

C/EBPalpha gene targeting constructs and uses thereof

Field of the invention

The present invention relates to DNA constructs encoding C/EBPalpha (C/EBP α) and to the use of said constructs for the inhibition or prevention of tumours. The invention further relates to the preparation of knock-in non-human mammals and hepatocytes expressing exogenous C/EBP α under the control of a tumour-specific promoter.

Background of the invention

10 The murine CCAAT/enhancer binding protein α (C/EBP α) is a 395 amino acid transcription factor encoded by a single-exon gene (Christy, R. J., et al., 1991, *Proc. Natl. Acad. Sci. USA* 88:2593-2597). This heat-stable transactivator is highly expressed in hepatic and adipose tissues but is detectable at lower levels in the brain, gut, and lungs (Birkenmeier, E. H., et al., 1989, *Genes*
15 *Dev.* 3:1146-1146; Friedman, A. D., et al., 1989, *Genes Dev.* 3:1314-1322; Johnson, P. F., et al., 1987, *Genes Dev.* 1:133-146). A member of the bZIP family of proteins, C/EBP α recognizes and binds to specific DNA sequences through a basic DNA-binding domain and a leucine zipper dimerization domain. A peptide sequence of twenty-four predominantly basic amino acids
20 serves as the DNA contact region. Immediately adjacent to this region is a thirty-five residue α helical coil containing four leucines each spaced seven amino acids apart. These leucines align on the same side of the α helix and interdigitate with other proteins carrying the same leucine motif to "zip" the proteins together. C/EBP α can form both homodimers as well as
25 heterodimers. The consensus binding site for C/EBP α homodimers is T(T/G)NNG(C/T)AA(G/T) although tests *in vitro* indicate that highly divergent sequences also can be bound (Vinson, C. R., et al., 1989, *Science* 246:911-916; McKnight, S. L., et al., 1989, *Genes Dev.* 3:2021-2024).

Two important functions had long been ascribed to C/EBP α based on numerous *in vitro* studies. The first is that of a transcriptional regulator that induces cellular growth arrest and terminal differentiation in hepatocytes and adipocytes (Freytag, S. O. and Geddes, T. J., 1992, *Science* 256:379-382; 5 Mischoulon, D., et al., 1992, *Mol. Cell. Biol.* 12:2553-2560). The second is that of a regulator that transactivates energy-related genes to establish and maintain energy homeostasis (Kaestner, K. H., et al., 1990, *Proc. Natl. Acad. Sci. USA* 87:251-255; Park, E. A., et al., 1990, *Mol. Cell. Biol.* 10:6264-6272; 10 McKnight, S. L., et al., 1989, *Genes Dev.* 3:2021-2024).

Early investigations into the functions of C/EBP α had identified an inverse correlation between its expression and cellular proliferation. Work in 3T3-L1 adipoblasts demonstrated that premature expression of C/EBP α could induce 15 cellular growth arrest and terminal differentiation (Umek, R. M., et al., 1991, *Science* 251:288-292; Freytag, S. O. and Geddes, T. J., 1992, *Science* 256:379-382). Observations in rat primary hepatocytes and partial hepatectomies revealed a greater than 80% drop in C/EBP α transcripts as the cells progressed into the growth phase (Mischoulon, D., et al., 1992, *Mol. Cell. Biol.* 12:2553-2560.). 20 Western analyses showed cultured hepatoma cells to contain approximately 5% of the amount of C/EBP α as the normal rat liver (Friedman, A. D., et al., 1989, *Genes Dev.* 3:1314-1322). Proliferation of hepatoma cells and transformed hepatocytes is inhibited by transfection of constitutively expressing C/EBP α genes (Hendricks-Taylor, L. R. and 25 Darlington, G. J., 1995, *Nucleic Acids Res.* 23:4726-4733; Diehl, A. M., et al., 1996, *J. Bio. Chem.* 271:7343-7350).

Other *in vitro* experiments showed C/EBP α to transactivate the promoters of many energy-related genes including the following: albumin which binds and 30 transports long chain fatty acids in serum, the lipid-binding protein (422/aP2)

which binds and transports long chain fatty acids in adipocytes, stearoyl acyl-CoA desaturase 1 (SCD1) which desaturates fatty acids for transport and storage in adipocytes, the insulin-responsive glucose transporter (GLUT4) which controls entry of glucose into adipocytes for lipogenesis, and
5 phosphoenolpyruvate carboxykinase (PEPCK) which is a key enzyme involved in gluconeogenesis (Kaestner, K. H., et al., 1990, *Proc. Natl. Acad. Sci. USA* 87:251-255; Park, E. A., et al., 1990, *Mol. Cell. Biol.* 10:6264-6272). The ability of C/EBP α to transactivate these genes led to the proposal that its primary function is that of a regulator of energy metabolism (McKnight, S. L.,
10 et al., 1989, *Genes Dev.* 3:2021-2024).

The principal organ involved in the maintenance of energy homeostasis in mammals is the liver. Hepatic C/EBP α mRNA is detectable on the thirteenth day of the twenty-one day mouse gestation period; it rises by day 17 and
15 reaches a maximum near the time of birth (Birkenmeier, E. H., et al., 1989, *Genes Dev.* 3:1146-1146; Kuo, C. F., et al., 1990, *Dev.* 109:473-481). High C/EBP α expression during the prenatal term coincides with the initiation of hepatic glycogen storage.

20 Rodents, in a time frame analogous to humans, gain the capacity to synthesize and store large amounts of hepatic glycogen during the last trimester of fetal life, setting the stage for the shift from fetal metabolic processes to those of the neonate (Jones, C. T., 1982, *The Development of the Metabolism in the Fetal Liver*. New York: Elsevier Biomedical Press).
25 Mobilization of this stored glycogen at birth acts to maintain blood glucose homeostasis. Gluconeogenesis begins within hours thereafter. Attendant with these metabolic changes is the up-regulation in expression of the energy-related liver enzymes, PEPCK, tyrosine aminotransferase (TAT), acetyl-CoA carboxylase, albumin, glucose-6-phosphatase (G6Pase), and glycogen
30 synthase (Park, E. A., et al., 1992, *J. Biol. Chem.* 267:613-619; Grange, T., et

al., 1991, *Nucleic Acids Res.* 19:131-139.; Tea, H. J., et al., 1994, *J. Biol. Chem.* 269:10475-10484; Wang N. D., et al., 1995, *Science* 269:1108-1112). Members of the C/EBP family of transcription factors have been implicated in the transactivation of these genes.

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US 5,545,563 ('563) discloses a vector for transient transfection in mammals expressing C/EBP α under the control of exogenous CMV or other exogenous tumour-specific promoter. '563 further discloses a replacement or insertion vector, capable of expressing a mutant C/EBP α , for the preparation of knock-out (or deletion) animals, which possess predetermined mutations and alleles in their chromosomal C/EBP α genes. According to this method, the naturally present gene sequence of a cell of the animal is replaced with an analog sequence capable of causing the expression of an analog C/EBP α that is a sequence which has been altered compared to the naturally occurring C/EBP α . The hepatocytes of the knock-out animal prepared with the method disclosed in US 5,545,563 are prone to immortalization and accordingly the animals are predisposed to cancer.

Other knock-out mice have been disclosed in the art (Wang, N. D., et al., 1995, *Science* 269:1108-1112) and used for the analysis of the role of C/EBP α in promoting tissue maturation as well as in the production and maintenance of life-sustaining fuel levels in the neonate. The absence of C/EBP α in these mice gave rise to conditions that greatly resemble the symptoms of the human premature infant including growth retardation, severe hypoglycemia, insufficient hepatic glycogen storage and insufficient adipose fat storage.

The problem related to the knock-out animals disclosed in the prior art is that these knock-out animals have abnormal liver function, experience severe hypoglycaemia, and die within a few hours post-partum. Furthermore, their

hepatocytes have been shown to be prone to tumourigenesis (Soriano, H. E., et al., 1998, *Hepatology* 27:392-401). Accordingly, these knock-out animals are not useful for producing, or for testing, potential therapeutic applications of C/EBP α -expressing cells and tissues. Another problem associated with the
5 US 5,545,563 disclosure is its suggestion to express C/EBP α under the regulation of the α -fetoprotein (AFP) promoter in a mammalian vector. Because the normal regulation of AFP is complex and its complete array of regulatory sequences may not be known for some time to come, expression systems that rely on the use of cloned AFP promoter DNA run the risk of
10 rendering inaccurate AFP regulation. Unintended gene expression is a risk inherent in all current gene therapy strategies.

It would therefore be desirable to identify and provide new and different means for the generation of tumour-resistant cells and animals which could be
15 useful in the treatment of tumours.

Summary of the invention

The present invention seeks to address the problems above, and in particular
20 to provide new and useful means for the generation of tumour-resistant cells and non-human animals.

One aspect of the invention is a DNA construct for the expression of a functional C/EBP α , comprising a DNA molecule encoding C/EBP α .

The DNA construct according to the invention preferably comprises, in a 5' to
25 3' end direction reading frame, the following components:

- (i) a first region having homology with an endogenous tumour-specific promoter gene sequence of a gene locus;

- (ii) a DNA molecule encoding the C/EBP α ; and
- (iii) a second region having homology with a region within the gene locus.

The DNA molecule encoding the C/EBP α and a second region of homology is positioned downstream of the tumour-specific promoter sequence.

- 5 Such a construct allows the expression of the exogenous C/EBP α under the control of the endogenous tumour-resistant promoter of a host cell or animal, for example a mammal.

The DNA construct according to the invention allows the expression of C/EBP α derived from any organism, preferably a mammal, such as human or
10 non-human C/EBP α . For example, a mouse C/EBP α .

Preferably, the first region has homology with an endogenous tumour-specific promoter of a gene locus, which is activated in liver and lung tumours. More preferably, the first region is homologous with an endogenous mammalian α -fetoprotein (AFP) promoter of the AFP gene locus and the second region is
15 homologous with a region within the AFP gene locus. The second region being downstream of the AFP promoter.

Accordingly, an embodiment of the invention is a DNA construct for expression of a functional mammalian C/EBP α comprising, in a 5' to 3' direction, the following components:

- 20 (i) a first region having homology with an endogenous AFP promoter gene sequence of the AFP locus;
- (ii) a DNA molecule encoding the C/EBP α gene; and
 - (iii) a second region having homology with a region within the endogenous AFP gene locus.

- The first and second regions of homology with the endogenous tumour-specific promoter (for example AFP promoter) of the AFP locus and within the tumour-specific promoter gene locus are also indicated as "upstream" and "downstream" sequence, respectively, as they are positioned, in the DNA construct, upstream and downstream to the DNA molecule encoding the exogenous C/EBP α of interest. The upstream and downstream sequences allow the positioning of the DNA molecule encoding the exogenous C/EBP α of interest into the endogenous tumour-specific promoter locus (for example, into the AFP locus).
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- 10 The invention also provides an expression vector comprising the DNA construct.

- Another aspect of the invention relates to host cells comprising the DNA construct or the expression vector. The host cell may be a procaryotic cell. In particular, a mammal cell. For example, a human or non-human animal hepatocyte or pneumocyte. The human or non-human animal cell in which the DNA construct is inserted becomes a tumour-resistant cell. Accordingly, the present invention provides tumour-resistant cell(s), preferably hepatocyte(s) or pneumocyte(s), which can be used in therapy. For example, tumour-resistant hepatocytes, hepatocyte aggregates or hepatocyte grafts according to the invention can be administered to an area in need of transplant in a mammal, such as a human or non-human animal.
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- The tumour-resistant cells can be prepared by transfecting host cells with the DNA construct and/or the expression vector according to the invention. These tumour-resistant cells can also be isolated from knock-in non-human animals which have been created using the DNA construct, vector and/or cell of the invention.
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The tumour-resistant cells, according to the invention, can also be human or non-human animal pluripotent embryonic or adult stem cells transfected with

the DNA construct and/or vector of the invention. Preferably, such transfected pluripotent embryonic or adult stem cells are cultivated and differentiated into tumour-resistant hepatocytes or pneumocytes.

5 The invention provides a process for preparing a hepatocyte graft for administration to an area in need of transplant, wherein the process comprises making the hepatocytes of the graft tumour-resistant by transfecting these hepatocytes with the DNA construct.

10 Also provided is a method for transplanting hepatocytes, hepatocyte aggregates or hepatocyte grafts, comprising preparing hepatocytes, hepatocyte aggregates or hepatocyte grafts transfected with the DNA construct, and transplanting the prepared transfected hepatocytes, hepatocyte aggregates or hepatocyte grafts to an area in need of transplant.

The invention also relates to bioreactors, preferably, bioartificial livers, comprising the use of the tumour-resistant cells according to the invention.

15 A further aspect of the invention is a knock-in non-human animal transfected or modified with the DNA construct according to the invention. In such a knock-in non-human animal, the exogenous C/EBP α transgene is specifically transcribed by the animal's endogenous tumour-specific promoter. Preferably, the endogenous tumour-specific promoter is the AFP promoter, which
20 transcribes the exogenous C/EBP α transgene specifically in the fetal liver at a point earlier than normal or in developing cancer cells where the AFP promoter is reactivated.

The invention provides a method for the preparation of knock-in non-human animals modified with the DNA construct of the invention. For example,
25 pluripotent non-human animal embryonic stem cells modified by the DNA construct of the invention could be used in blastocyst microinjections to produce chimeric animals that can propagate the modified allele.

Alternatively, somatic cells of an animal could be modified in culture by transfection with the DNA construct of the invention and used as donors for nuclear transfer to generate viable animals carrying the modified allele.

Also provided are tumour-resistant cells isolated from these knock-in non-
5 human animals. Such tumour-resistant cells are preferably tumour-resistant hepatocytes or pneumocytes isolated from these knock-in animals.

These tumour-resistant hepatocytes can be used to populate bioartificial livers that provide essential liver function for patients with liver failure.

The present invention also provides a method for in vivo screening and/or
10 modulation of tumours comprising administering one or more carcinogens to a non-human animal transfected with the DNA construct of the invention and identifying the tumours which are inhibited or suppressed by the expression of transgenic C/EBP α .

The invention further provides a method for *in vitro* tumour screening
15 comprising: modifying a cell or cell culture to express the DNA construct of the invention; administering to the cell or cell culture one or more carcinogens; and identifying one or more tumour cell, wherein the tumour(s) is inhibited and/or suppressed by the function of C/EBP α protein

20 A further aspect of the invention is a method for treating symptoms associated with liver glycogen-insufficiency in a human premature infant or a premature animal comprising administering into, injecting into or transfecting transiently the liver of the human premature infant or the premature animal with the circular (non-linearized) form of the DNA construct of the invention. The
25 circular form of the construct is less likely to integrate into the host genome. Transient transfection of the host cell is desirable because the need for

artificial C/EBP α expression is only necessary until the host's own endogenous C/EBP α expression becomes activated.

A still further aspect is a method of treating cancer in human or non-human animals whose cells produce elevated levels of AFP comprising treating the
5 human or animal cancer with the circular form of the DNA construct of the invention.

The invention also provides a pharmaceutical preparation comprising the construct of the invention and, optionally, a pharmaceutically acceptable carrier and/or diluent.

10 Further, the invention relates to the use of a DNA construct and or vector according to the invention for the manufacturing of a pharmaceutical preparation for use in medicine. For example, for the treatment of symptoms associated with glycogen-insufficiency in a human premature infant or premature non-human animal; and for the treatment of cancer in humans or
15 non-human animals whose cells produce elevated levels of AFP (treating the human or non-human animal cancer with the circular form of the DNA construct of the invention).

Brief description of the figures

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Figure 1 is an illustration of the AFP-C/EBP α gene targeting construct. The C/EBP α transgene is striped. The stippled regions represent the homologous sequences that help mediate homologous recombination. The ID tag is an artificially inserted sequence containing a HindIII restriction enzyme site; it
25 assists in differentiating transcripts produced by the transgene from endogenous C/EBP α mRNA.

Figure 2 shows a restriction enzyme map of the AFP-C/EBP α gene targeting plasmid. * - indicates approximate location of restriction enzyme site.

Figure 3A-C is the sequence of the AFP-C/EBP α gene targeting plasmid. The letter "N" denotes undetermined sequence. Sequence data for the AFP upstream and downstream sequences was obtained from GenBank NCBI (National Center for Biotechnology Information) to synthesize PCR probes used to screen a 129SvJ murine genomic lambda-phage library purchased from Stratagene. Nucleotides 1-5548 comprises the upstream promoter and regulatory sequences of AFP (also shown in SEQ ID NO:1). Nucleotides 8707-9612 comprises the AFP downstream gene sequence starting from the inside of intron 1 up to part of intron 3 (also shown in SEQ ID NO:2). The section of nucleotides 757-4285 represents the subsection of the AFP gene that has not been sequenced.

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Figure 4 is a schematic of the process used to create the C/EBP α gene knock-in mouse.

Figure 5 is a restriction enzyme map of the wildtype C/EBP α allele and the knock-in allele. The 11.0 kb fragment in PstI digested DNA and a 3.7 kb fragment in HindIII digested DNA indicate the presence of the knock-in allele. The 3.4 kb PstI fragment and 8.0 kb HindIII fragment show the presence of the endogenous C/EBP α gene.

Figure 6 shows Southern blots revealing the genotypes of the knock-in (KI) mice versus that of wildtype (WT) mice using PstI digested DNA (Panel A) and HindIII digested DNA (Panel B).

Figure 7 shows a graph of the expression pattern of the knock-in C/EBP α (KI C/EBP α) gene as compared to normal C/EBP α expression.

Figure 8 shows a graph of real-time PCR data illustrating the expression profiles of C/EBP α and glycogen synthase (GS) in knock-in (KI) and wildtype mice. The data shows early and higher expression of KI C/EBP α as well as
5 higher expression of KI GS in comparison to wildtype mice.

Figure 9 shows that mRNA levels of C/EBP α were higher in the livers of knock-in versus wildtype mice between 12.5 and 15.5 days of gestational age.

10 Figure 10 shows that mRNA levels of glycogen synthase were higher in the livers of knock-in versus wildtype mice between 12.5 and 15.5 days of gestational age.

Figure 11 (A, B, C, D) shows liver sections of mice at 15.5 days gestational
15 age. Slides treated with or without Diastase were stained with PAS. A) KI PAS – the pink hue (darker cells) shows the presence of hepatic glycogen in the knock-in liver, B) KI PAS Diastase – diastase has cleared the slide of glycogen, C) WT PAS – wildtype animals have not yet deposited glycogen at this time point, D) WT PAS Diastase

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Figure 12 shows cultures of fetal primary hepatocytes derived from knock-in mice were more likely to die in response to exposure to genotoxic agents than cells from wildtype mice.

25 Figure 13 shows knock-in and wildtype cells cultured in the absence of genotoxic agents have similar survival rates.

Figure 14 (A, B, C, D) shows livers from carcinogen treated wildtype mice exhibit pronounced tumour formation (A and B), and livers from carcinogen

treated knock-in mice (C and D) exhibit smaller and fewer tumour growths than the wildtype mice.

5 Detailed description of the invention

One aspect of the present invention relates to novel constructs that can be used to transform host cells so that they express and secrete (i.e., into the host cell environment or into the knock-in organism) exogenous C/EBP α .

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Preferably, the DNA construct according to the invention is a DNA construct for the expression of a functional C/EBP α comprising, in a 5' to 3' end direction reading frame, the following components:

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- (i) a first region having homology with an endogenous tumour-specific promoter gene sequence of a gene locus;
- (ii) a DNA molecule encoding C/EBP α ; and
- (iii) a second region having homology with a region within the gene locus.

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The DNA molecule encoding the C/EBP α and a second region of homology is positioned downstream of the tumour-specific promoter sequence.

Such a DNA construct allows the expression of the exogenous C/EBP α under the control of the endogenous tumour-specific promoter.

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The DNA construct according to the invention allows the expression of C/EBP α derived from any organism, preferably mammal, for example human or non-human animal C/EBP α . For example, mouse C/EBP α .

The construct of the present invention produces results that are particularly advantageous over the construct suggested in the prior art. For example, the vector suggested by US 5,545,563 which relies on expression from a cloned exogenous promoter has the problem of lacking the complete set of regulatory elements leading to imprecise gene expression.

The DNA molecule can be any gene molecule from any organism encoding any C/EBP α known in the art or any derivative or mutation thereof.

Examples of known C/EBP α genes are for example those disclosed in the NCBI UniGene web page (<http://www.ncbi.nlm.nih.gov/>). For example, but not limited to, C/EBP α genes of *Mus musculus*, UniGene cluster Mm.34537; *Homo sapiens*, UniGene cluster Hs.76171; *Rattus norvegicus*, Unigene cluster Rn.22163 (all of them herein incorporated by reference).

The human C/EBP α gene has also been disclosed in US 5,545,563 (herein incorporated by reference).

Sequence data for the AFP upstream and downstream sequences was obtained from GenBank NCBI (National Center for Biotechnology Information) to synthesize PCR probes used to screen a 129SvJ murine genomic lambda-phage library purchased from Stratagene.

Accordingly, the DNA construct according to the invention comprises a DNA molecule encoding C/EBP α of any organism, preferably, mammal or more preferably, human or non-human animal, for example mouse, C/EBP α .

The C/EBP α of the present DNA construct is preferably a naturally functional C/EBP α . However, any functional C/EBP α can be used. It is within the knowledge of a skilled person in the art how to prepare any functional C/EBP α analog or mutant obtained by a deletion, insertion and/or substitution of at least one nucleotide of the naturally occurring C/EBP α .

The C/EBP α gene used in the preparation of the DNA construct can be isolated according to a variety of standard techniques, for example, isolated from human or animal cDNA libraries. Human or animal cDNA libraries can be obtained from any human or animal cell, for example, from a hepatoma or
5 other cell that actively expresses C/EBP α . The DNA molecule may be cloned into any suitable prokaryotic or eukaryotic vector and the vectors then are screened using a probe whose sequence is the same as, or complementary to, that of the desired C/EBP α or to a fragment thereof.

10 Suitable vectors, as well as the methods for performing such hybridization screenings are described, for example, in Sambrook, J. and Russel D.W., In: Molecular Cloning A Laboratory Manual, 3rd Edition, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (2001), herein incorporated by reference). Alternatively, amplification procedures, such as PCR are used to
15 facilitate the isolation of the C/EBP α -encoding DNA. PCR methods are reviewed by Love, S. et al., 1992, Neuropath. Appl. Neurobiol. 18:95-111.

According to another alternative, synthetic chemical methods may be used to synthesize the C/EBP α gene molecule. Thus, for example, the well-known
20 phosphodiester or phosphotriester methods may be used.

The first region of the DNA construct of the invention has homology with the sequence of an endogenous tumour-specific promoter of a gene locus. The second region of the DNA construct is a region having homology with a region
25 within the tumour-specific promoter gene locus that lies downstream of the tumour-specific promoter. Within the DNA construct, this second region of the DNA construct lies downstream of the C/EBP α gene.

Usually, the promoter locus would include all of the gene regulatory elements
30 upstream (5') of the coding sequence. For the second region of homology (the

region within the gene locus), it is convenient to use a homologous sequence that is at least 500 base-pairs long or longer. The purpose of the two homologous regions is to allow DNA cross-over events to insert construct sequences into the genome. The greater the length of homology, the more efficient gene targeting should be. For the invention's construct, particular care was taken to retain the portion of AFP's intron 1 sequence believed to contain additional AFP gene regulatory elements.

The first and second regions can be chosen to have homology to suitable endogenous mammalian promoters (i.e. promoters that are more active in tumour cells than in non-tumour cells). Examples of such a promoter include, the α -fetoprotein (AFP) promoter, the amylase promoter, the cathepsin E promoter, the M1 muscarinic receptor promoter, the gamma-glutamyl transferase promoter, the CMV promoter or the like. Preferably, the first and second regions of the DNA construct have homology with the AFP promoter and a region within the AFP locus.

The first and second regions of homology with the endogenous tumour-specific gene (for example AFP) will preferably flank the DNA molecule encoding C/EBP α . These first and second regions are also indicated as "upstream" and "downstream" sequences, respectively, as they are positioned, in the DNA construct, upstream and downstream to the DNA molecule encoding the exogenous C/EBP α of interest. The upstream and downstream sequences allow the positioning of the DNA molecule encoding the exogenous C/EBP α of interest into the endogenous tumour-specific locus (for example, into the AFP locus) and in particular to be under the control of the endogenous tumour-specific promoter (ex. AFP promoter). The positions of the upstream and downstream regions and the DNA molecule encoding C/EBP are shown in Figure 1.

Suitable C/EBP α and AFP sequences can be prepared from Stratagene's Mouse Lambda Genomic Library – 129SvJ (Catalog #946313).

The fetal oncoprotein, α -fetoprotein (AFP), is synthesized primarily in the embryonic liver and yolk sac (Sellem, C. H., et al., 1984, *Dev. Biol.* 102:51-60; Dziadek, M. A. and Andrews, G. K., 1983, *EMBO J.* 2:549-554). AFP messenger RNA is detectable in the hepatic primordia which form on the 10th day of the 21-day mouse gestation period (Dziadek, M. A. and Andrews, G. K., 1983, *EMBO J.* 2:549-554; Kaufman, M. H., 1992, *The Atlas of Mouse Development*. London: Academic Press). Expression peaks in the fetal liver by the 19th day and decreases to negligible amounts after birth (Sellem, C. H., et al., 1984, *Dev. Biol.* 102:51-60). The AFP prenatal expression pattern precedes fetal C/EBP α expression in the liver by 2-3 days where C/EBP α mRNA is detectable on day 13, peaks near the time of birth, and then also drops after birth (Birkenmeier, E. H., et al., 1989, *Genes Dev.* 3:1146-1146; Kuo, C. F., et al., 1990, *Dev.* 109:473-481).

AFP transcription normally remains off in the adult; in contrast, C/EBP α transcription rises to a maximum again as the animal enters the adult stage of development at six weeks of age (Darlington, G. J., et al., 1995, *Curr. Opinion Genet. Dev.* 5:565-570). The increased production of C/EBP α in adulthood is consistent with the decreased rate of hepatocyte proliferation at this stage. Interestingly, during periods of coerced hepatic cell division such as in partial hepatectomies and tumorigenesis, C/EBP α transcription becomes suppressed while AFP expression becomes reactivated (Mischoulon, D., et al., 1992, *Mol. Cell. Biol.* 12:2553-2560; Tomizawa, M., et al., 1998, *Biochem. Biophys. Res. Comm.* 249:1-5).

This inverse relationship in gene activation between C/EBP α and AFP represents an ideal condition for artificially expressing C/EBP α under the

regulatory elements of the AFP gene in order to enlist C/EBP α as an inhibitor of tumour growth during chemically induced carcinogenesis.

According to a preferred embodiment, the first and second regions of the DNA
5 construct of the invention have homology with the endogenous human or
animal AFP promoter of a gene locus and with a downstream sequence
region within the gene locus. Accordingly, with standard gene targeting
techniques, a single-copy of the exogenous C/EBP α transgene is placed
under the control of the endogenous mammal, human or non-human animal,
10 tumour-specific promoter (ex. AFP promoter) to create a cell or a knock-in
non-human animal capable of producing exogenous C/EBP α at a time
different from the endogenous C/EBP α . For example, when the exogenous
C/EBP α transgene is placed under the control of the endogenous AFP gene
promoter to create, for example, a C/EBP α gene knock-in mouse strain, the
15 exogenous C/EBP α is transcribed specifically in the fetal liver at a time point
earlier than normal as well as in developing liver tumour cells.

The DNA construct of the invention preferably further comprises at least one
selectable marker gene sequence.

As dominant gene sequence, the neomycin gene selectable marker can be
20 used for positive selection (for instance PGKneo). The neomycin resistance
gene confers a survival advantage to a cell cultured in the presence of
neomycin or G418.

Other examples of selectable marker gene sequences include the hprt gene,
25 the tk gene (preferably the MC1tk cassette, that is, the Herpes Simplex Virus
type 1 thymidine kinase), the nptII gene (Thomas, K. R. et al., 1987, Cell
51:503-512; Mansour, S. L. et al., 1988, Nature 336:348-352, both herein
incorporated by reference), or other genes which confer resistance to amino
acid or nucleoside analogs, or antibiotics, etc. The selectable or detectable

marker gene may be any gene which can complement for a recognizable cellular deficiency. Most preferably, the DNA construct may contain flanking gene sequences capable of "negative selection" as well as "positive selection," such as the tk gene that encodes thymidine kinase, or the hprt gene that encodes hypoxanthine phosphoribosyl transferase. Cells
5 expressing active thymidine kinase are able to grow in media containing HATG, but are unable to grow in media containing nucleoside analogs such as 5-azacytidine (Giphart-Gassler, M. et al., 1989, Mutat. Res. 214:223-232). Cells containing an active HSV-tk gene are incapable of growing in the
10 presence of gancyclovir or similar agents (Giphart-Gassler, M. et al., 1989, Mutat. Res. 214:223-232). Cells which express an active HPRT enzyme are unable to grow in the presence of certain nucleoside analogs (such as 6-thioguanine, 8-azapurine, and the like), but are able to grow in media supplemented with HAT (hypoxanthine, aminopterin, and thymidine).
15 Conversely, cells which fail to express an active HPRT enzyme are unable to grow in media containing HATG, but are resistant to analogs such as 6-thioguanine, and the like (Littlefield, J. W., 1964, Science 145:709-710).

The DNA construct according to the invention can be introduced into a cell, for
20 example a hepatocyte, a proliferating somatic cell, a pluripotent stem cell or an adult stem cell, by any method which allows the introduced molecule to undergo recombination at its regions of homology. Examples of such standard methods are electroporation, microinjection, calcium phosphate transformation and the like.

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After transformation, the cells are cultured in medium that selects for transfectants that have received and integrated the positive selectable marker gene sequence. The culturing conditions are then altered, such that they can select against recipient cells that have acquired the negative gene. A positive
30 and negative selection may also be accomplished simultaneously. Since all of

these sequences are present on the same targeting vector molecule, such dual selection requires a recombinational event to occur.

5 The survivors of the selection are then screened either for the function of the chromosomal allele, or more preferably, by Southern blot analysis for clones in which the selectable marker gene sequence has been introduced into the chromosomal allele.

After selection for cells which have incorporated the desired DNA molecule (for example by selection for G418 resistant cell), the cells are cultured, and
10 the presence of the introduced DNA molecule is confirmed as described above. Any of a variety of methods may be used to identify cells which have undergone the desired recombinational event. Direct screening of clones, use of PCR, use of hybridization probes, etc., may all be employed for this purpose.

15 The DNA construct of the invention may also comprise an identification (ID) tag, which is used to differentiate transcripts produced by the transgenic C/EBP α from the endogenous C/EBP α mRNA, for example, by introducing a novel restriction enzyme site into the sequence, the transgenic C/EBP α cDNA can be distinguished from the endogenous gene product through the use of
20 PCR and/or restriction enzyme digestion. The positioning of an ID tag incorporating a novel HindIII site in one embodiment of the DNA construct of the invention is shown in Figure 1.

The DNA construct of the invention may be incorporated into vector molecules. The term vector, as used herein is intended to denote any viral or
25 plasmid molecule that is capable of being introduced (as by transformation, electroporation, transfection, or the like) and/or propagated (i.e. replicated) in a prokaryotic or eukaryotic cell.

Preferred prokaryotic vectors include plasmids such as those capable of replication in *E. coli* such as, for example, pBR322, ColE1, pSC101, pACYC 184, pVX. Such plasmids are, for example, disclosed by Sambrook, J. and Russel D.W., 2001, In: Molecular Cloning, A Laboratory Manual, 3rd Ed., Cold Spring Harbor Press, Cold Spring Harbor, N.Y. Bacillus plasmids include pC194, pC221, pT127, and the like. If desired, yeast and fungal vectors may be used. Examples of suitable yeast vectors include the yeast 2-micron circle, the expression plasmids YEP13, YCP and YRP, etc., or their derivatives. Such plasmids are well known in the art (Botstein, D., et al., 1982, Miami Wntr. Symp. 19:265-274). Examples of mammalian vectors are disclosed by Sambrook, J. and Russel D.W., 2001, as above.

The C/EBP α -encoding sequences may be expressed in mammalian cells, using eukaryotic vectors (especially, a eukaryotic viral or retroviral vector). Typically, such vectors are designed to include a prokaryotic replicon, and selectable marker, such that the propagation of the vector in bacterial cells can be readily accomplished. In order to replicate in mammalian cells, however, the vectors will also contain a viral replicon, such as the replicon of Epstein-Barr virus, bovine papilloma virus, parvovirus, adenovirus, or papovirus (i.e. SV40 or polyoma virus).

Accordingly, the invention relates to any kind of expression vector comprising the DNA construct of the invention.

An example of an expression vector is the mouse AFP-C/EBP α gene targeting plasmid disclosed in Figure 2 and Figure 3A-C. The expression vector according to the invention may comprise or consist of the plasmid construct of Figure 2 and Figure 3A-C. Essential portions of the nucleotide sequence of the plasmid APF α NeoTK are shown in SEQ ID NO:1 and SEQ ID NO:2.

The DNA construct or expression vector according to the invention allows the positioning of the exogenous C/EBP α inside the chromosome of the host cell, under the control of the host cell endogenous tumour-specific promoter.

5 Preferably, the DNA construct comprises the upstream and downstream regions homologous to the AFP promoter and gene locus. Using standard gene targeting techniques, for example as described in A. L. Joyner's Gene Targeting: A Practical Approach, 1993, Oxford University Press, a single-copy of the exogenous C/EBP α transgene is placed under the control of the

10 endogenous human or animal tumour-specific promoter (ex. AFP promoter) to create a mammalian host cell or a knock-in non-human animal capable of producing exogenous C/EBP α at a time different from the endogenous C/EBP α . For example, when the exogenous C/EBP α transgene is placed under the control of the endogenous AFP gene to create, for example, a

15 C/EBP α gene knock-in mouse strain, the exogenous C/EBP α is transcribed specifically in the fetal liver at a time point earlier than normal as well as in developing liver tumour cells.

According to another aspect, the invention relates to a host cell, preferably a eukaryotic cell, comprising the DNA construct or any expression vector

20 according to the invention. The host cell is preferably a mammal, human or animal cell and more preferably, a hepatocyte.

The host cell transfected with the DNA construct or expression vector of the invention is indicated as a tumour-resistant cell.

Accordingly, the invention provides tumour-resistant cells in which the

25 exogenous C/EBP α transgene is specifically transcribed by the endogenous tumour-specific promoter, which is reactivated in tumours (where the normal expression of endogenous C/EBP α is turned off) initiating transcription of exogenous C/EBP α and inhibiting cell proliferation. Accordingly, the

exogenous C/EBP α introduced into the host cells with the DNA construct is only produced in transfected hepatocytes rendering them less prone to tumourigenesis and carcinogenesis than normal hepatocytes. Such tumour-resistant cells are useful for transplant or therapy. For example, hepatocytes containing the C/EBP α knock-in allele could be used to provide transplant patients with a cell source that is more resistant to tumourigenesis than normal liver cells and thus give the patient a level of protection against future liver cancer. Additionally, the tumour-resistant hepatocytes also can be used to populate bioartificial liver systems. The tumour-resistant hepatocytes in such a system would be less likely to secrete harmful tumourigenic substances into the body of the patient than non-transfected hepatocytes in traditional bioartificial systems.

The tumour-resistant host cell of the invention may be a single cell, a cell culture, a cell aggregate or a cell graft for transplant.

The tumour-resistant host cell may also be isolated from knock-in non-human animals modified by the DNA construct or expression vector of the invention as disclosed below.

The tumour-resistant host cell may also be isolated from human or non-human animal pluripotent embryonic (ES) or adult stem cells transfected with the DNA construct of expression vector. Such transfected embryonic or adult stem cells may be used in therapy to regenerate a tissue or organ in need of transplant or regeneration. Any ES or adult stem cells known in the art can be used. They can be, for example, human or animal ES or adult stem cells, of a normal karyotype and capable of continued proliferation in an undifferentiated state or capable of differentiating into a specific tissue cell. For example, those described in US 5,843,780, US 6,200,806, US2001024825 or US2003008392 (herein incorporated by reference). Also Thomson J., et al., 1995, Proceedings of the National Academy of Sciences of USA, Vol.92,

No.17:7844-7848; Reubinoff B.E. et al., April 2000, Nature Biotechnology, Vol.18, pages 399-404 (both of them herein incorporated by reference).

5 They can also be used for the preparation of transgenic animals, as explained below.

The invention provides a mammalian, human or non-human animal, hepatocyte graft for administration to an area in need of transplant, wherein the cells of the hepatocyte graft are tumour-resistant cells and comprise the DNA construct of the invention. The graft may be an autograft or allograft.

10 Also provided is a process for preparing a hepatocyte graft for administration to an area in need of transplant, wherein the process comprises making the hepatocytes of the graft tumour-resistant by transfecting these hepatocytes with the DNA construct of the invention.

15 Further, it is provided a method for transplanting hepatocytes, hepatocyte aggregates or hepatocyte grafts, comprising preparing hepatocytes, hepatocyte aggregates or hepatocyte grafts modified with the DNA construct of the invention, and transplanting the modified prepared hepatocytes, hepatocyte aggregates or hepatocyte grafts to an area in need of transplant.

20 According to another aspect, it is provided a method of treating cancer in mammal, human or non-human animals, whose cancers produce elevated levels of AFP comprising treating the mammal cancer with the DNA construct of the invention. In fact, the inverse relationship in gene activation between C/EBP α and AFP presents an ideal solution for artificially expressing C/EBP α under the regulatory elements of the AFP gene to suppress hepatic tumour
25 growth.

Hepatocellular carcinoma (HCC) is one of the most common cancers in the world and its prognosis remains poor (Nakatsura T., et al., 2003, Biochem

Biophys Res Commun., 306(1):16-25). Because of insufficient quantities of transplantable livers, alternatives for intact tissue transplantation have been developed including cellular hepatocyte transplantation (Ohashi K., et al., 2001, J Mol Med, 79:617-630) and, for short durations, the use of bioartificial
5 livers. A major concern regarding these alternatives is the potential of the cells in these systems to undergo neoplastic transformation. Transplanted hepatocytes that become malignant pose obvious dangers to the recipient; malignant cells in bioartificial livers also present serious problems as tumourigenic factors may be secreted by these cells into the body of the
10 patient. To reduce these risks in hepatocyte transplantation and bioartificial liver systems, genetic modifications in hepatocytes that minimize the occurrence of tumourigenesis are highly desirable.

According to a further aspect, it is therefore provided tumour-resistant hepatocyte cultures or hepatocyte aggregates prepared according to any
15 embodiment of the invention for use in bioartificial livers (also known as hepatocyte bioreactors).

Hepatocyte bioreactors or bioartificial livers are known in the art (for example as disclosed in US 5,270,192 herein incorporated by reference).

A hepatocyte bioreactor or bioartificial liver may comprise a containment
20 vessel having a perfusion inlet and a perfusion outlet; a matrix within the containment vessel so as to entrap hepatocyte aggregates within the containment vessel while allowing perfusion of the matrix. Examples of bioartificial livers known in the art are: Extracorporeal Liver Assist Device (ELAD®), Vitagen; HepatAssist 2000, Circle Biomedical; Bioartificial Liver
25 Support System (BLSS®), Excorp Medical, Inc.; LIVERX2000 System, Algenix Inc.; Modular Extracorporeal Liver System (MEL®), Charite Virchow Clinic Berlin, etc.

Tumour-resistant host cells, preferably hepatocytes, transfected with the DNA construct of the invention are preferably isolated from knock-in animals prepared as described below.

- 5 Experimental results using cultured primary liver cells derived from newborn knock-in mice indicate that the cells experience a significantly greater degree of growth inhibition than normal cells when exposed to chemically induced DNA damage. DNA damage is one of the initiators of HCC (Bralet M., et al., 2002, Hepatology, 36(3):624-630). This observation suggests that the
10 cultured knock-in liver cells possess a greater resistance to tumourigenesis than normal liver cells.

- Another embodiment of the invention is to provide a method for the treatment of symptoms in premature human infants or premature animals associated
15 with liver glycogen-insufficiency.

- The capacity to synthesize and store glycogen is one of the hallmarks of mature hepatocytes. Normal hepatocytes do not gain this ability until near the time of birth. As described later, the present authors have found that after
20 birth, the C/EBP α gene knock-in mice develop completely normally and exhibit no adverse effects from carrying the C/EBP α transgene. Accordingly, if the accelerated maturation of hepatocytes is introduced into prematurely born human infants or prematurely born non-human animals, a large portion of the symptoms related to energy insufficiency in the premature infants or
25 animals would be alleviated. This goal is achieved by administering to, injecting into, or transfecting the liver of a premature infant or animal, showing symptoms associated with liver glycogen-insufficiency, with the AFP-C/EBP α construct of the invention. Transient transfection of the DNA construct into premature infants or animals accelerates the maturation process of their
30 underdeveloped livers. Furthermore, as activation of the AFP promoter is

specific to the fetal liver, inadvertent transfection of non-liver cells would not result in unwanted C/EBP α expression.

Also, the AFP-C/EBP α construct of the invention can be used in a new gene therapy strategy to combat hepatocellular carcinoma in humans or animals. Transient transfection of the DNA construct into patients with liver cancer would suppress growth only of cancerous cells without affecting normal cells because the majority of liver cancer cells reactivate the AFP promoter while normal cells do not.

10

In order to provide a "gene therapy" for the cancer, the DNA construct of the invention is preferably introduced into a suitable vector, most preferably, viral or retroviral vectors are employed for this purpose. Examples of suitable vectors, in addition to those identified above, are discussed by Fletcher, F. A. et al., 1991, J. Exper. Med. 174:837-845; Makela, T. P. et al., 1992, Gene 118:293-294; Porgador, A. et al., 1992, Canc. Res. 52:3679-3686; Yoshimura, K. et al., 1992, Nucl. Acids Res. 20:3233-3240.

15

The principles of gene therapy are disclosed by Oldham, R. K. (In: Principles of Biotherapy, Raven Press, NY, 1987), and similar texts. Disclosures of the methods and uses for gene therapy are provided by Boggs, S. S. (Int. J. Cell Clon. 8:80-96 (1990)); Karson, E. M. (Biol. Reprod. 42:39-49 (1990)); Ledley, F. D. (In: Biotechnology, A Comprehensive Treatise, volume 7B, Gene Technology, VCH Publishers, Inc. NY, pp 399-458 (1989)); all of which references are incorporated herein by reference. Such gene therapy can be provided to a recipient in order to treat (i.e. suppress or attenuate) an existing condition, or to provide a prophylactic gene therapy to individuals who, due to inherited genetic mutations, or somatic cell mutation, contain cells having impaired gene expression (for example, only a single functional allele of the C/EBP α gene).

25

30

Adenovirus vectors may be employed to deliver the exogenous C/EBP α -encoding sequences to the liver (as by injecting such vectors directly into the recipient's portal vein). The nature of the blood flow to the liver makes it likely
5 that any vector delivered via the portal vein will come to reside within the hepatocytes and tumours of the liver, but is unlikely to extend outside of that organ to other tissues. In sum, the vectors are likely to become "trapped" in the liver tissue which serves as a filter for the body.

10 In a similar manner as above, constructs containing a lung-specific promoter in place of the AFP promoter can be used to accelerate maturation of underdeveloped lung tissues leading to improved survival and quality of life for these infants as well as to treat pulmonary cancer in adult human or animals. Some promoters which may be used in conjunction with C/EBP α for
15 inhibiting lung cancer are: GRP (gastrin-releasing peptide), NSE (neuron specific enolase), and CEA (carcinoembryonic antigen).

Another embodiment of the invention relates to the preparation of knock-in non-human animals, preferably mammals, transfected with the DNA construct
20 or expression vector of the invention.

The present inventors have successfully used genetic engineering processes to create a C/EBP α gene knock-in mouse model that expresses the C/EBP α transgene specifically in liver cells in a bid to inhibit the development of
25 hepatocellular carcinoma. Standard gene targeting techniques (as described in A. L. Joyner's Gene Targeting: A Practical Approach, 1993, Oxford University Press) enabled the present authors to place a single copy of the C/EBP α transgene under the regulation of one of the two endogenous alleles of the mouse AFP gene promoter. However, as already mentioned, other

tumour-specific promoters other than the AFP promoter may be used. Also other knock-in non-human animals other than mice can be created.

Knock-in non-human animals can be prepared according to any standard
5 technique known in the art.

An outline of an example of the preparation of knock-in mice is shown in Figure 4.

10 The DNA construct or expression vector according to any embodiment of the invention can be used for the preparation of non-human knock-in animals. Preferably, the DNA construct and/or vector is linearized and introduced into pluripotent recipient cells (preferably embryonic stem ("ES") cells), or equivalent (Robertson, E. J., In: Current Communications in Molecular
15 Biology, Capecchi, M. R. (ed.), Cold Spring Harbor Press, Cold Spring Harbor, N.Y. (1989), pp. 39-44, which reference is incorporated herein by reference). The transfected pluripotent cell may be cultured in vivo, in a manner known in the art (Evans, M. J. et al., Nature 292:154-156 (1981)) to form a transgenic animal.

20

Any ES cell may be used in accordance with the present invention. It is, however, preferred to use primary isolates of ES cells. For example, those prepared according to US 5,843,780, US 6,200,806, US2001024825 or US2003008392 (herein incorporated by reference). Also Thomson J., et al.,
25 1995, Proceedings of the National Academy of Sciences of USA, Vol.92, No.17:7844-7848; Reubinoff B.E. et al., April 2000, Nature Biotechnology, Vol.18, pages 399-404 (both of them herein incorporated by reference).

Such isolates may also be obtained directly from embryos such as the CCE cell line disclosed by Robertson, E. J., In: Current Communications in
30 Molecular Biology, Capecchi, M. R. (ed.), Cold Spring Harbor Press, Cold

Spring Harbor, N.Y. (1989), pp. 39-44), or from the clonal isolation of ES cells from the CCE cell line (Schwartzberg, P. A. et al., Science 246:799-803 (1989), which reference is incorporated herein by reference). Such clonal isolation may be accomplished according to the method of E. J. Robertson (In: Teratocarcinomas and Embryonic Stem Cells: A Practical Approach, (E. J. Robertson, Ed.), IRL Press, Oxford, 1987) which reference and method are incorporated herein by reference. The purpose of such clonal propagation is to obtain ES cells which have a greater efficiency for differentiating into an animal.

10

The DNA construct comprising the exogenous C/EBP α may be introduced into the pluripotent cell by any method which allows the introduced molecule to undergo recombination at its regions of homology.

15 After electroporation, the ES cells are cultured in medium that selects for transfectants that have received and integrated the positive selectable marker gene sequence. The culturing conditions are then altered, such that they can select against recipient cells that have acquired the negative selection gene.

20 The survivors of such selection are then screened either for the function of the chromosomal allele, or more preferably, by Southern blot analysis for clones in which the selectable marker gene sequence has been introduced into the originally present chromosomal allele.

25 After selection for cells which have incorporated the desired DNA molecule (for example by selection for G418 resistant cells), the cells are cultured, and the presence of the introduced DNA molecule encoding exogenous C/EBP α under the control of the endogenous tumour-specific promoter is confirmed as described above. Any of a variety of methods may be used to identify cells
30 which have undergone the desired recombination event. Direct screening of

clones, use of PCR, use of hybridization probes, etc., may all be employed for this purpose.

ES cells that have undergone recombination may be used to produce the knock-in non-human transgenic animals. The ES cells are preferably cultured on stromal cells (such as STO cells (especially SNC4 STO cells) and/or primary embryonic fibroblast cells) as described by E. J. Robertson (In: Teratocarcinomas and Embryonic Stem Cells: A Practical Approach, (E. J. Robertson, Ed.), IRL Press, Oxford, 1987, pp 71-112), which reference is incorporated herein by reference. The stromal (and/or fibroblast) cells serve to eliminate the clonal overgrowth of abnormal ES cells.

In a preferred embodiment, the present authors have used gene targeting techniques to place a single-copy C/EBP α transgene under the control of the endogenous mouse α -fetoprotein (AFP) gene promoter to create a C/EBP α gene knock-in mouse strain. The transgene is expressed in the fetal liver several days prior to endogenous C/EBP α production and then shuts down at the time of birth in accordance to AFP regulation.

The experiments have demonstrated that early activation of C/EBP α in the knock-in mouse liver induces accelerated maturation of the tissue leading to glycogen deposition earlier than that found in wildtype mice. Accordingly, the transient transfection of C/EBP α -expressing constructs of the invention can be used in gene therapy to ameliorate the physiologic dysfunction of the human premature infant or premature animals.

Additionally, C/EBP α is known to be necessary for maintaining the non-transformed hepatic phenotype. Because the AFP promoter is reactivated in hepatocellular carcinoma, where normal C/EBP α expression is turned off, our

knock-in mice gave us a unique opportunity to test C/EBP α as an inhibitor of liver tumour formation *in vivo*.

This novel non-human animal knock-in model may be used as an *in vivo* platform for identifying the types of carcinogens and the varieties of tumours
5 that can be inhibited and suppressed by expression of transgenic C/EBP α .

More in particular, these novel non-human animal knock-in model or host cells, preferably hepatocytes or pneumocytes obtained from such knock-in
10 animal, may be used as an *in vivo* platform for identifying the types of carcinogens and the varieties of tumours that can be inhibited and suppressed by expression of transgenic C/EBP α .

The host cells, in particular hepatocytes or pneumocytes, derived from this
15 knock-in non-human animal may also be used as an *in vitro* assay for carcinogens that are susceptible to C/EBP α treatment.

Carcinogens are generally indicated as substances that increase the risk of initiating development of neoplasms in humans or animals. A carcinogen may
20 be a chemical, a form of electromagnetic radiation or an inert solid body. Both genotoxic chemicals, which affect DNA directly, and nongenotoxic chemicals, which induce neoplasms by other mechanism, are included. DEN is an example of a genotoxic agent known to produce hepatocellular carcinomas (Bralet, M., et al., 2002, *Hepatology* 36(3):624-630).

25

Accordingly, it is also provided a method for *in vivo* screening and/or modulation of tumours comprising administering one or more carcinogens to a non-human animal modified with the DNA construct of the invention and identifying the tumours which are inhibited or suppressed by the expression of
30 transgenic C/EBP α .

The knock-in non-human animal according to the invention may be used to supply liver cells that are highly resistant to tumourigenesis for use in models testing hepatocyte transplantation and for populating bioartificial liver systems.

- 5 One common concern regarding both hepatocyte transplantation and bioartificial livers is the potential of their cells to undergo neoplastic transformation. The presence of the C/EBP α transgene in the hepatocytes would greatly reduce the risk of this complication.
- 10 Accordingly, it is provided: a non-human knock-in animal modified with the DNA construct or expression vector of the invention; a non-human knock-in animal, wherein the animal's endogenous tumour-specific promoter transcribes the exogenous C/EBP α transgene specifically in the fetal liver at a point earlier than normal; a knock-in animal, wherein the tumour-specific
- 15 promoter is AFP; a knock-in animal, wherein the exogenous C/EBP α transgene expresses C/EBP α mRNA prior to endogenous C/EBP α production; a tumour-resistant cell isolated from the knock-in animal according to the invention; a tumour-resistant cell isolated from the knock-in animals, wherein the tumour-resistant cell is a hepatocyte or pneumocyte; a human or animal
- 20 pluripotent embryonic or adult stem cell transfected with the DNA construct of claim; a tumour-resistant cell, wherein the tumour-resistant cell is isolated from a human or animal pluripotent embryonic or adult stem cell transfected with the DNA construct; a method for preparing a knock-in non-human animal comprising introducing the DNA construct or expression vector of the
- 25 invention into a non-human animal pluripotent embryonic or adult stem cell, and breeding the cell culture.

The present invention also relates to a medical or pharmaceutical composition comprising any DNA construct and/or expression vector of the invention and optionally a pharmaceutically acceptable carrier and/or diluent.

Said medical or pharmaceutical composition may be used for any medical treatment, and in particular for any of the treatments indicated, such as but not limited to: the treatment of cancer, preferably for the treatment of cancer in a human or animal whose cancer cells produce elevate levels of AFP; the
5 treatment of symptoms associated with liver glycogen-insufficiency in a human premature infant or a premature animal; the treatment of conditions associated with underdeveloped lung tissue in a human or animal.

The invention further relates to the use of any DNA construct and/or vector according to the invention for the preparation of a medical or pharmaceutical
10 composition for the treatment of (but not limited to): the treatment of cancer, preferably for the treatment of cancer in human or animal whose cancer cells produce elevate levels of AFP; the treatment of symptoms associated with liver glycogen-insufficiency in a human premature infant or a premature animal; the treatment of conditions associated with underdeveloped lung
15 tissue in human or animal.

Having now generally described the invention, the same will be more readily understood through reference to the following examples which are provided by way of illustration, and are not intended to be limiting of the present
20 invention.

Examples

25 Example 1

Method used to produce the C/EBP α gene knock-in mouse

Stratagene's Mouse Lambda Genomic Library – 129SvJ (Catalog #946313) was used to isolate the necessary AFP and C/EBP α sequences. Routine
30 molecular biology techniques (for example Sambrook J. and Russel D. W.,

Molecular Cloning, A Laboratory Manual, 3rd edition, 2001, Cold Spring Harbor Laboratory Press) were used to build the AFP-C/EBP α gene targeting construct detailed in Figures 1-3.

- 5 A 5.5kb EcoRI-TaqI fragment of the AFP promoter and upstream sequence was spliced onto the NruI-SpeI C/EBP α gene sequence in a pBlueScript vector. An extra T nucleotide at position 5549 was retained between the AFP fragment and the C/EBP α fragment which facilitated the construction process. An oligonucleotide sequence containing the HindIII identification tag and two
- 10 polyadenylation signals was ligated into the SpeI site at the end of the C/EBP α gene sequence from position 7021-7056. A PGKneo expression cassette containing the neomycin gene and a PGK promoter (purchased from Lexicon Genetics Incorporated) was placed downstream of the C/EBP α polyadenylation signals by blunt-end ligation into an EcoRI site that was
- 15 engineered into the identification tag oligonucleotide sequence. An additional 900bp of AFP sequence comprising a portion of intron 1 through intron 3 isolated by SacI digestion was blunt-end ligated downstream of the neomycin cassette to provide sufficient homologous sequence to promote efficient gene targeting into the AFP locus. MC1tk containing the thymidine kinase gene and
- 20 MC1 promoter (purchased from Lexicon Genetics Incorporated) was placed at the 3' end of the cloned sequence again by blunt-end ligation into the original 3' end SacI site. The obtained construct (25 μ g/ml) was linearized using EcoRI and electroporated into mouse embryonic stem cells (1.8×10^6 cells/ml) (as adequately explained in A. L. Joyner's Gene Targeting: A Practical
- 25 Approach, 1993, Oxford University Press and Hogan et al.'s Manipulating the Mouse Embryo: A Laboratory Manual, 1994, CSHL Press). Positive drug selection using G418 (active ingredient, 0.18mg/ml) and negative selection using gancyclovir (2 μ mol/L) were employed to enrich the yield of gene targeted clones. Correctly modified stem cells were identified by PCR and
- 30 Southern blotting. These cells were then used to generate chimeric founder

mice carrying the altered allele in their germ cells. The founders were bred to produce gene targeted knock-in mice.

The complete process for generating the knock-in mice is shown in Figure 4.

5

The wildtype C/EBP α gene locus and the expected knock-in gene locus are illustrated in Figure 5. A C/EBP α probe would be expected to detect DNA fragments of different sizes in wildtype versus knock-in mice (as illustrated).

- 10 Southern blots probed with a C/EBP α sequence demonstrated the presence of the altered allele in the knock-in mice (Figure 6). Under PstI digestion, knock-in mouse DNA shows an extra 11kb C/EBP α fragment that is absent in the wildtype animal. Under HindIII digestion, the extra C/EBP α fragment is 3.7kb in size.

15

As the genetic modification was designed to place a single copy of the C/EBP α transgene under the regulation of one of the two endogenous alleles of the mouse α -fetoprotein (AFP) gene promoter, we considered that the transgene would yield an expression pattern like the one illustrated in Figure

20 7.

Example 2

Determination of levels of C/EBP α mRNA and glycogen synthase mRNA

25

The levels of fetal C/EBP α mRNA have been assessed by using real-time PCR in a LightCycler. Oligonucleotide primers used were 5'-TGGACAAGAACAGCAACGAGTA-3' (SED ID NO:3) and 5'-GCAGTTGCCATGGCCTTGA-3' (SEQ ID NO:4).

30

We found C/EBP α mRNA in the knock-in mice to be detectable earlier and at higher amounts than in the wildtype animals (Figure 8).

Additionally, to evaluate the functionality of the newly expressed transgene,
5 we measured the levels of glycogen synthase mRNA also using real-time
PCR. Oligonucleotide primers used were 5'-
CCATGAACAGCAAGGGTTGTAA-3' (SEQ ID NO:5) and 5'-
TGGAAGTGGGCAACCACTATA-3' (SEQ ID NO:6). C/EBP α mRNA levels
were determined to be between 100% to 300% higher in the knock-in mice
10 from 12.5 – 15.5 days of gestation than in the wildtype and glycogen synthase
mRNA was 25%-50% higher over the same time period (Figure 9).

Earlier studies had shown C/EBP α to be a transcriptional activator of the
glycogen synthase gene (Wang, N. D., et al., 1995, *Science* 269:1108-1112).
15 Glycogen synthase mRNA was found to be expressed at higher levels than in
the wildtype mice (Figure 10).

Definitive proof of the functionality of the transgene product came in the form
of histology sections of the livers from the mice. Periodic acid-Schiff (PAS)
20 and PAS-diastase treated slides showed the presence of glycogen in the
knock-in mice on day 15.5 of gestation when wildtype mice had not yet begun
to deposit liver glycogen (Figure 11). Under PAS staining, glycogen produces
a pinkish hue, which is seen as darker cells in Figure 11A. Figure 11A shows
that glycogen is present in the liver of the knock-in mouse fetus at 15.5 days
25 gestational age. Diastase treatment digests away any glycogen that may be
present on the slide. Figure 11B shows that diastase treatment followed by
PAS staining does not produce the pink colour in the knock-in mouse liver
indicating that the pink colour had indeed originated from liver glycogen.
Figure 11C (PAS staining) and 11D (PAS-diastase) show that wildtype fetal

livers at 15.5 days gestational age do not normally contain detectable glycogen.

5 Example 3

Resistance to tumourigenesis

We further verified that our genetic modification could confer upon hepatocytes a degree of resistance to tumourigenesis.

10

We exposed cultures of primary hepatocytes derived from 18.5 day old mouse fetuses to 3.2mM n-nitrosopiperidine, a genotoxic agent. Fetal livers were minced with a scalpel under sterile conditions in calcium-free Earle's Balanced Salt Solution (Gibco-BRL) containing 50mmol/L EDTA to obtain
15 tissue fragments. These tissue fragments were then incubated in Earle's Balanced Salt Solution with calcium (Gibco-BRL) and 0.02mg/ml soybean trypsin inhibitor (Sigma) and 1.6mg/ml collagenase (Sigma) and incubated at 37°C, 5% CO₂. The EDTA/collagenase treatments were performed twice. For long-term observation, the control cells were grown in Hepatocyte Culture
20 Medium (HCM) (Clonetics) and the experimental groups were grown in HCM with 3.2mM n-nitrosopiperidine (Sigma) dissolved directly into it. Our experiments showed that the cultures of the knock-in hepatocytes experienced a greater degree of growth inhibition and cell death than the wildtype cells (Figures 12). Figure 13 shows that, in the absence of
25 carcinogen, the knock-in cells demonstrated survival rates comparable to that of wildtype cells.

Lastly, after a year-long experiment in which twelve day old juvenile knock-in and wildtype mice were given intraperitoneal injections of 10µg
30 diethylnitrosamine (DEN) per gram of body weight, we were able to observe

the effects of our transgene on the development of liver tumours in live animal models. DEN is another genotoxic agent known to produce hepatocellular carcinomas (Bralet, M., et al., 2002, *Hepatology* 36(3):624-630).

- 5 The mice were maintained on a 12 hour light and 12 hour dark daily cycle and had unrestricted access to food and water. Figure 14 shows that while the wildtype livers (A, B) contained many tumourous growths, knock-in livers (C, D) had dramatically fewer tumours. A visual survey of the liver surfaces for nodules and tumours revealed markedly fewer numbers in the knock-ins (19.8
10 ± 12.6 ; n = 10) than the wildtypes (41.0 ± 23.3 ; n = 5) (Student t test, $P > 0.05$). These data provide compelling evidence that C/EBP α expressed under the regulation of the AFP promoter results in liver cells that are much less likely to undergo tumourigenesis than normal hepatocytes.

Claims

1. A DNA construct comprising:
 - a first region having homology with an endogenous tumour-specific promoter gene sequence of a gene locus;
 - 5 a DNA molecule encoding C/EBP α , the DNA molecule located 3' of the first region; and
 - a second region having homology with a region within the gene locus, the second region located 3' of the DNA molecule;
 - wherein the construct is operable to express a functional
 - 10 C/EBP α protein.
2. The construct of claim 1, wherein the DNA molecule encodes human C/EBP α .
- 15 3. The construct of claim 1, wherein the DNA molecule encodes mouse C/EBP α .
4. The construct of claims 1-3, wherein the tumour-specific promoter gene comprises the AFP promoter gene and the gene locus comprises the
- 20 AFP gene locus.
5. The construct of claims 1-4, further comprising a selectable marker, and identification tag, or a combination thereof.
- 25 6. An expression vector comprising the construct of claims 1-5.
7. The vector of claim 6, wherein the expression vector further comprises:
 - a DNA molecule having the sequence of SEQ ID NO:1; and
 - a DNA molecule having the sequence of SEQ ID NO:2.

8. A host cell comprising the construct of claims 1-5 and/or the vector of claims 6-7.
- 5 9. The host cell of claim 8, wherein the host cell is present in a cell culture.
- 10 10. The host cell of claims 8-9, wherein the host cell is a mammal hepatocyte or pneumocyte.
- 11 11. The host cell of claims 8-10, wherein the host cell is a human or mouse cell.
- 15 12. The host cell of claims 8-11, wherein the host cell is a tumour-resistant cell.
- 20 13. The host cell of claims 8-12, wherein the host cell is a hepatocyte and/or pneumocyte suitable for grafting in an area in need of transplant.
- 25 14. The cell of claims 8-13, wherein the cell is a tumour resistant cell.
15. The cell of claim 14, wherein the tumour resistant cell is isolated from a knock-in non-human mammal including the DNA construct and/or the vector.
16. The cell of claims 14-15, wherein the cell is a hepatocyte or pneumocyte.

17. The cell of claims 14-16, wherein the cell is a pluripotent, embryonic, or adult stem cell.
18. The cell of claims 14 and 16-17, wherein the cell is a human cell.
- 5 19. The cell of claims 14-18, wherein the cell is derived from a stem cell.
20. A hepatocyte bioreactor comprising the construct of claims 1-5 and/or the vector of claims 6-7.
- 10 21. A pharmaceutical composition comprising the construct of claims 1-5 and/or the vector of claims 6-7.
- 15 22. The pharmaceutical composition of claim 21, further comprises a pharmaceutically acceptable carrier, a diluent, or a combination thereof.
- 20 23. The construct of claims 1-5 or the vector of claims 6-7 for use in medicine.
- 25 24. Use of the DNA construct of claims 1-5 or the vector of claims 6-7 for the preparation of a pharmaceutical composition for the treatment of any one or a combination of: symptoms associated with glycogen-insufficiency and treatment of cancer.
- 25 25. The use of claim 24, wherein the symptoms associated with glycogen-insufficiency are displayed in a human premature infant or a premature non-human animal.

26. The use of claims 24-25, wherein the cancer is in human or non-human animal whose cells produce elevated levels of AFP.
27. The use of claims 24-26, wherein the human or non-human animal was
5 born prematurely and suffers from liver glycogen-insufficiency.
28. The use of claims 24-25, wherein the human or non-human animal exhibits one or more conditions associated with underdeveloped lung tissue.
- 10 29. The use of claims 24-25, wherein the treatment further comprises accelerating maturation of underdeveloped lung tissues.
- 15 30. A process for introducing a construct into an isolated cell, cell culture or graft comprising the steps of:
 preparing a DNA construct operable to express functional mammalian C/EBP α protein, the construct comprising:
 a first region having homology with an endogenous tumour- specific promoter gene sequence of a gene locus;
 a DNA molecule encoding C/EBP α , the DNA molecule located 3' of the first region; and
 a second region having homology with a region within the gene locus, the second region located 3' of the DNA molecule; and
25 administering the construct to an isolated cell, cell culture or graft.
- 30 31. The process of claim 30, wherein the cell or cell culture is hepatocyte(s) and/or pneumocyte(s) and the insertion of the construct

modify the hepatocyte(s) and/or pneumocyte(s) to render the hepatocyte(s) and/or pneumocyte(s) tumour-resistant.

- 5 32. The process of claims 30-31, further comprising administering the hepatocyte and/or pneumocyte to a graft.
33. The process of claims 30-31, further comprising preparing hepatocyte and/or pneumocyte aggregate(s).
- 10 34. The process of claims 30-33, further comprising preparing hepatocyte and/or pneumocyte grafts.
35. The process of claims 30-34, wherein the graft is autograft or allograft.
- 15 36. A non-human knock-in mammal comprising the DNA construct of claims 1-5 and/or the vector of claims 6-7.
37. The mammal of claim 36, wherein the mammal is a mouse.
- 20 38. The mammal of claims 36-37, wherein the construct is transcribed in the fetal liver at an earlier point than normal.
39. The mammal of claims 36-38, wherein the construct expresses C/EBP α mRNA prior to endogenous C/EBP α production.
- 25 40. A method for *in vivo* tumour screening comprising:
 modifying a non-human animal to express the DNA construct of claims 1-5;
 administering to the animal one or more carcinogens; and

identifying one or more tumours, wherein the tumour(s) is inhibited and/or suppressed by the function of C/EBP α protein.

5 41. The method of claim 40, further comprising modulating growth of one or more tumours.

42. A method for *in vitro* tumour screening comprising:
modifying a cell or cell culture to express the DNA construct of claims 1-5;
10 administering to the cell or cell culture one or more carcinogens; and
identifying one or more tumour cell, wherein the tumour(s) is inhibited and/or suppressed by the function of C/EBP α protein.

15 43. A process for introducing the construct of claims 1-5, the vector of claims 6-7, the cell of claims 8-19 or the pharmaceutical composition of claims 21-22 into an area in need of a mammal subject, comprising administering the construct, vector, cell or composition into the area in need of the mammal subject.

20 44. The method of claim 43, wherein the further comprising:
modifying hepatocytes to include the construct, vector, cell or composition, wherein the modifying renders the hepatocytes tumor-resistant; and
administering the hepatocytes to an area in need of transplant.

25 45. The method of claim 44, wherein administering the hepatocytes further comprises supplying an autograft or allograft.

30 46. The method of claims 43-45, further comprising preparing hepatocyte aggregates.

47. The method of claims 43-46, further comprising preparing hepatocyte grafts.
- 5 48. The method of claim 47, wherein the mammal is born prematurely and suffers from liver glycogen-insufficiency.
49. The method of claims 43-48, further comprising:
administering the construct, vector, cell or composition into the
10 mammal; and
treating liver glycogen insufficiency in the mammal.
50. The method of claims 43-47, wherein the mammal comprises a cancer,
and wherein the cancer produces elevated levels of AFP.
15
51. The method of claims 43-47, further comprising:
administering the construct into the mammal; and
treating a cancer in the mammal, wherein the cancer produces
elevated levels of AFP.
20
52. The method of claims 43-47, wherein the mammal exhibits one or more
conditions associated with underdeveloped lung tissue.
53. The method of claims 43-47, further comprising:
25 administering the construct into the mammal; and
treating one or more symptoms of underdeveloped lung tissue in the
mammal.
54. The method of claim 53, further comprising accelerating maturation of
30 underdeveloped lung tissues.

55. The method of claims 43-53, wherein the mammal is a human or non-human animal.