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Mice with humanized liver endothelium

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Chapter 1

General Introduction

The Liver

The liver is one of the largest organs in the human body accounting for approximately 2-5% of total body weight (1). It plays an important role in essential liver functions including detoxification of metabolites, production of various proteins such as clotting factor VIII, storage of vitamins and minerals and the production of bile. Another remarkable characteristic of the liver is its capacity to regenerate to its original size and volume following injury. The regenerative capacity of the liver allows it to restore lost mass without jeopardizing viability of the entire organ. In this chapter, background information will be given about the various characteristic features of the liver, which demonstrate that the liver is a vital and unique organ making it impossible to reconcile life without its presence.

Liver Anatomy and Function

The healthy human liver has a brown reddish color and is situated in the peritoneal cavity. The liver is divided into lobes, which in turn are further divided into lobules. The majority of the cells in the liver are the hepatocytes. They make up approximately 75% of the total liver mass (1). Hepatocytes are polarized cells that are arranged along cords of one cell thickness. They excrete bile into canaliculi on the apical surface of the hepatocytes. Bile from the canaliculi is drained into bile ducts lined by cholangiocytes and further transported to the gall bladder for storage (2, 3). The basolateral microvillus covered surface of the hepatocytes is exposed to the space of Disse. The space of Disse is separated from the blood flow by the sinusoidal liver endothelium as shown in the enlargement of figure 1. The space of Disse is continuous with lymphatic vessels found at the portal triad. Plasma collected at the space of Disse flows back to the lymph vessels at the portal vein (4).

Even though hepatocytes are key players in the exertion of many liver functions, they can only function properly alongside the cholangiocytes, Kupffer cells, hepatic stellate cells and endothelial cells, which are termed the non-parenchymal cells. Cholangiocytes are epithelial cells that contribute to bile secretion through release of bicarbonate and water (5). Stellate cells, which were first recognized by Toshio Ito in 1950, reside in the peri-sinusoidal space of Disse, store high levels of vitamin A in lipid droplets and contribute to synthesis of extracellular matrix (6, 7). Also located in the space of Disse are the Kupffer cells, which were first described by Karl Wilhelm Von Kupffer in 1876. They are specialized macrophages and play an important role in local liver innate immunity through phagocytosis of antigens and

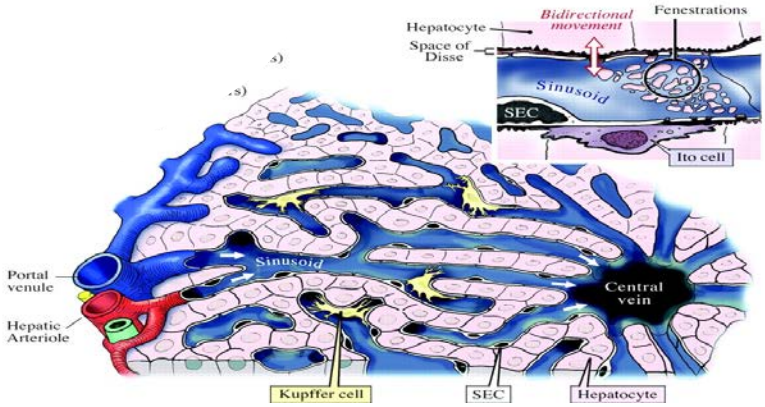

Figure 1. Microscopic presentation of hepatic architecture of a lobule and sinusoid.
In healthy liver, hepatocytes are arranged along cords and surrounded by fenestrated endothelium. The space of Disse is located between the hepatocytes and the sinusoidal endothelial cells (SEC). Kupffer cells reside within the sinusoids (8).

micro-organisms and production of cytokines (9). Finally, the largest group of non-parenchymal cells is formed by the endothelial cells, which make up approximately 3% (10) of the total liver cell mass, or 10-20% of total number of liver cells (11, 12). This cell type plays an important role in this thesis and will be discussed in more detail below.

Liver Sinusoidal Endothelial Cells

Endothelial cells are present throughout the human body and form the inner lining of blood and lymph vessels. Endothelial cells display phenotypic heterogeneity and can be divided in three major groups (figure 2). The first group is termed continuous endothelium and contains endothelial cells that are tightly connected to each other and are surrounded by a basement membrane. The basement membrane consists of collagen IV, collagen VI, fibronectin and tenascin amongst others (13). In continuous fenestrated endothelium, endothelial cells have fenestrae, which are cytoplasmic pores that extend through the full thickness of the cell. The fenestrae in this second group of endothelial cells possess a diaphragm across their opening. The third group is called the discontinuous endothelium (14). Liver sinusoidal endothelial cells (LSEC) constitute the sinusoidal wall and belong to this type of endothelium. Morphologically they are characterized by the presence of fenestrae, which are arranged in sieve plates. The fenestrae of liver sinusoidal endothelial cells lack, in contrast to continuous

fenestrated endothelium, a diaphragm and the basement membrane is poorly formed or absent. The diameter of the fenestrae is dynamic, between 100-200 nm, and influenced by hormones, drugs, toxins and diseases (15). Furthermore, while the size of the fenestrae decreases from the periportal to the centrilobular region, the number of fenestrae increases (16). The fenestrae act as a sieve, allowing bidirectional exchange of fluids and particles between the sinusoidal blood and the hepatocytes via the space of Disse. Endothelial cell transport can occur through receptor-mediated endocytosis and transcytosis (17). Hyaluronic acid and acetylated low-density lipoprotein are among the macromolecules that are taken up by the liver sinusoidal endothelial cells.

Phenotypic characterization of liver sinusoidal endothelial cells by the expression of cell membrane proteins to distinguish them from other (endothelial) cells remains problematic. Liver sinusoidal endothelial cells have been shown to express CD31 or platelet adhesion molecule 1 (PECAM-1), a transmembrane glycoprotein belonging to the immunoglobin superfamily. It is expressed on all types of endothelial cells and some hematopoietic cells, functioning as an adhesion and signaling receptor (18). Liver sinusoidal endothelial cells can be isolated from the human liver using magnetically labeled antibodies directed against CD31 as demonstrated by our experiments described in the present thesis. *In vitro* studies have shown that liver sinusoidal endothelial cells also express CD34, CD105, and CD144, also known as lymphatic vessel endothelial receptor 1 (LYVE-1) (19-21). Since the above mentioned markers are also expressed by other cell types, caution must be used in the identification of liver sinusoidal endothelial cells by cell surface markers.

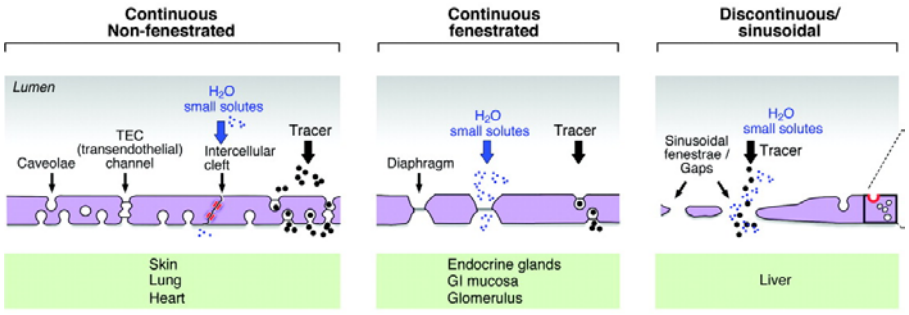

Figure 2. Phenotypic heterogeneity of endothelium.
Endothelial cells can be divided in three major groups, continuous non-fenestrated, continuous fenestrated and discontinuous fenestrated endothelium (14).

The role of liver sinusoidal endothelial cells in immunity

The liver is exposed to pathogens as well as to dietary antigens derived from the gastro-intestinal tract. While pathogenic micro-organisms must be efficiently cleared from the liver, unnecessary activation of the systemic immune system should be avoided in order to prevent possible damage to hepatocytes. Instead of inducing a systemic immune response, the liver seems to favor the induction of tolerance to antigens (20, 22). Interestingly, the liver seems to be capable of actively inducing peripheral tolerance as systemic specific-antigen tolerance is achieved following intra-portal antigen application (23-25).

Liver sinusoidal endothelial cells are exposed to a variety of antigens and play an important role in the induction of tolerance by the liver through local regulation of the immune response (22). Leucocytes that pass through the sinusoids adhere to liver sinusoidal endothelial cells by interacting with CD54 and CD106 (26, 27). Liver sinusoidal endothelial cells can control leucocyte adhesion under influence of the local microenvironment resulting in increased leucocyte adhesion by strong inflammatory stimuli.

Besides leucocytes, T cells also pass the liver allowing for direct interaction with Kupffer cells and liver sinusoidal endothelial cells (28, 29). Similar to Kupffer cells and stellate cells, liver sinusoidal endothelial cells act as antigen presenting cells in the liver. They present antigen to CD8+ T cells and CD4+ T cells via major histocompatibility complex (MHC) class I and II molecules respectively (30) alongside the expression of CD40, CD80 and CD86 (31). Even though antigen presentation is a feature shared by other types of endothelial cells (32-34), liver sinusoidal endothelial cells are unique as they are able to endocytose, process and present antigen to T cells without prior stimulation with pro-inflammatory molecules such as interferon-γ (35, 36). Furthermore, liver sinusoidal endothelial cells have been found to express CD4 (37), the mannose receptor (38) and low levels of CD11c (39) making them resemble dendritic cells.

Sinusoidal endothelial MHC class I interaction with CD8+ T cells is believed to result in immune tolerance to prevent excessive immunological response to various dietary antigens from the gastro-intestinal tract (22). Previous studies showed that naïve murine host CD8+ T cells stimulated by liver sinusoidal endothelial cells failed to develop towards cytotoxic effector cells, finally leading to antigen specific tolerance (40). Furthermore, murine host CD4+ T cells, which were primed by donor liver sinusoidal endothelial cells, failed to differentiate into

effector Th1 cells. Instead they expressed high levels of IL-4 and IL-10, which are immune suppressive mediators (31). This mechanism of tolerance induction may in part explain the relatively high tolerance of transplanted donor liver by the allogeneic host (20, 41).

Liver Development

Development of the liver, also known as hepatogenesis, is an evolutionary conserved process that involves the interaction of endodermal and mesodermal germ layers and begins in the mouse at embryonic day 8 (42). In the earliest stages of embryonic development, ectodermal, endodermal and mesodermal germ layers are established. The endodermal germ layer forms the primitive foregut tube, which is subdivided in the foregut, midgut and hindgut region. The embryonic liver originates from the ventral foregut endoderm as a thickening of the ventral endodermal epithelium, following signals from the adjacent cardiac mesoderm, forming the hepatic diverticulum (1, 43) (figure 3). In the next step, cells from the hepatic diverticulum invade the adjacent septum transversum mesenchyme to form the liver bud (figure 3) (44, 45).

The caudal part of the hepatic diverticulum will develop into the extrahepatic bile ducts, the cystic duct and the gall bladder, while the cranial part of the hepatic diverticulum will eventually give rise to bipotential fetal hepatoblasts (46, 47). The hepatoblasts of the hepatic diverticulum will later differentiate in either hepatocytes or cholangiocytes, while the septum transversum mesenchyme contributes the fibroblasts and stellate cells of the adult liver (46, 48).

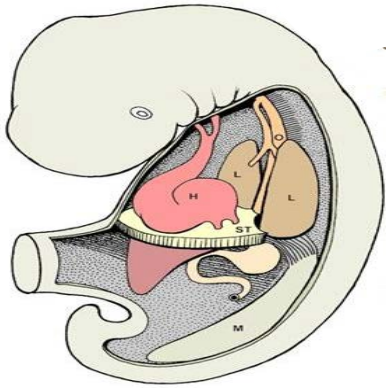

Figure 3. Embryonic development of the liver. Schematic overview of an embryo at the fourth week of gestation. At this stage cells from the hepatic diverticulum invade the adjacent septum transversum mesenchyme to form the liver bud. L = lung, H = heart, ST = septum transversum, M = mesonephros. www.bionalogy.com/respiratory_system.htm

Development of liver vasculature

In the embryo, *de novo* development of the vasculature occurs through a process called vasculogenesis by angioblasts. Angioblasts are endothelial progenitor cells (EPC) that have the capacity to differentiate into mature endothelial cells. Peichev *et al.* were able to isolate endothelial progenitor cells from human fetal liver and characterized them based on the expression of cell surface markers CD34, vascular endothelial growth factor receptor 2 (VEGFR-2) and CD133 (49). However, the primary vasculature is extended by sprouting of new capillaries from pre-existing mature endothelial cells by a process termed angiogenesis (50-52). As the liver bud is further generated by expansion of hepatoblasts, the hepatic vasculature starts to develop by vasculogenesis as well as angiogenesis. At this stage, the general architecture of the adult liver, which is crucial for normal liver function, is established. Mouse studies have shown that endothelial cells play a crucial role in controlling the growth of the hepatic diverticulum before the embryonic liver vessels, that supply blood and oxygen, are even developed (53). The development of the centrilobular veins and portal arteries, through vasculogenesis, is preceded by the development of portal veins and sinusoids (54). Portal veins and sinusoids differentiate from pre-existing vessels through angiogenesis. The portal veins are derived from vitelline veins, which are developmental veins that run from the yolk sac and form a plexus around the duodenum before entering the sinus venosus of the heart (54).

The sinusoids either develop from cells that originate in the capillary vessels of the septum transversum or from the disruption of pre-existing vitelline veins (46, 48, 54, 55). They acquire their characteristic features through a multistage process starting with non-fenestrated continuous endothelial cells lining at the fourth week of gestation. In humans, between the 10^{th} and 17^{th} week of gestation, the subendothelial basement membrane is lost, endothelial fenestrations develop and the composition of extracellular matrix changes being rich in tenascin and poor in laminin (55-57). After this first phase of liver sinusoidal differentiation, which ends approximately around the 12^{th} week of gestation, a second differentiation phase takes place. The second phase ranges from the 12^{th} to the 20^{th} week of gestation and is characterized by the appearance of cell surface markers such as CD4, a glycoprotein involved in immune mechanisms (48). The final and third stage of liver sinusoidal differentiation takes place from the 20^{th} week of gestation up to the perinatal period, when definitive structural differentiation of the sinusoidal endothelium is achieved (58).

Liver Hematopoiesis

The structural changes that occur in the liver sinusoidal endothelial cells during the first phase of sinusoidal differentiation coincide with the beginning of the hematopoietic function of the fetal liver (46, 48). Human hematopoiesis starts outside the embryo in the yolk sac and from cells originating in the embryonic aorta and vitelline artery (59, 60). Hematopoiesis then transiently proceeds to the liver and is ultimately established and stabilized in the bone marrow. The yolk sac mainly sustains primitive erythropoiesis enabled by mesoderm-derived blood islands, whereas hematopoietic stem cells derived from within the embryo are in addition capable of lymphopoiesis (61-63). The fetal liver is colonized by hematopoietic progenitors twice, first by stem cells from the yolk sac followed one week later by stem cells from within the embryo (figure 4).

Hematopoiesis however can only exist through dynamic interaction with the parenchymal compartment. Fetal liver stromal cells – which is a heterogeneous population of cells including hepatoblasts, fibroblasts and endothelial cells – have been shown to enhance hematopoietic stem cell proliferation through Wnt signaling, whereas after inducing hepatoblasts to differentiate to a mature form using oncostatin M, the ability to support hematopoiesis is diminished (64, 65). Interestingly, the structure of the sinusoids of the fetal liver resembles that of the hematopoietic bone marrow as both tissues contain sinuses that are lined by fenestrated discontinuous endothelium (66) and are devoid of a basement membrane containing laminin (48).

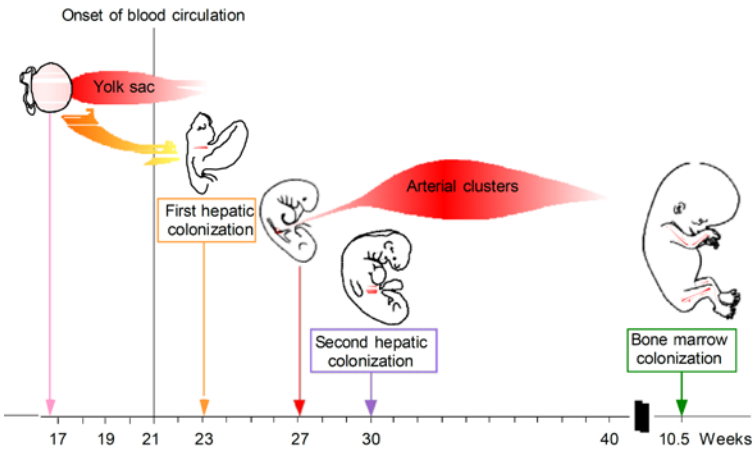

Figure 4. Chronology of appearance of hematopoietic stem cells in the developing human embryo. The fetal liver is first colonized by stem cells from the blood islands of the yolk sac followed by colonization of intra-embryonic stem cells (61).

Vascular origin of hematopoietic cells

Hematopoietic stem cells give rise to myeloid (including monocytes, macrophages, erythrocytes and dendritic cells amongst others) and lymphoid (T-cells, B-cells and natural killer (NK) cells)) cells. They are known to express cell surface markers CD34 (67), CD31 (68, 69), CD90, CD117 and CD133 (70, 71), In keeping with their immaturity, they do not express lineage-specific antigens as CD38 and CD45RA for example and are referred to as lineage negative cells (Lin^- cells) (70). The first human hematopoietic stem cells are produced in the yolk sac in the third week of development (72). Fusion of multiple blood islands in the yolk sac ultimately forms the capillary network of the yolk sac. Hematopoietic cells emerge in close physical association with endothelial cells in the yolk sac. Hematopoietic cells are generated at the center of the blood islands, whereas endothelial cells are produced from endothelial progenitor cells at the periphery of the blood islands (73). In addition to this physical association, hematopoietic stem cells and endothelial progenitor cells share several cell surface markers such as CD31, CD34, VEGFR-2 and CD133 suggesting a common mesodermal ancestor called the "hemangioblast".

The existence of the hemangioblast was first postulated by Sabin and Murray in the early 20^{th} century (74, 75). Later studies using embryonic stem (ES) cell cultures amongst others confirmed the existence of the hemangioblast (76-78). Besides angioblasts and hematopoietic stem cells, the hemangioblast can also give rise to a subset of vascular endothelial cells, which have a transient blood-forming capacity in the blood islands of the yolk sac and embryonic aorta (79-81). These blood-forming endothelial cells are also called hemogenic endothelium. The emergence of multilineage hematopoietic stem cells from endothelium was demonstrated by lineage-tracing studies and *in vivo* imaging amongst others (82-84). Blood-forming endothelial cells have also been isolated from embryonic liver and fetal bone marrow (60, 85-88). In an attempt to distinguish hemogenic endothelium from non-hemogenic endothelium, studies showed that hemogenic endothelium expresses CD143, Angiotensin Converting Enzyme (60, 85, 89).

Liver Regeneration

The liver has the remarkable capacity to regenerate, after loss of up to 75% of hepatic cell mass complete regeneration can occur. Liver regeneration has been studied extensively in mice, after partial hepatectomy, a surgical procedure which removes 2/3 of the liver mass, liver mass is restored within 5-7 days. In humans

regeneration is slower, with complete regeneration after hepatectomy occurring after 8-15 days (90). Liver regeneration is a tightly controlled process, which involves all cell types of the liver and which is coordinated by signals from various growth factors, including epidermal growth factor (EGF), hepatocyte growth factor (HGF) and insulin that are produced within the liver as well as released from extra-hepatic organs. Following 2/3 partial hepatectomy, hepatocytes are the first to proliferate during the first 3 days starting in the periportal areas and then proceeding to the pericentral areas forming clumps of small hepatocytes. These clumps are then invaded by stellate cells initiating the restoration of the normal hepatic architecture (90-92). The hepatic vasculature is then remodeled by existing liver sinusoidal endothelial cells through angiogenesis (93). Proliferation of hepatocytes is initiated by VEGFR-2 activation at the cell surface of liver sinusoidal endothelial cells with subsequent up-regulation of transcription factor Id1 in endothelial cells and secretion of HGF and Wnt2 angiocrine factors (94). Ding *et al*. have shown that besides the production of HGF and Wnt, direct contact between hepatocytes and liver sinusoidal endothelial cells is necessary for the first wave of hepatocyte proliferation to occur. Furthermore, their study shows that the VEGFR-2 - Id1 pathway is important in the restoration of liver vasculature. Regeneration is eventually terminated by apoptosis of hepatocytes to correct for over-shooting of the regenerative response (95).

In the normal liver, regeneration following injury is accomplished by quiescent hepatocytes that re-enter the cell cycle from G0 to G1. Studies have shown that when the remaining mature hepatocytes are incapable of proliferation due to injury or chemical inhibition, liver regeneration is performed by hepatic stem cells, also known as the oval cells in rodents (96-98). Oval cells are small cells with a large nucleus, which have the potential to differentiate in either hepatocytes or cholangiocytes and reside in the smallest and most peripheral branches of the biliary tree, the canals of Hering (2).

However, despite the capacity of the liver to regenerate following injury, inadequate liver regeneration forms an unresolved problem and may have a lifelong impact on both recipients and living donors (99). Studies that increase our understanding of the regeneration process and aim at improving the regeneration capacity of the liver would thus greatly benefit this group of patients.

Liver Disorders

Considering the large amount of functions that are performed by the liver, many different liver disorders have been described. These liver disorders form an important health problem and can be divided into acquired and inherited liver disorders. Among the acquired disorders viral hepatitis and (non-)alcoholic steatohepatitis are the most prevalent causes of liver disease with an increased risk of developing cirrhosis and subsequently hepatocellular carcinoma. Inherited liver disorders are less common and can be grouped in disorders that primarily affect the liver or disorders that lead to extra-hepatic manifestations. Examples of disorders that cause primary liver disease are Wilson's disease, α1-antitrysin deficiency and progressive familial intrahepatic cholestasis (PFIC). These rare disorders are caused by genetic mutations in the hepatocytes that lead to accumulation of toxic substances, which in turn damage the hepatocytes. Inherited disorders such as the hemophilia's and Crigler-Najjar syndrome (100) are characterized by the fact that even though the genetic mutation affect liver function, patients suffering from these disorders exhibit mainly extra-hepatic symptoms (101, 102).

Treatment of Liver Disorders

Nowadays, the only curative treatment option for many patients suffering from an inherited liver disorder is a whole liver transplantation, also referred to as orthotropic liver transplantation (OLT). In the years from 1968 to 2010 approximately 100,000 transplantations have been performed in Europe. According to the European Liver Transplant Registry, 6% of patients were transplanted because of a metabolic liver disease. Even though the number of successful transplantations has increased considerably over the past years, shortage of donor organs remains. Not all patients can benefit from this procedure and die while waiting for transplantation (103). Furthermore, between 10-20% of transplanted patients require a retransplant (104). Therefore, curative treatment options besides whole liver transplantation need to be investigated.

Cell transplantation

Instead of whole liver transplantation, patients suffering from a liver disorder could also be helped with liver cell transplantation. Cell transplantation offers several advantages over whole liver transplantation. Cells from a single donor can be used for multiple recipients, the procedure is less invasive and cells may be less immunogenic than solid organs. First experiments exploring this technique were

performed in 1976 (105) and hepatocyte transplantation was first applied in humans in 1992 (106). Transplantation studies have shown that the liver is the most optimal site for transplanted hepatocytes to survive and function properly (107). Hepatocytes can be delivered to the liver by intrasplenic or intraportal infusion (108, 109). Approximately 70-80% of transplanted cells that reach the sinusoids are destroyed within a day or two. The remaining fraction of transplanted cells has to cross the sinusoidal barrier before they can engraft into the hepatic parenchym of the host (110). Transplantation of hepatocytes in rats with normal healthy livers leads to an engraftment of only 0.5-3% (111, 112). Following engraftment, replacement of host hepatocytes by transplanted donor cells, a process known as repopulation, can only occur if the transplanted cells have a growth advantage over the host hepatocytes. This occurs in diseased animal models such as the multidrug resistance (mdr2) knockout mouse, a model of PFIC III. The hepatocytes of these mice have a defective export pump that belongs to the superfamily of the ATP-binding cassette (ABC) transporters on their canalicular membrane. As a consequence, these mice fail to secrete phospholipids in their bile causing chronic bile-salt induced damage to hepatocytes when fed with a high bile salt diet. When these mice are transplanted with healthy hepatocytes, the liver is repopulated by the transplanted cells (113, 114). Extensive repopulation of transplanted hepatocytes also occurs in fumarylacetoacetate hydrolase (FAH) deficient mice, which is a model for hereditary tyrosinemia type I in humans. Accumulation of toxic metabolites of the tyrosine catabolic pathway in the hepatocytes of these mice can be prevented by administration of NTBC (2-(2-nitro-4-trifluoromethyl-benzoyl)-1,3-cyclohexanadione). Transplantation of healthy mouse or human hepatocytes in FAH (immune)-deficient mice and discontinuation of NTBC treatment causes cell death in host hepatocytes, but not in transplanted cells leading to near total liver replacement of donor cells (115, 116).

However, repopulation of transplanted cells does not occur in liver disease where the liver remains unaffected as is the case in disorders as hemophilia A or Crigler-Najjar syndrome. To ensure repopulation in these disorders, transplanted cells need a selective growth advantage over the host hepatocytes. In experimental models, this situation can be created by inducing liver injury to the host hepatocytes prior to transplantation using a variety and combination of chemicals such as galactosamine, carbon tetrachloride and monocrotaline (111, 117, 118). Transplantation experiments performed in rodents have shown that a combination

of treatment with retrorsine or monocrotaline followed by 2/3 partial hepatectomy led to repopulation by the transplanted hepatocytes (118, 119). Retrorsine and monocrotaline are naturally occurring structurally related pyrrolizide alkaloids, which are metabolized to bioactive compounds in the liver were they can inhibit proliferation of host hepatocytes by alkylating proteins and DNA (120, 121). Additional toxicology studies have shown that liver sinusoidal endothelial cell damage precedes hepatocyte injury (122, 123). Monocrotaline causes endothelial cell death leading to denudation of liver sinusoids and enhancing engraftment of transplanted hepatocytes (124, 125). Unfortunately, most of these drugs are toxic and not suitable for clinical application.

The success of hepatocyte transplantation in animal models led to several clinical trials of hepatocyte transplantations in patients suffering from inborn errors of metabolism as well as chronic (106, 126) and acute liver diseases (127, 128). The most successful outcome of hepatocyte transplantation has been for patients that suffer from an inborn error of metabolism (129). In a study performed by Dhawan *et al*. factor VII deficiency was treated with intraportal hepatocyte infusions in two brothers which reduced the need for exogenous factor VII by 80% (130). Other examples of inborn errors of hepatic metabolism that were treated with hepatocyte transplantation are familial hypercholesterolemia (131-133), α1-antitrypsin deficiency (134) and Crigler-Najjar Syndrome type I (135, 136). Unfortunately, the long-term results of these clinical studies have thus far been disappointing.

Stem and progenitor cells as alternative sources for liver-directed cell transplantation

Major obstacles in clinical implementation of hepatocyte transplantation are the low engraftment yield, the limited availability of donor cells and the difficulty of manipulating hepatocytes *in vitro*. These problems have encouraged the use of stem cells as an alternative cell source with the aim to generate mature and functionally active hepatocytes. Stem cells can be isolated from the bone marrow, but also from the adult and fetal liver. The human fetal liver is a rich source of various stem and progenitor cells including the hepatoblasts. As mentioned earlier, hepatoblasts can differentiate into either mature hepatocytes or cholangiocytes (47). Several groups studied the engraftment and repopulation capacity of hepatoblasts isolated from human fetal livers (137-139). Following transplantation of hepatoblasts from human fetal liver at 11 to 13 weeks of gestation in a non-

conditioned athymic mouse, a repopulation success of 10% was found (137). However, transplantation studies performed by other groups showed only marginal capacities of repopulation by human fetal liver hepatoblasts (140-142). These varying results emphasize the need for further studies into the potential of hepatoblasts as an alternative cell source.

The human fetal liver is an important source of hematopoietic stem cells consisting for up to 60% of blood cells (43). Hematopoietic stem cells isolated from the human fetal liver have proven to be very useful in developing mice with a humanized immune system (143, 144). Studies have explored the capacity of bone marrow-derived hematopoietic stem cells to differentiate into a variety of non-hematopoietic cell tissues such as heart and brain (145, 146). Furthermore, some studies have suggested that the bone marrow is an alternative source of (oval) stem cells that can give rise to mature hepatocytes (147, 148). Bone marrow-derived cells have been shown to promote liver regeneration and decrease hyperbilirubineamia in the Gunn rat (149). Subsequent studies, however, have shown that the apparently normal bone marrow-derived hepatocytes were the result of fusion between hematopoietic stem cells and host hepatocytes (98, 150).

The presence of recipient endothelial cells in donor liver biopsies several months after liver transplantation has raised the question whether circulating (endothelial) progenitor cells contribute to repair of damaged liver endothelium (151, 152). Ashara *et al*. were the first to demonstrate that damaged vascular endothelium can be repaired by endothelial progenitor cells, which are derived from hematopoietic stem cells and circulate in the peripheral blood (153). Besides peripheral blood, endothelial progenitor cells have also been isolated from cord blood and bone marrow (154, 155). Endothelial progenitor cells were able to improve chronic liver injury in mice and rats (156-162). According to these studies, endothelial progenitor cells differentiated into endothelial cells and incorporated in the liver vasculature. In contrast, recent studies in hemophilia A mice did find phenotypic correction following transplantation of healthy bone marrow, but this was not due to differentiation of the transplanted cells in endothelial cells, but to donor bone marrow derived mononuclear and stromal cells, which expressed FVIII mRNA and protein (163).

Gene Therapy

Another treatment option for patients suffering from a liver disorder is gene therapy. The goal of gene therapy is permanent cure of disease by introducing therapeutic genes in (diseased) target cells. This genetic modification of cells can be achieved by gene transfer using viral vectors. The vectors can be administered directly into the patient, a procedure known as *in vivo* gene therapy. In *ex vivo* gene therapy, target cells from normal donors or the patient's own cells are isolated and genetically modified *in vitro* and then administered to the patient.

Lentiviral vectors

Lentiviral vectors, which are derived from the human immune deficiency virus (HIV-1), belong to the group of integrating viral vectors and have the ability to transduce non-dividing cells efficiently (164, 165). Because they integrate, they are especially useful for long-term gene therapy in cells that can undergo replication. Lentiviral vectors are retroviruses and like all retroviruses, the RNA genome of the lentiviral vector is converted in double-stranded linear DNA by reverse transcription and integrated in the host genome (166). Over the years, lentiviral vectors have undergone multiple modifications to build in several safety features that minimize the risk of generating replication-competent virus (167, 167-169, 169, 170, 170). In third generation lentiviral vectors, the genes coding for the viral proteins are separated from the transfer vector (171). The transfer vector expresses the transgene sequence and the elements necessary for integration of the therapeutic gene into the target cells (169, 172, 173). The sequences for the viral proteins are divided over three plasmids. The *gag* and *pol* genes code for the structural and enzymatic proteins, which are required to form functional vector particles and are present on a single plasmid. The Rev protein regulates the nuclear export of the vector transcript and is coded by the *rev* gene present on a separate plasmid. The third plasmid contains the sequence for the envelope protein (171) (figure 5). Using this methodology, viral particles can be generated that transduce host cells and integrate into the host genome, but are incapable of viral replication.

Vector targeting

Viral infection of the target cell is dependent on the interaction between the envelope protein and cell surface molecules present on the target cells. The vascular stomatitis virus G (VSV-G) envelope protein has broadened lentiviral vector tropism, facilitating transduction of various cell types (174). In the past

years, other glycoproteins have been used to design different types of envelope proteins for lentiviral vectors, a process known as pseudotyping (175). While broad tropism of lentiviral vectors is suitable for *in vitro* cell transduction, *in vivo* gene therapy requires restricting gene transfer to the desired cell type relevant for a particular therapeutic application in order to improve safety and efficacy. Efficient long-term correction by gene therapy is hampered by an activated immune response following systemic administration of the viral vector. The immune system becomes activated as it recognizes the viral vector and/or the therapeutic protein as foreign, but also because of transduction of antigen presenting cells resulting in elimination of the transduced cells (176, 177). Studies modifying the Sendai virus fusion (SV-F) envelope protein improved hepatocyte specific transduction both *in vitro* as well as *in vivo* (178, 179). Restricted tropism of lentiviral vectors was also accomplished by fusion of the measles hemagglutinin envelope protein with a single chain variable fragment (scFv) with a high affinity for the target receptor of a variety of cell types (180-183). Further studies are needed to determine whether restricting vector delivery and transgene expression to the target cell type prevents the activation of the host immune response.

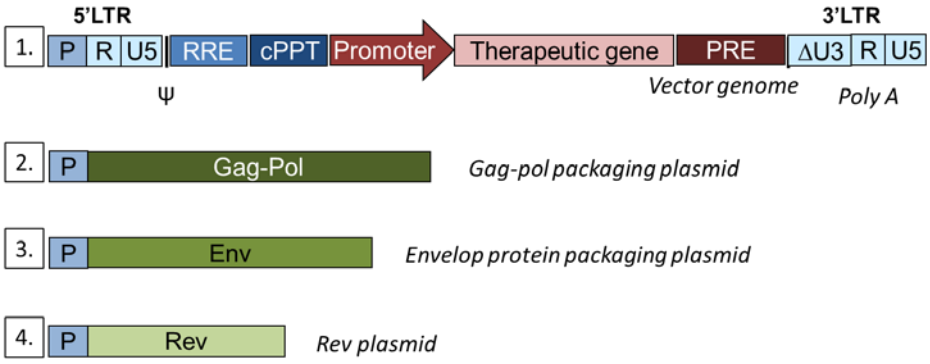

Figure 5. Lentiviral vector plasmid system

In third generation lentiviral vectors, the genes coding for the viral proteins are separated from the transfer vector. 1) The transfer vector expresses the transgene sequence and the elements necessary for integration of the therapeutic gene into the target cells. 2) The *gag* and *pol* genes code for the structural and enzymatic proteins, which are required to form functional vector particles and are present on a single plasmid. 3) The Rev protein regulates the nuclear export of the vector transcript and is coded by the *rev* gene present on a separate plasmid. 4) The fourth plasmid contains the sequence for the envelope protein.

Regulated gene therapy

The ability to control the level and timing of expression of a therapeutic gene is a desired feature for clinical implementation of gene therapy. Several controllable transgene expression systems have been developed (184-187). Among these systems, the tetracycline inducible system has been extensively studied with proven efficacy both *in vitro* as well as *in vivo* (188-191) and with successful incorporation in different viral vectors (192-194) (figure 6). In the original design of this system, expression of the transgene was only possible in the absence of tetracycline or its analog doxycycline. In the absence of tetracycline, the bacterial tetracycline repressor protein (TetR) binds to a specific DNA sequence, the tetracycline operator sequence (TetO), also referred to as the *tet* response element (TRE) with expression of the transgene situated downstream of the TetO. The TetR protein was fused to the VP16 activation domain from Herpes Simplex Virus generating the transcriptional activator protein (tTA) suitable for mammalian cells (195). Further modifications of the tTA protein led to the reverse phenotype, reverse tTA (rtTA), in which binding to the TetO with subsequent expression of the transgene was only possible in the presence of tetracycline or doxycycline (195). This system is also known as the "Tet-on" system. Additional improvements made the system more suitable for *in vivo* application with an increased sensitivity and responsiveness to low concentrations of doxycycline (196). Initially, the TetO and rtTA proteins were expressed from two separate constructs requiring co-transduction of cells making it difficult to work efficiently *in vivo*. This problem was solved combining all the elements into a single vector in an autoregulatory loop (197). The low basal expression of rtTA has proven to be sufficient to initiate the autoregulatory loop after doxycycline administration (197-199). rtTA is based on a bacterial gene and even though improvements have been made in the basal activity and sensitivity of rtTA, animal studies have detected immune responses against rtTA leading to clearance of transduced cells followed by loss of transgene expression (200-202).

Liver-directed cell and gene therapy

Cell and gene therapy can be combined in *ex vivo* liver gene therapy as an alternative approach in providing long-lasting treatment of liver disorders. Transplantation of genetically modified autologous hepatocytes not only overcomes limitations of shortage of donor hepatocytes, but also reduces the need for immunosuppression and raises the possibility of using freshly isolated – instead of cryopreserved- hepatocytes. In the Watanabe heritable hyperlipidemic

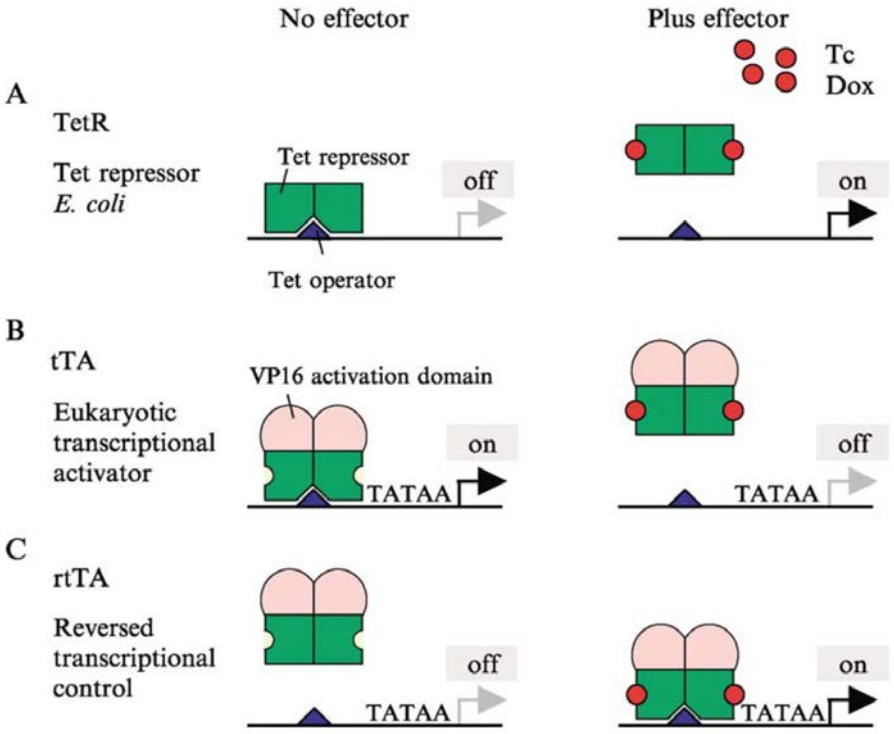

Figure 6. Regulation of gene expression using the tetracycline inducible system
A) In the absence of tetracycline, the bacterial tetracycline repressor protein (TetR) binds to the tetracycline operator sequence (TetO). B) The TetR protein was fused to the VP16 activation domain generating the transcriptional activator protein (tTA). C) The Tet-on regulatory system. Gene expression requires the addition of tetracycline or doxycycline (203).

rabbit, an animal model for familial hypercholesterolemia (FH), a decrease in serum cholesterol level of 30-50% was achieved following transplantation of autologous hepatocytes that were genetically corrected *ex vivo* with a recombinant retroviral vector (204). The positive results obtained in this study led to a clinical trial in patients with FH, which was carried out approximately 20 years ago. In this pilot study, three of the five patients showed a significant and prolonged reduction in low-density lipoprotein (LDL)-cholesterol following transplantation of autologous hepatocytes transduced *ex vivo* with a retroviral vector expressing the human LDL receptor (205). Even though the feasibility and safety of liver-directed *ex vivo* gene therapy were demonstrated, the observed transgene expression in the transplanted hepatocytes of these patients was at a low level (<5%) ending further clinical *ex vivo* liver gene therapy trials (131).

One of the obstacles that limited the clinical outcome in these studies was the low transduction efficiency of the transplanted hepatocytes by the oncoretroviral vector due to low *in vitro* proliferative ability of adult hepatocytes. In order to overcome this problem, the feasibility of *ex vivo* gene therapy was recently explored in the Gunn rat, which is the animal model for Crigler-Najjar syndrome type 1 using a lentiviral vector instead of the oncoretroviral vector. Hepatocytes of the Gunn rats lack bilirubin UDP-glucuronosyltransferase (UGT1A1) enzyme activity, leading to high serum levels of unconjugated bilirubin. Following transplantation of *ex vivo* transduced hepatocytes using a lentiviral vector, hyperbilirubinemia in Gunn rats was ameliorated by a decrease in bilirubin serum levels of 30% lasting at least 8 months (206). Significant progress has been made in *ex vivo* liver-directed gene therapy over the past years, leading to biosafety and efficacy studies in non-human primates (207-209). Unfortunately, low engraftment of transplanted genetically modified hepatocytes was found in these studies, emphasizing the need for additional studies before routine clinical implementation is achieved.

Scope and outline of the thesis

The only curative treatment option for many patients that suffer from a liver disorder remains whole liver transplantation. This is an invasive procedure, which is accompanied by increased morbidity and mortality. In search of alternative treatment options, several research groups have performed animal studies investigating liver-directed cell and gene therapy for the treatment of acute liver failure, chronic liver disease and inherited metabolic liver disorders (100, 210-212). The successful results encouraged scientists to perform clinical studies (129). Even though these clinical trials have shown that liver-directed cell and gene therapy is feasible, long-term outcome is disappointing and not as successful as the animal studies.

A problem in translating results of animal studies to a treatment for patients with liver disease is the difference in liver physiology between humans and rodents. A pre-clinical step usually involves testing new therapy strategies on primates, which is both expensive and ethically difficult. To avoid this, we set out to develop a mouse with a “humanized liver”, which would serve as an excellent *in vivo* model for studies on liver-directed cell and gene therapy.

A mouse with a humanized liver can be developed by transplanting human liver cells into the spleen of immune deficient mice. In the first set of experiments performed for this thesis, we found that human fetal liver sinusoidal endothelial cells were able to engraft and repopulate the mouse liver. This serendipitous finding set the basis for subsequent experiments with the aim to:

1) Identify cells suitable for liver engraftment
2) Use mice with humanized liver endothelium in gene therapy

In the present thesis we first show in **chapter 2** that transplantation of human fetal liver sinusoidal endothelial cells in immune deficient mice is feasible and provide a good alternative cell transplantation therapy. We compared the engraftment and repopulation capacity of human adult liver endothelial cells to fetal liver endothelial cells and performed transplantation experiments assessing the engraftment and repopulation potential of other types of endothelial cells.

As mentioned in the general introduction, the liver has a remarkable capacity to regenerate following injury and like hepatocytes, most of the regeneration of the liver vasculature is due to mitotic division of preexisting endothelial cells (213). However, the presence of recipient endothelial cells in donor liver biopsies several months after liver transplantation has raised the question whether circulating bone marrow-derived progenitor cells contribute to repair of damaged liver endothelium (151, 152). Since livers are well tolerated following transplantation, it has been hypothesized that replacement of donor endothelium by recipient progenitor cells may play a role in decreasing graft rejection (214). The human fetal liver is a rich source of progenitor cells. However, it is unknown whether human hematopoietic progenitor cells from the fetal liver have the capacity to differentiate into endothelial cells *in vivo.* In **chapter 3** we therefore induced angiogenesis by monocrotaline administration to immune deficient mice and subsequently analyzed the capacity of human hematopoietic progenitor cells to regenerate damaged mouse liver endothelium.

Adult liver cell transplantation is hampered by numerous factors such as poor engraftment of donor hepatocytes in the host liver and loss of hepatocytes after transplantation, despite the use of immunosuppression. Fetal liver cells are able to proliferate in cell culture and could therefore present an alternative source of cells for transplantation. The human fetal liver contains a mixture of cells including liver precursor cells called the hepatoblasts. In **chapter 4** we examined which fetal liver cell type can be most efficiently transplanted and used for liver-directed cell and gene therapy.

Clinical implementation of *ex vivo gene* therapy will require the ability to regulate the expression of genes to maintain expression levels within a therapeutic window (188) amongst others. The second objective of **chapter 4** was to investigate whether the use of human fetal liver cells in long-term *ex vivo* gene therapy is feasible by performing transplantation experiments using tetracycline induced regulated transgene expression.

Research in surface targeting of lentiviral vectors has recently led to the development of a lentiviral vector specifically targeting human endothelial cells (180). In this thesis we show that human liver endothelial cells can engraft and repopulate the liver of immunodeficient mice. This mouse model with a humanized liver endothelium makes it possible to test human liver endothelial cell directed gene therapy *in vivo.* **In chapter 5** we performed experiments to investigate whether human CD105-targeted lentiviral vectors can specifically transduce human endothelium following systemic administration.

Finally, in **chapter 6** the experiments and their results will be discussed and perspectives are given.

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