Title: NON-NATIVE LIVER GENERATION IN AN ANIMAL WITH IMPAIRED NATIVE LIVER FUNCTION BY CELL IMPLANTATION

Abstract

Animals with impaired native liver function harboring a functioning non-native liver derived from implanted non-native cells, and a method of producing such animals are described.
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Description

Non-Native Liver Generation in an
Animal With Impaired Native Liver Function,
by Cell Implantation

Technical Field

The invention relates to non-native functioning liver
 generation in animals with impaired native liver function, and
to (transgenic) animals harboring functioning non-native livers.

Background Art

There is a current notable shortage of donor livers. In
1990, illustratively, 2,656 liver transplants were performed, but
10 times more people died of liver disease. The treatment of
liver disease by the implantation of healthy liver cells has long
been envisioned as a potential solution to liver diseases.
Hepatocyte transplantation could be used to treat patients with
congenital hepatic enzyme deficiencies to replace liver lost
through disease (e.g. viral hepatitis, alcohol-related cirrhosis
or fibrosis), or provide a temporary support system where acute
liver dysfunction may be resolved by regeneration over a period
of time. Hepatocyte transplantation also offers the opportunity
to create animal models with human liver function. Such animals
would be very useful for the study of viral infections affecting
the liver (e.g. human hepatitis virus or cytomegalovirus
infection) and/or test the effectiveness and safety of treatment
or therapies for such infections. They would also be useful for
the study of chemical (e.g. alcohol) toxicity in the human liver
and chemical-induced cirrhosis and fibrosis and to test the
effectiveness and safety of treatments or therapies for
chemical-induced liver damage.

Historically, several methods have been pursued in an
attempt to create an auxiliary liver. Transplantation of liver
fragments had been carried out as early as the 1930s. However,
in most cases the transplanted liver tissue either degenerated
or disappeared within a few days or weeks.


In addition to problems in hepatocyte isolation, the method and site of implantation have been investigated for their effect on transplantation. Direct infusion of hepatocyte suspensions into the portal vein of rats has been investigated. However, contrary to initial beliefs, the portal blood supply was not found to be paramount to hepatocyte survival. Demonstrations have been made for hepatocyte growth following intramuscular injection, abdominal cavity injection, spleen, liver and kidney inoculation, and injection into celiac or renal arteries. While the above-mentioned methods have been able to produce small amounts of viable implanted hepatocytes, the resulting hepatic contribution to function has been relatively small and often temporary.

Another currently investigated approach to liver transplant resides in the implantation of new liver cells on scaffolds of biodegradable polymers that are hoped to regenerate into masses and take over at least some of the native liver's functions. Although it has been reported that with this approach liver cells on a matrix can survive and function in rats six months to a year after transplantation, implanted cell survival is low, and, in some animal trials, too much connective tissue grew into the matrix, binding tightly to the polymer and forming scar tissue preventing the liver cells from dispersing through the matrix

In a different vein, transgenic animal technology has provided the means to create and analyze animal models of genetic diseases affecting virtually any organ to which transgene expression can be directed. Because of its involvement in many diseases of medical importance, the liver has been a frequent target for this type of analysis.

Among transgenes reported to be associated with liver lesions are those encoding growth hormone, which alters hepatocyte size and nuclear characteristics; oncogenes, which induce hepatic tumors; the hepatitis B virus large envelope polypeptide, which induces hepatocellular necrosis and carcinoma formation in adults; transforming growth fact α, which causes both tumors and excessive liver growth; and a variant form of α₁-antitrypsin (AAT), which reproduces some characteristics of AAT deficiency disease in humans, including neonatal and adult hepatitis.

Because of the liver's large synthetic and secretory capability, targeting transgene expression to this organ also serves as a way to determine the systemic influence of overproduction of biologically potent molecules. This approach has previously been used to study the consequences of inappropriate plasminogen activator expression in vivo by introducing into mice a transgene bearing the urokinase-type plasminogen activator (uPA) coding sequence fused to the albumin enhancer/promoter (Heckel et al. Cell (19900 62:447-456).


In many transgenic founder mice the albumin-urokinase type plasminogen activator (Alb-uPA) construct directed high-level uPA expression to hepatocytes, resulting in elevated plasma uPA and fatal hemorrhaging in newborns. Two lines of Alb-uPA transgenic mice were established from surviving founder mice that expressed lower levels of uPA. In these lines, about half of the transgenic neonates bled into either the intestine or abdomen and died within four days after birth, whereas the remaining transgenic offspring appeared normal and survived. Both bleeding and non-bleeding transgenic neonates displayed marked hypofibrinogenemia and unclottable blood, and it was concluded that any injury sufficient to initiate bleeding was rapidly fatal in affected mice (Heckel et al. (1990), supra).

A general expectation in experiments of this type is that transgene expression will be stable over time in targeted cells. Unexpectedly, surviving mice in both of the Alb-uPA lines showed a gradual decrease in the level of plasma uPA activity accompanied by a restoration of clotting function that was complete between 1 and 2 months of age (Heckel et al., (1990) supra). This phenomenon has been explained by a report that uPA is cytotoxic for hepatocytes and that inactivation of transgene expression by DNA rearrangement in isolated hepatocytes in Alb-uPA mice is followed by repopulation of the entire liver by cells that no longer produce uPA (Sandgren et al, Cell (1991) 66:245-256). Thus, Alb-uPA expression provides a transgenic mouse in which endogenous transgene-expressing hepatocytes have a selected disadvantage relative to hepatocytes (native or non-native) that are not expressing the transgene. In addition, the livers in these mice provide an environment for growth of
hepatocytes (native or non-native) that are not expressing the transgene. These cells grow out at the expense of transgene-expressing cells and the result is livers with apparently normal architecture.

Disclosure of the Invention

Despite progress made in the implantation and development of mature hepatocytes, present technology has not made possible the generation of a (functioning) non-native liver into an animal, particularly one with defective liver function. In humans, such technology would be useful in liver disease therapy and/or treatment. In non-human animals such technology would have considerable value, e.g. to provide models of (diseased) human liver function useful to study potential treatment and/or therapies for human liver disease.

Accordingly, one object of the invention is to provide a method of correcting defective liver function in an animal host by implanting particular non-native cells (or corresponding tissue) into the liver region of a host animal with impaired native liver function to thereby generate a functioning non-native liver in the host and correct the impaired liver function. Another object of the present invention is to provide transgenic, non-human animals (e.g. rodents such as mice) harboring a transgene that encodes a product that is disadvantageous, but not lethal, to native liver cells and thus impairs native liver function. These transgenic, non-human animals are useful as a host system for non-native (e.g. human) liver. Another object of the present invention is to provide non-human host animals that can both maintain and expand a fully-functioning non-native (e.g. human) liver tissue useful for (1) modeling liver disease, (2) liver tissue banking (e.g. maintenance and expansion of liver tissue for later analysis and re-implantation back into donors), (3) testing pharmaceuticals for human liver toxicity in vivo, and/or (4) genetic manipulation
prior to re-implantation (i.e., hepatocyte-directed gene therapy). Another object of the invention is to provide a method of generating a functioning non-native liver in a host animal by implanting non-native liver cells (or tissue) into a host animal with a genetically established defective liver function. Another object of the invention is to provide non-human animals harboring a functioning non-native liver. Another object of the invention is to provide a novel method for maintaining full-differentiated, full-functioning donor (e.g. human) hepatocytes in an in vivo setting for genetic manipulation prior to return to the donors. The genetic manipulation could include, for example, introducing expression vectors that direct the production of medically important proteins in the transplant recipient.

It has been discovered by the inventors that the above objects of the invention and other objects which will become apparent from the description of the invention given hereinbelow are satisfied by implanting non-native cells (e.g., fetal, neonatal or adult, non-hematopoietic liver stem, progenitor or mature cells; fetal, stem, progenitor or adult bile duct cells; endodermal (e.g. pancreas or gut) cells; (cultured) totipotent stem cells; or cultured animal cells), or corresponding tissue, into the liver region of a host animal having impaired native liver function, such that the implanted cells (or tissue) colonize the host animal and develop therein a functioning non-native liver.

**Best Mode for Carrying Out the Invention**

Any animal having impaired native liver function may be used in accordance with the invention as a suitable host animal. Suitable host animals therefore include fish, fowl (e.g. chickens, ducks, geese, turkeys, etc.), or mammals such as rodents (e.g. mice or rats), guinea pigs, pigs, dogs, cats, rabbits, goats, sheep, horses, ruminants such as cows, monkeys, other non-human primates, as well as humans.
Impairment of native liver function in the host animal may have occurred accidentally, e.g. through disease, such as cirrhosis, hepatitis, fibrosis or a genetic liver deficiency disease such as deficiencies in Factor IX, Factor VIII, LDL receptor, or one of varied metabolic enzymes (e.g. phenylalanine hydroxylase), or any of the other hundreds of known metabolic diseases that affect the liver.

In a preferred embodiment, the invention is applied as therapy and/or treatment to such animals, including humans, suffering from liver failure. In this context, the invention is used as an alternative to liver transplant to restore liver function.

In another embodiment, a non-native liver is generated in a non-human animal in which liver insufficiency has been induced to obtain an animal model system with a functioning non-native (e.g. human or other animal) liver. In these non-human animals, impairment of native liver function may be purposefully achieved by compromising the native hepatocyte genetically so that they are at a selective disadvantage towards implanted non-native cells (e.g. hepatocytes).

In accordance with the invention, during normal turnover of hepatocytes, the implanted non-native hepatocytes have a proliferative advantage over the accidentally or purposely disadvantaged native hepatocytes and ultimately replace all of the native hepatocytes in the host. This provides a host harboring substantially few, if any native hepatocytes, but a functioning, substantially non-native, liver generated from the implanted non-native cells. (The liver generated is substantially non-native insofar as it is likely to comprise some non-hepatocyte cells found in the native liver and perhaps some native hepatocytes.)

Genetic inducement of liver insufficiency in a non-human
mammal may be achieved in accordance with this embodiment of the present invention with a transgenic, non-human animal harboring a transgene which encodes a product that is disadvantageous, but not immediately lethal, to native liver cells. Generally, any deleterious transgene product may be used that impairs cellular function to the extent that hepatocytes expressing the gene have a distinct growth disadvantage relative to cells that do not express a transgene (e.g. the implanted non-native hepatocytes).

Examples of such transgene products include any type of plasminogen activator, such as an urokinase-type or tissue-type plasminogen activator, or bacterial plasminogen activators (e.g. streptokinase) or toxins such as an attenuated diphtheria toxin. Other examples of transgene products useable in accordance with this embodiment of the present invention include agents that adversely affect the metabolism or growth potential of the native liver cells. Illustrative agents are agents which compromise the ability of the native liver cells to transmit growth factor signals (e.g. with dominant and negative transgenes affecting steps in signal transduction pathway) or by producing mutant proteins that clog circulatory pathway(s).

In an illustrative genetic inducement of liver failure, the expression of high levels of urokinase-type plasminogen activator (uPA) has been reported by the inventors to cause impaired liver function in mammals. Sandgren et al in Cell (1991) 29:245-256. Surviving transgenic animals harboring Alb-uPA are born with a liver consisting of smooth, pale to nearly white tissue, "white liver," within which develop multiple reddish nodules which expand in size with age. The white areas represent original transgenic liver tissue while the red areas represent post-natal changes in some aspects of liver function and structure which give rise to red nodular growth. The white tissue samples express high levels of uPA mRNA whereas all samples of the red nodules lack detectable transgene expression.
This white liver tissue contains small hepatocytes with altered rough endoplasmic reticulum morphology but is neither necrotic or fibrotic. Expression of the uPA transgene appears to be deleterious to hepatocytes, but not immediately lethal. This kind of genetic impairment of native liver function provides a suitable format for establishing regenerative liver outgrowths from either donor animals (e.g. humans) or endogenous liver cells that spontaneously cease uPA transgene expression through DNA recombination and loss of all functional copies of the transgene tandem array.

In the later case, heterozygous Alb-uPA mice frequently develop regenerative liver nodules with normal reddish liver color that grow out at expense of surrounding "white" liver tissue and eventually reconstitute the liver. These "red" liver nodules are clonal outgrowth of single hepatocytes based on analysis of the transgene remnants left behind following transgene recombination. Hepatocytes that have deleted all functional transgenes, and lack detectable transgene express, acquire a selected advantage and quickly expand and replace the transgene-expressing hepatocytes in the surrounding "white tissue". An embodiment that is an extension of these observations is that non-native (e.g. human) liver cells can be transplanted into these mice (or their functional equivalent) and, as a result of their inherent growth advantage over native, transgene-expressing liver cells, they grow out to reconstitute the liver at the expense of endogenous liver cells which do die or disappear.

According to this general genetic inducement method, a transgene that encodes a product which is disadvantageous, but not immediately lethal, to the liver cells of the host animal is constructed. The resulting fusion construct is inserted using known techniques into fertilized eggs followed by implantation into pregnant or pseudopregnant females. The progeny are then bred to produce offspring which express the construct, and which
have impaired native liver function.

A variety of different non-native cells (or corresponding tissue) may be used for implantation into the host in accordance with the invention. These cells (or tissue) may be fetal, neonatal and/or adult stem, progenitor and/or mature non-hematopoietic liver cells. Preferably non-hematopoietic liver progenitor cells and more preferably non-hematopoietic liver stem cells are used. In this context, hepatocytes, which constitute about 60% of the mammalian liver, may be used as the liver cells.

The non-native hepatocytic stem cells which may be used in the invention are distinct from fetal hematopoietic stem cells. Fetal hematopoietic stem cells are precursors to the hematopoietic system, and have previously been used to generate a non-native hematopoietic system in a host.

Other non-native cells (or tissue) which may be used in accordance with the invention include (i) stem, progenitor and/or mature bile duct cells, (ii) endodermal tissue cells such as pancreas or gut cells, (iii) optionally cultured totipotent stem cells including (ES) cells or embryonic carcinoma (EC) cells, and/or (iv) cultured animal cells.

In a particularly preferred embodiment, totipotent animal stem cells are first cultured under conditions which control their differentiation, such as by using particular growth factors in the cell culture medium, and these cultured cells are then used to colonize the host animal. Totipotent animal stem cells can also (alternatively) be first treated or cultured with growth factors that enhance survival, promote replication, and/or selectively enrich for stem cells.

Animal cells (or tissue) from other sources, including but not limited to embryonic stem cells or fetal endoderm, can also
be treated by growth factors or other substances to bring about differentiation of a particular cell lineage with characteristics of hepatic stem cells. Illustratively, embryonic stem cells or embryonal carcinoma cells can be stimulated to differentiate by retinoic acid or another inducer. Subsequent differentiation in culture along the endodermal or hepatocyte cell lineage can be achieved by stimulation with growth factors and/or selection, using known techniques and materials.

In an embodiment of the present invention, the non-native cells (or tissue) may be genetically engineered (using known techniques) to produce, in vivo in the host, a physiologically effective amount of one or more medically relevant protein and then transplanted in accordance with the present invention. This technique can be used to improve hepatocyte function in an animal, e.g., by producing Factor IX, LDL receptors, phenylalanine hydroxylase, etc. or to produce proteins of systemic value, e.g. Factor VIII, adenosine deaminase, or peptide hormones.

Thus, non-native cells (or tissue) to be implanted may be isolated from a donor animal, as noted below, and optionally cultured, using known techniques. For example, in accordance with an embodiment of the invention, non-hematopoietic liver stem, progenitor and/or mature cells are first isolated from the terminal or transitional bile ductules of a mature donor animal other than the host animal, using known techniques. In another illustrative embodiment, hepatic cells may be isolated from the donor animal using known procedures, e.g. by extraction through a biopsy needle. Alternatively, hepatic cells may be isolated from the donor animal by collagenase perfusion of the portal vein.¹

The donor animal used in accordance with the invention may

be an animal of the same species or of a different species as compared to the host animal. Generally it is a different individual. The donor animal may be any animal harboring a normal functioning liver or an animal with a congenital or squired liver disease. In a preferred embodiment the donor animal is a human.

The non-native cells (or tissue) are then introduced into the host organism in a manner which provides for implantation of a sufficient number of the non-native cells into the liver region of the host animal to permit their development into a non-native liver. The present invention does not require the use of a polymeric matrix for supporting the implanted cells. Once implanted into the liver region of the host animal, the injected cells (or tissue) are subjected therein, by virtue of their location, to a variety of native biological signals to which the native liver is normally subjected and which provide for the growth and maintenance of implanted non-native liver cells in the host.

Thus, cells from a variety of sources may be used in accordance with the invention, since such cells are comprised of a mixture of cells of different degree of development (i.e., stem, progenitor and mature cells). These mixtures contain cells having a sufficiently primitive degree of development so that, when subjected to the biological environment of the host’s liver area, these cells will establish a functioning non-native liver.

Illustratively, other cells, particularly of endodermal origin, may be used to form a new liver. For example, Scarpelli et al in Laboratory Investigation (1990) 62:552 reported identifying cells in the pancreas of hamsters, treated with carcinogens, which have the appearance of hepatocytes. These cells and others may form functional hepatocytes when placed in the appropriate environment (e.g. a failing liver) in accordance with the present invention.
Implantation into the host animal’s liver area may be achieved using different techniques. Illustratively, a suspension of from $1 \times 10^3$ to $1 \times 10^7$, preferably $1 \times 10^4$ to $1 \times 10^6$, non-native cells, optionally together with additional adjuvants to promote the transplantation of the non-native cells (i.e., substances which are known to enhance non-native cell survival and/or division$^2$), are administered (e.g. injected through a syringe needle) into any of several different sites where colonization of the non-native cells may be observed.

Suitable sites include the abdominal cavity, muscle tissue, kidney, pancreas, celiac artery, fat pads and/or subcutaneous area of the host animal. Preferred sites of administration in accordance with the invention, and which are preferably effectuated by injection, include the portal vein, the spleen, directly into the native liver, and the umbilical vein of the fetal host which leads to the fetal liver.

In accordance with the invention, if the host and donor are two different animals steps are taken prior to implantation to suppress potential host immune rejection of the implanted non-native cells. This may be achieved in a variety of ways, such as by treating the host animal with cyclosporin or any other material known to suppress the host’s immunological system, e.g. agents that are known to selectively destroy subgroups of the immune cell population of the host, such as T-cell destroying antibodies. Alternatively, tolerance in the host can be induced by transferring non-native cells to the thymus of the host before implantation (see, Rosselt et al, *Science* (1990) 249:1293-1295), or cells from an animal of the same strain as the host can be used. Still further, an immunodeficient host can be used, such as inbred strains of an animal (e.g. SCID mice or nude mice) bred to act as recipients of non-native cells.

$^2$For example, cells can be introduced together with growth factors or other substances that enhance survival, implantation, and for growth.
In an illustrative and preferred embodiment, the recipient for the non-native cells is a transgenic mouse with defective liver function, and human non-hematopoietic liver cells are used to generate the non-native liver. A suitable transgenic mouse is described by Sandgren et al in *Cell*, (1991) 66:245-256, which is hereby incorporated by reference. The transgenic mouse described in this publication of the inventors exhibits a high expression of albumin-urokinase-type plasminogen activator (Alb-uPA) fusion construct. This mouse with impaired liver function is a good recipient for human non-hematopoietic liver cell implantation (but mice with liver impairment resulting from other causes may be used).

The transgenic mouse with impaired liver function is injected with non-native, preferably human non-hematopoietic liver cells such that the injected human hepatocytic stem cells colonize the native liver at the expense of the endogenous "white tissue" of the transgenic mouse. The human liver cells proliferate to form nodules which ultimately reconstitute the entire native liver of the host with human hepatocytes and establish human liver function. This includes the replacement of most mouse plasma proteins with their corresponding human plasma proteins.

The resulting mouse or any other non-human host produced in accordance with the invention, is well suited for (1) modeling study of human liver function, including screening pharmaceutical agents for treating human liver disease, (2) modeling human congenital diseases affecting the liver (including those in which the cause is unknown) by implanting patient liver cells into host mice, (3) liver cell banking (e.g. maintenance and expansion of liver tissue for later analysis or re-implantation into the donor), (4) genetic manipulation of full-functioning, full-differentiated hepatocytes for later re-implantation into donors (i.e., hepatocyte-directed gene therapy such as hepatitis virus resistant human liver cells that express antisense against
the virus), and/or (5) modeling human liver for analysis of therapeutic agents that can promote liver regeneration.

More particularly, the present invention provides:

(i) Methods for maintaining and generating fully-functional, full-differentiated liver tissue for future analysis and therapeutic uses;

(ii) Therapeutic uses including: (a) re-implantation of healthy liver tissue into donor patients once an infectious agent or diseased tissue has been eliminated, and (b) genetic modification of donor cells and subsequent re-implantation into donor patients (e.g. hepatocyte-directed gene therapy);

(iii) Animal model systems with functioning, non-native liver (e.g. human hepatocytes) for the study of viral infections (e.g. human hepatitis virus or cytomegalovirus infection) and to test the effectiveness and safety of treatments or therapies for such infections;

(iv) Animal model systems with functioning, non-native (e.g. human) liver for the study of chemical (e.g. alcohol) toxicity in the liver and chemical-induced cirrhosis and fibrosis and to test the effectiveness and safety of treatments or therapies for chemical-induced liver damage;

(v) Methods for generating non-human animal models of congenital human liver disorders (even when the cause is not known) by reconstitution of host animal livers with human liver hepatocytes collected from patients diagnosed with liver disease;

(vi) Animal model systems with functioning, non-native (e.g. human) liver which models congenital human liver disorders (e.g., glycogen storage diseases, a,-antitrypsin deficiency, coagulation factor disorders and deficiencies) for use in testing
the effectiveness and safety of treatments and therapies;

(vii) Methods for generating non-human animals in which the liver-derived plasma proteins (which comprise >90% of the total plasma proteins) are derived from transplanted, non-native (e.g. human) liver cells;

(viii) Animal systems with a functioning, non-native (e.g. human) liver in which to generate and study normal or abnormal donor-liver derived plasma proteins;

(ix) Animal systems with a functioning, non-native (e.g. human) liver for testing the effectiveness and safety of treatments or therapies for disorders affecting donor liver-derived plasma proteins; and

(x) Systems for generating in large quantities of recombinant plasma proteins for therapeutic or diagnostic use. Hepatocytes are equipped to assemble, and appropriately modify, copious amounts of plasma proteins; thus, transplanted hepatocytes, or their genetically engineered derivatives, may be used to generate valuable donor proteins or other derived proteins.

* * * * *

Obviously, numerous modifications and variations of the present invention are possible in light of the above teachings. It is therefore to be understood that within the scope of the appended claims, the invention may be practiced otherwise than as specifically described herein.
Claims

1. A non-human animal harboring substantially no native functioning hepatocytes and a functioning substantially non-native liver generated from implanted non-native cells or tissues.

2. The non-human animal of Claim 1, wherein said substantially functioning non-native functioning liver is a human liver.

3. A primate according to Claim 1.

4. A primate according to Claim 2.

5. A mammal according to Claim 1.

6. A mammal according to Claim 2.

7. A rodent according to Claim 1.

8. A rodent according to Claim 2.

9. A non-human animal harboring a functioning substantially non-native liver, obtained by:

   (i) implanting into the liver area of a non-human animal host having impaired native liver function, cells which are non-native to said non-human animal and which are at least one member selected from the group consisting of (i) non-native fetal, neonatal and adult non-hematopoietic liver stem, progenitor and mature cells, (ii) stem, progenitor and mature bile duct cells, (iii) non-native fetal, neonatal or adult stem, progenitor and mature endodermal tissue cells, (iv) totipotent stem cells, and (v) cultured animal cells, and
(ii) allowing the non-native cells to develop into a functioning substantially non-native liver in said non-human animal host and thereby obtaining a non-human animal harboring substantially no native functioning hepatocytes and a functioning substantially non-native liver.

10. The non-human animal of Claim 9, wherein said non-native cells are fetal stem, progenitor or mature non-hematopoietic liver cells.

11. The non-human animal of Claim 9, wherein said non-native cells are stem, progenitor or adult bile duct cells.

12. The non-human animal of Claim 9, wherein said non-native cells are stem, progenitor or mature endodermal tissue cells.

13. The non-human animal of Claim 9, wherein said non-native cells are totipotent stem cells.

14. The non-human animal of Claim 9, wherein said animal and said non-native cells are from the same species.

15. The non-human animal of Claim 9, wherein said animal and said non-native cells are from different species.

16. The non-human animal of Claim 9, wherein said non-native cells are human cells.

17. The non-human animal of Claim 9, wherein said non-human animal is a transgenic animal harboring an albuminplasminogen activator transgene or its functional equivalent with regard to native liver function.

18. A rodent according to Claim 17.
19. A mouse according to Claim 17.

20. A non-human animal harboring substantially no native functioning hepatocytes and a functioning substantially non-native liver, obtained by implanting into the liver area of a non-human animal host suffering from impaired native liver function, non-native fetal, neonatal or adult stem, progenitor or mature non-hematopoietic liver cells and allowing said non-native cells to develop into a non-native liver in said non-human animal host.

21. A method for restoring liver function in a human host suffering from impaired native liver function, comprising implanting into the liver area of said human, fetal, neonatal or adult human non-hematopoietic liver stem, progenitor or mature cells which are non-native to said human host, and allowing said non-native cells to develop into a functioning non-native human liver in said human host.

22. The method of Claim 21, wherein said human host suffering from impaired native liver function has been diagnosed as suffering from cirrhosis or fibrosis of the liver.

23. The method of Claim 21, wherein said human host suffering from impaired native liver function has been diagnosed as suffering from hepatitis.

24. The method of Claim 21, wherein said human host suffering from impaired native liver function has been diagnosed as suffering from a genetic deficiency disease causing said impaired native liver function.

25. The method of Claim 21, wherein said human host suffering from impaired native liver function has suffered a trauma causing said defective liver function.
26. The method of Claim 21, wherein said implantation is achieved by injecting said non-native cells into the portal vein of said human host suffering from impaired native liver function.

27. The method of Claim 21, wherein said implantation is achieved by injecting said non-native cells into the spleen of said human host suffering from impaired native liver function.

28. The method of Claim 21, wherein said implantation is achieved by injecting said non-native cells into the liver of said human host suffering from impaired native liver function.

29. The method of Claim 21, wherein said implantation is achieved by injecting said non-native cells into the celiac artery of said human host suffering from impaired native liver function.

30. A method of restoring liver function in an animal with impaired liver function, said method comprising:

(a) implanting into the liver area of a host animal in need of restoration of liver function, cells which are non-native to said host animal and which are at least one member selected from the group consisting of (i) fetal, neonatal and adult non-hematopoietic liver stem, progenitor and mature cells, (ii) stem, progenitor and adult bile duct Cells, (iii) fetal, neonatal and adult stem, progenitor and mature endodermal tissue cells, and (iv) totipotent stem cells, and (v) cultured animal cells; and

(b) allowing said non-native cells to develop into a functioning non-native liver in said host animal.

31. The method of Claim 30, wherein said implantation is achieved by injecting said non-native cells into the portal vein, spleen, liver, or celiac artery, of said host animal.
32. A method for obtaining an animal with a functioning non-native liver, said method comprising:

(a) implanting into a host animal with impaired native liver function, cells which are non-native to said host animal and which are least one member selected from the group consisting of (i) fetal, neonatal and adult non-hematopoietic liver stem, progenitor and mature cells, (ii) stem, progenitor and adult bile duct cells, (iii) fetal, neonatal and adult stem, progenitor and mature endodermal tissue cells, (iv) totipotent stem cells, and (v) cultured animal cells; and (b) allowing said non-native cells in said host to develop into a functioning non-native liver.

33. The method of Claim 34, wherein said host animal is a transgenic animal harboring a transgene which compromises native hepatocytes.

34. A method for screening a pharmaceutical agent for treatment or therapy of a human liver disorder or disease comprising using the animal of Claim 2.

35. A method for screening a pharmaceutical agent for treatment or therapy of a human liver disorders or disease comprising using the primate of Claim 4.

36. A method for screening a pharmaceutical agent for treatment or therapy of a human liver disorder or disease comprising using the mammal of Claim 6.

37. A method for screening a pharmaceutical agent for treatment or therapy of a human liver disorder or disease comprising using the rodent of Claim 8.

38. A method of using the animal of Claim 1, comprising using said animal for maintaining and generating fully-functional, fully-differentiated liver tissue for future analysis.
or therapeutic use.

39. A method of using the animal of Claim 9, wherein said animal is used for maintaining and generating fully-functional, fully-differentiated liver tissue for future analysis or therapeutic use.

40. The method of Claim 39, wherein said therapeutic use comprises: the reimplantation of healthy liver tissue into a donor patient from whom said cells which were non-native to said non-human animal were obtained, once an infectious agent or disease tissue has been eliminated from said donor patient.

41. A method for hepatocyte-directed gene therapy, comprising obtaining hepatocytes from a donor animal, genetically modifying said cells to enable them to produce a physiologically effective amount of at least one medically relevant protein, implanting said genetically modified cells into the non-human animal of Claim 1 to generate fully-functional, fully-differentiated and genetically modified liver tissue, and reimplanting said generated liver tissue into said donor animal.
**INTERNATIONAL SEARCH REPORT**

**A. CLASSIFICATION OF SUBJECT MATTER**

<table>
<thead>
<tr>
<th>IPC(S)</th>
<th>C12N 15/00</th>
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<tbody>
<tr>
<td>US CL</td>
<td>800/2</td>
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</tbody>
</table>

According to International Patent Classification (IPC) or to both national classification and IPC.

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

| U.S. | 800/2 |

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

| none |

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

| APS, Dialog, hepatocytes, transplantation |

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>X</td>
<td>Science, volume 233, issued 12 September 1986, Demetriou et al., &quot;Replacement of liver function in rats by transplantation of microcarrier-attached hepatocytes&quot;, pages 1190-1192, see entire article.</td>
<td>1,5,7</td>
</tr>
<tr>
<td>Y</td>
<td>Cell, volume 66, issued 26 July 1991, Sandgren et al., &quot;Complete hepatic regeneration after somatic deletion of an albumin-plasminogen activator transgene&quot;, pages 245-256, see entire article.</td>
<td>1-41</td>
</tr>
</tbody>
</table>

| [X] Further documents are listed in the continuation of Box C. | [ ] See patent family annex. |

- * Special categories of cited documents:
  - "A" document defining the general state of the art which is not considered to be part of particular relevance
  - "E" earlier document published on or after the international filing date
  - "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
  - "O" document referring to an oral disclosure, use, exhibition or other means
  - "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

- "Y" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

- "&" document member of the same patent family

Date of the actual completion of the international search: 17 SEPTEMBER 1993

Date of mailing of the international search report: 02 NOV 1993

Name and mailing address of the ISA/US

Commissioner of Patents and Trademarks

Box PCT

Washington, D.C. 20231

Facsimile No. NOT APPLICABLE

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SUZANNE ZISKA

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<table>
<thead>
<tr>
<th>Category</th>
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<th>Relevant to claim No.</th>
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</thead>
<tbody>
<tr>
<td>X</td>
<td>Cancer Research, volume 44, issued June 1984, Reddy et al., &quot;Response of hepatocytes transplanted into syngeneic hosts and heterotransplanted into athymic nude mice to peroxisome proliferators&quot;, pages 2582-2589, see entire article.</td>
<td>1,5,7</td>
</tr>
<tr>
<td>Y</td>
<td>Cancer Research, volume 50, issued 01 July 1990, Sell, &quot;Is there a liver stem cell&quot;, pages 3811-3815, see entire article.</td>
<td>2,6,8</td>
</tr>
<tr>
<td>Y</td>
<td>Laboratory Investigation, volume 65, no. 6, issued 1991, Modis et al., &quot;Hepatocytes modulate the hepatic microvascular phenotype&quot;, pages 661-670, see entire article.</td>
<td>1-41</td>
</tr>
<tr>
<td>Y</td>
<td>Proceedings of the National Academy of Sciences, volume 88, issued February 1991, Ponder et al., &quot;Mouse hepatocytes migrate to liver parenchma and function indefinitely after intrasplenic transplantation&quot;, pages 1217-1221, see entire article.</td>
<td>1,5,7</td>
</tr>
</tbody>
</table>
### Box I Observations where certain claims were found unserviceable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. □ Claims Nos.:  
   because they relate to subject matter not required to be searched by this Authority, namely:

2. □ Claims Nos.:  
   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. □ Claims Nos.:  
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

### Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. □ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. □ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. □ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. □ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

**Remark on Protest**  
□ The additional search fees were accompanied by the applicant's protest.  
□ No protest accompanied the payment of additional search fees.
BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

Unity of invention is lacking for the following reasons:

I. Claims 1-8, a first product, drawn to a non-human animal harboring substantially no native functioning hepatocytes and a functioning substantially non-native liver generated from implanted non-native cells or tissues, classified in Class 800, subclass 2, for example and a first method of using the first product, claims 33 and 34, drawn to a method for screening a pharmaceutical agent for treatment or therapy of a human liver disorder using the animal of claim 2, Class 424, subclass 9, for example.

This application contains claims directed to the following patentably distinct species of the claimed invention: species A, claims 3 and 4, drawn to primates; species B, claims 5 and 6, drawn to mammals; species C, claims 7 and 8, drawn to rodents. If Invention I is elected, Applicants must choose a species from species A-C.

Applicant is required to elect a single disclosed species for prosecution on the merits to which the claims shall be restricted if no generic claim is finally held to be allowable. Currently, claims 1 and 2 are generic.

Applicant is advised that a response to this requirement must include an identification of the species that is elected consonant with this requirement, and a listing of all claims readable thereon, including any claims subsequently added. An argument that a claim is allowable or that all claims are generic is considered nonresponsive unless accompanied by an election.

Upon the allowance of a generic claim, applicant will be entitled to consideration of claims to additional species which are written in dependent form or otherwise include all the limitations of an allowed generic claim. If claims are added after the election, applicant must indicate which are readable upon the elected species.

Should applicant traverse on the ground that the species are not patentably distinct, applicant should submit evidence or identify such evidence now of record showing the species to be obvious variants or clearly admit on the record that this is the case. In either instance, if the examiner finds one of the inventions unpatentable over the prior art the evidence or admission may be used in a negative statement concerning the other invention.

II. Claims 9-19, a second product drawn to a non-human animal harboring a functioning substantially non-native liver, obtained by implanting non-native cells into the liver area of a non-human animal having impaired native liver function, classified in Class 800, subclass 2, for example.

This application contains claims directed to the following patentably distinct species of the claimed invention: species A, claims 10-16, drawn to cells not genetically transformed; species B, claims 17-19, drawn to genetically modified cells. If Invention II is elected, Applicants are required to choose either species A or species B.

Applicant is required to elect a single disclosed species for prosecution on the merits to which the claims shall be restricted if no generic claim is finally held to be allowable. Currently, claim 9 is generic.

Applicant is advised that a response to this requirement must include an identification of the species that is elected consonant with this requirement, and a listing of all claims readable thereon, including any claims subsequently added. An argument that a claim is allowable or that all claims are generic is considered nonresponsive unless accompanied by an election.

Upon the allowance of a generic claim, applicant will be entitled to consideration of claims to additional species which are written in dependent form or otherwise include all the limitations of an allowed generic claim. If claims are added after the election, applicant must indicate which are readable upon the elected species.

Should applicant traverse on the ground that the species are not patentably distinct, applicant should submit evidence or identify such evidence now of record showing the species to be obvious variants or clearly admit on the record that this is the case. In either instance, if the examiner finds one of the inventions unpatentable over the prior art, the evidence or admission may be used in a negative statement concerning the other invention.

III. Claim 20, a third product, drawn to a non-human animal harboring substantially no native functioning hepatocytes and a functioning substantially non-native liver, obtained by implanting into the liver area of a host suffering from impaired native liver function, fetal, neonatal, adult stem progenitor or mature non-hematopoietic liver cells, classified in Class 800, subclass 2, for example.
IV. Claims 21-29, a second method, drawn to a method for restoring liver function in a human host, comprising implanting into the liver area of said human non-hematopoietic liver cells non-native to said host, classified in Class 424, subclass 93, for example.

V. Claims 30-31, a third method, drawn to a method of restoring liver function in an animal with impaired liver function comprising, classified in Class 424, subclass 93, for example.

VI. Claim 32, a fourth method, drawn to a method of obtaining an animal with a functioning non-native liver, classified in Class 424, subclass 93C, for example.

VII. Claim 35, a fifth method, drawn to a method for screening a pharmaceutical agent for treatment or therapy of a human liver disorder or disease comprising using the primate of claim 4, classified in Class 424, subclass 9, for example.

VIII. Claim 36, a sixth method, drawn to a method for screening a pharmaceutical agent for treatment or therapy of a human liver disorder or disease comprising using the rodent of claim 8, classified in Class 424, subclass 9, for example.

IX. Claim 37, a seventh method, drawn to a method for screening a pharmaceutical agent for treatment or therapy of a human liver disorder or disease comprising using the rodent of claim 8, classified in Class 424, subclass 9, for example.

X. Claims 38-40, an eighth method, drawn to a method of using the animal of claim 1 or claim 9, comprising using said animal for maintaining and generating fully functional, fully differentiated liver tissue, classified in Class 424, subclass 572 or Class 435, subclass 240.2, for example.

XI. Claim 41, a ninth method, drawn to a method for hepatocyte-directed gene therapy, classified in Class 424, subclass 93, for example.

Each grouping of claims forms a separate invention which are not so linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single inventive concept. PCT Rules 13.1 and 13.2 do not permit multiple distinct products and methods within a single inventive concept.