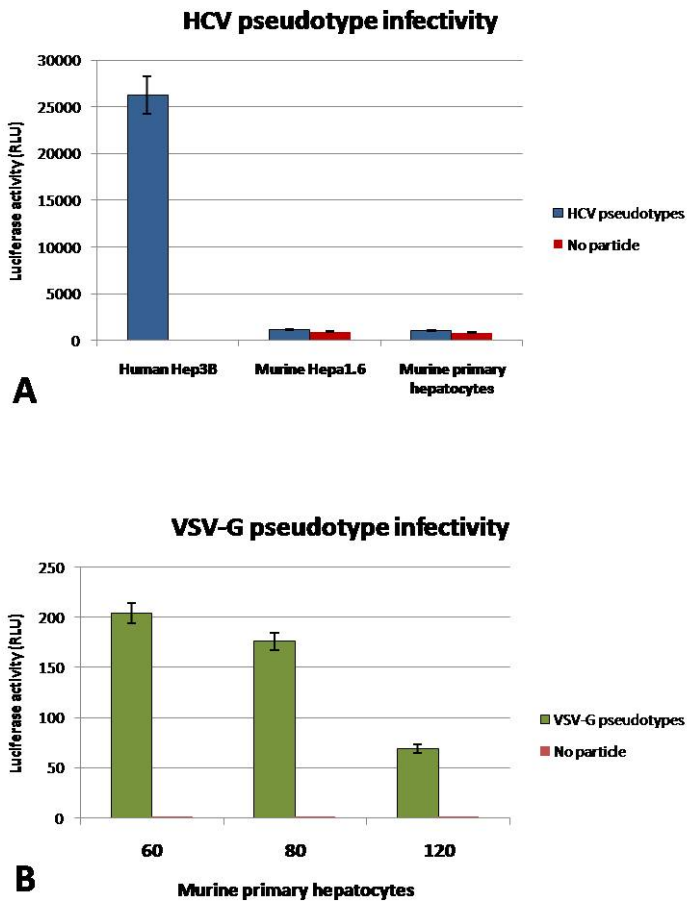

Fig 36 – HIV pseudo-particles infectivity. Panel A: HIV pseudotypes bearing strain H native E1E2 HCV glycoproteins (blue bars) were tested for their ability to infect human Hep3B, murine Hepa1.6 and wild type murine primary hepatocytes. No virus particle inoculation was performed for each line as control (red bars). All infections were performed in triplicate, on 120 x 10^3 cells, and the mean luciferase activity (RLU) is shown. Human Hep3B resulted permissive to HCV pseudotypes entry, while murine Hepa1.6 and wild type primary hepatocytes were resistant to particles entry. Panel B: VSV-G pseudotype particles were used to infect wild type murine primary hepatocytes (green bars); the infection was performed in triplicate, on 60, 80 and 120 x 10^3 cells, and luciferase activity ever resulted significantly higher than no virus particle controls (red bars).

5. CONCLUSIONS

Investigation of HCV replication and pathogenesis, such as development of HCV-specific antivirals and vaccines, has been hampered by the lack of small animal models of HCV infection and propagation.

In the last few years, genetic engineering advances have increased the ability to modify mouse genome and create inbred mouse models for studying biological processes *in vivo*.

In our study we designed a gene targeting strategy to produce novel humanized mouse strains expressing human-specific HCV entry receptors. Early results of receptor-mediated HCV entry suggested a critical role for human CD81 and SR-BI (Pileri et al. 1998, Dubuisson et al. 2003). Recent studies have identified human occludin as another HCV entry receptor and provided evidence that CD81 and occludin are the human factor essential to overcome the cross-species barrier at the level of entry (Ploss et al. 2009). Therefore, we generated three new knock-in mouse strains respectively for human CD81, SR-BI and occludin, as *in vivo* model systems to analyze HCV entry. We introduced the human genes for these receptors in the mouse genome by homologous recombination in ES cells. The human coding sequence of HCV receptors was specifically inserted in murine orthologues loci to achieve their native expression patterns. These modifications were successfully established in the genome of murine ES cells, which were used to generate inbred genetically modified mice. So, we produced humanized knock-in mouse strains for all three HCV receptors. Molecular analyses of genomic DNA extracted from mice tail biopsies confirmed the presence and germ line propagation of the human genes. Then, the effective expression of the inserted human genes was demonstrated at the transcription level analyzing messenger RNA extracted from different mice tissues including liver.

Until now, we produced heterozygous humanized mice for all three HCV receptors, and their inter-crosses will generate homozygous humanized mice for CD81 and SR-BI. Instead we reported prenatal lethality for homozygous humanized occludin mice. We demonstrated that homozygous condition for occludin locus humanization lead to mouse mortality in the early embryonic development, between morula and blastocyst stages. Since occludin null mice, though viable, have several defects (Saitou et al. 2000), we asked whether the observed embryo lethality could be due to a not sufficient expression of human gene. We performed quantitative analysis of occludin transcripts in heterozygous mice and we demonstrated that the expression level of human occludin was comparable than the expression level of murine occludin. We also analyzed occludin protein expression and demonstrated that humanized occludin heterozygous than wild type mice did not express lower levels of liver occludin protein and no different molecular weight protein was generated in liver. A critical role of occludin in preimplantation mouse embryo development was reported (Kim et al. 2004). Furthermore, mouse blastocyst formation was regulated by post-translational control of occludin (Sheth et al.

2000). Occludin amino acidic substitutions introduced to humanize the mouse could compromise interactions with its partners, such as ZO-1, and post-translational modifications of the protein, such as phosphorylation and glycosylation. These putative molecular alterations could impair the blastocyst development and explain embryo lethality observed in our homozygous occludin humanized mice. Further experiments have to be performed to correlate human occludin homozygous expression to the observed mortality, and to verify the molecular causes of this phenotype.

To study HCV entry into our humanized mice cells, we successfully optimized isolation and culture of murine primary hepatocytes and HCV pseudotype particles infection assay. We will perform this assay on primary hepatocytes isolated from heterozygous and homozygous mice expressing different combination of human entry factors. The heterozygous expression of human receptors could be sufficient to mediate HCV entry into the hepatocyte. However we could generate homozygous mice for both human CD81 and SR-BI, whereas only heterozygous humanized occludin mice are available.

Although CD81 and occludin represent the minimal human specific entry factors while murine SR-BI can mediate HCV entry in cell culture model systems, we included human SR-BI in our *in vivo* model since it has been demonstrated that HCV pseudoparticles entry in NIH3T3 murine cell line co-transfected with human entry receptors was enhanced by the overexpression of human SR-BI in the context of the other human proteins (Ploss et al. 2009). Furthermore, as HCV circulates in the serum associated to lipoproteins and SR-BI interacts with lipoproteins, this receptor could have *in vivo* human-specific entry properties undetermined by the *in vitro* studies.

Our humanized mice could be an interesting tool for analyzing HCV infection *in vivo*. HCV pseudotypes infection assay will demonstrate if the specific expression of chosen human receptors in knock-in mouse renders its cells permissive to HCV entry. These next results will provide the evidences to define our humanized mice as a new inbred model for the *in vivo* study of HCV-host interactions.

6. ACKNOWLEDGEMENTS

I thank Prof. Mario De Felice for giving me the opportunity to work at this project in his lab, for guiding me with patience and helpfulness in this scientific experience. I am grateful to the researchers Dr. Concetta Ambrosino, Dr. Donatella Malanga, Dr. Francesca Rizzo and Dr. Geppino Falco for their invaluable assistance and friendship. I would like to express my thanks to all colleagues and students who have worked with me in these years, for their friendship and professional collaboration. Most thank go to my friends Pina and Viviana.

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