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(54) Title: TRANSGENIC ANIMALS FOR ASSESSING DRUG METABOLISM AND TOXICITY

(57) Abstract: The present invention relates to transgenic non-human animals, tissues or cells derived therefrom and methods of producing them. The transgenic non-human animals or tissues or cells derived therefrom provide a system capable of expressing human proteins responsible for drug metabolism in place of the homologous endogenous non-human animal proteins and for the controlled expression of human genes introduced into the animal so that the expression of the human genes is regulated in a manner more closely analogous to that seen in vivo in humans. One aspect of the invention relates to the use of a human DNA sequence comprising at least part of introns 6 and/or 7 of the human PXR gene.

Transgenic Animals for Assessing Drug Metabolism and Toxicity

The present invention relates to transgenic non-human animals, tissues or cells derived therefrom and methods of producing them. The transgenic non-human animals or tissues or cells derived therefrom provide a system capable of expressing human proteins responsible for drug metabolism in place of the homologous endogenous non-human animal proteins and for the controlled expression of human genes introduced into the animal so that the expression of the human genes is regulated in a manner more closely analogous to that seen *in vivo* in humans. The transgenic non-human animals or tissues or cells derived therefrom are for use, especially but not exclusively, in assessing xenobiotic or drug metabolism, toxicity or other properties or functions of the introduced human proteins such as metabolism and/or biosynthesis of endogenous compounds.

Background to the Invention

A significant proportion of therapeutic drug candidates fail to become marketable drugs because of adverse metabolism or toxicity discovered during clinical trials. These failures represent a very significant waste of development expenditure and consequently there is a need for new technologies that can more reliably, quickly and economically predict at the pre-clinical development stage the metabolic and toxicological characteristics of drug candidates in man. At present, most pre-clinical metabolic and toxicity testing of drug candidates relies on laboratory animals, human and/or mammalian cell lines and/or tissues in culture. However, none of these methods is completely reliable in predicting metabolism or toxicity in a human subject. Metabolic and toxicological data from animals can differ significantly from that obtained from a human subject due to species differences in the biochemical mechanisms involved. In addition, interpretation of data derived from *in vitro* human cell cultures or isolated human tissue studies can be problematic since such systems are not available for all organs and tissues or they fail to retain the same metabolic characteristics as they possess *in vivo*.

It is known in the prior art that the metabolism, distribution and toxicity of most drugs depends on their interactions with four distinct main classes of proteins:

a) Phase-1 drug-metabolising enzymes, such as the cytochromes P450 which generally add or expose polar groups on the xenobiotic molecule;

b) Phase-2 drug-metabolising enzymes, such as transferases, in particular the glucuronyl transferases, glutathione transferases, sulphonyl transferases and acetyl transferases which conjugate the polarised xenobiotic molecule to a hydrophilic group thereby facilitating its subsequent excretion;

c) Drug transporter proteins, such as the ATP-binding cassette proteins which include the multi-drug resistance proteins (MDRs) and multi-drug resistance-associated proteins (MRPs) and the organic anion transporting polypeptides (OATPs) which facilitate the transport of drugs and other xenobiotic molecules across plasma membranes;

d) Transcription factors, such as the pregnane X receptor (PXR) and the constitutive androstane receptor (CAR) which regulate the transcription of genes encoding proteins of the preceding classes, in particular the cytochromes P450.

Variation between species is known in each of these protein classes both with respect to the multiplicity of proteins within each class, the function of the proteins themselves and with respect to genetic regulation of their expression.

It is known from WO2004/007708 how to produce non-human transgenic animals expressing functioning human P450s in which the functions of endogenous cytochromes P450 have been annulled by deletion of individual P450 genes or by deletion of the cytochrome P450 reductase gene encoding the enzyme on which the function of all cytochromes P450 depends. However, the described animal model has limitations in that not only are the introduced P450s restricted to particular organs or tissues of the non-human animal but also there is no provision to regulate expression of the human P450s in a manner that is analogous to that seen in the human. One of the prior art models is also limited in that additional modifications are needed to provide cytochrome P450 reductase activity to the introduced human P450s without reactivating endogenous non-human P450s. A yet further disadvantage resides in the lack of provision to reproduce human phase-2 metabolism, thus the system is unable to provide an entire metabolic profile.

It is also known from the prior art to humanise the induction characteristics of cytochromes P450 in the mouse by expressing human PXR (Xie et al, Nature Vol 406, 435-9, 2000) or human CAR (Zhang et al, Science Vol 298, 422-4, 2002) in a mouse wherein the mouse PXR gene and/or mouse CAR gene respectively have been deleted. While such animals demonstrate induction patterns of endogenous P450s that reproduce those seen in the human they have undesirable characteristics because the cytochromes P450 whose expression is regulated analogously to the human are still non-human cytochromes P450. A further disadvantage is that because the PXR or CAR genes themselves are not regulated as they are in the human by virtue of the transgene being driven by a heterologous tissue-specific promoter (albumin promoter), over-expression of the heterologous gene can occur which can have the result that a normal metabolic pathway is bypassed. Moreover, the PXR and CAR transgenes are derived from a cDNA rather than a genomic clone, thus the transgenic non-human animals consequently lack the sequences necessary

correctly to reproduce all the transcriptional and post-transcriptional regulation of PXR or CAR expression hence their expression is restricted to the liver and may not be of a physiological level. In addition these models do not encode for splice variants of the human gene. Another drawback of the PXR/CAR models is that they are unsuitable to combine with modifications of other genes within one animal since the humanisation of each gene is achieved by two independent genomic alterations: (i) knock-out of the endogenous gene (ii) transgenesis with the human orthologue under control of the albumin promoter at a different genomic location.

Ma *et al.* (Drug Metab Dispos. 2007 Feb;35(2):194-200) introduced the complete human PXR gene, including 5' and 3' flanking sequences, into PXR knock-out mice by bacterial artificial chromosome (BAC) transgenesis. They observed selective expression of human PXR in the liver and intestine. Treatment of PXR-humanised mice with PXR ligands mimicked the human response, as both hepatic and intestinal Cyp3a11 mRNA and protein were strongly induced by rifampicin, a human-specific PXR ligand, but not by pregnenolone 16α-carbonitrile (PCN), a rodent-specific PXR ligand. In wild-type mice, Cyp3a11 mRNA was strongly induced by PCN, but not by rifampicin.

There is therefore a need for improvements in animal models of human metabolism that can control expression of the human genes introduced into the animal so that their expression is regulated in a manner more closely analogous to that seen in humans. There is also a need for more aspects of the human metabolic pathway to be reproduced. Effective animal models of human metabolism require not only expression of the relevant human proteins but also annulment of the functions of the homologous endogenous proteins.

One reason why the present invention embodies a surprising advance over the prior art is that many prior art researchers appear to be of the view that the problem posed by the need for models of drug metabolism is already solved. For example, Xie and Evans (2002, DDT7, p509) state that humanising PXR is "one of the rare examples where replacing a single transcriptional regulator allows conversion of species-specific gene regulation". Furthermore, it is evident that workers have turned their attention to techniques that differ markedly to those that utilise transgenic animal systems. For example, attempts are being made to humanise the mouse liver as an organ by using human hepatocytes, the aim being to obtain a mouse model for drug metabolism in humans. This work is labour-intensive though, and in the inventors' opinion is of dubious relevance to the situation in reality.

The present invention is the first methodology that takes into account all of the problems that prior art systems suffer from and that seeks to resolve these problems in a practical manner. The

inventors have recognised that in order to provide transgenic non-human animal models with humanised drug metabolism pathways that overcome the undesirable features of the animal models described in the prior art a number of criteria should ideally be satisfied:

a) Regulation of the expression of introduced human proteins such that patterns of expression in the human are reproduced;

b) Expression of multiple human proteins so that multiple aspects of human metabolism are reproduced;

c) Annulled expression or function of multiple endogenous genes so that interference from non-human metabolic pathways on the functions of introduced human proteins is significantly reduced.

In the present invention, we provide methods of producing non-human animal cell and non-human transgenic animals that incorporate at least some if not all of these desired qualities. Such non-human animal cell and non-human transgenic animals possess desirable characteristics not available in the prior art in that they can model entire human pathways of xenobiotic metabolism rather than just individual elements of pathways and that such models are provided for all tissues and organs. This is achieved through the application of technical approaches hitherto not available in the prior art with respect to obtaining regulation of transgene expression analogous to that seen in human cells through the use of extensive regulatory DNA sequences and with respect to annulment of endogenous metabolic pathways through deletion or gene exchange. A number of relevant human proteins are expressed in a single animal.

STATEMENT OF THE INVENTION

The present invention provides transgenic non-human animals, tissues, cells derived therefrom, nucleic acid targeting vectors, and methods as set forth in the claims and herein below.

In general terms the present invention provides a non-human animal, tissue or cells derived therefrom incorporating at least one human DNA sequence encoding at least one transcription factor under control of a transcription factor promoter and whose endogenous equivalent genes have optionally been annulled, the non-human animal, tissue or cells derived therefrom further incorporating at least one or more of the following further human DNA sequences selected from the group comprising:

(i) a DNA sequence encoding a phase-1 drug-metabolising enzyme;

(ii) a DNA sequence encoding a phase-2 drug-metabolising enzyme; and/or

(iii) a DNA sequence encoding a drug transporter protein;

and whose endogenous equivalent gene or genes have optionally been annulled.

Throughout the specification and the claims which follow, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising" will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers.

Reference herein to "endogenous equivalent gene" of the non-human animal is intended to include a gene or genes whose expression product retains the same, similar or identical function as the human counterpart gene. For example, the human transcription factor gene known as PXR (NR1I2 nuclear receptor subfamily 1, group I, member 2), Entrez GeneID: 8856, has a murine counterpart of the same name whose Entrez GeneID is 18171. The proteins encoded by these genes have an equivalent function in the organisms from which they are derived.

Generally, the introduced transcription factor gene, the phase-1 drug-metabolising enzyme gene, the phase-2 drug-metabolising enzyme gene and/or the drug transporter protein gene will share a degree of homology with the endogenous gene with which it is equivalent. Preferably, the degree of homology will be greater than 30%, greater than 40%, greater than 50%, greater than 60%, greater than 70%, greater than 80%, greater than 90%, or even greater than 95%.

In the case of drug-metabolising enzyme genes, equivalence between genes can be assessed by a combination of substrate specificity, mode of regulation (for example, by transcription factors or exogenous drugs), sequence homology and tissue distribution. Certain genes have exact equivalents; examples of such genes are CYP2E1, CYP1A1, CYP1A2. CYP2B6 and CYP2D are examples where there is only one gene in the human, but numerous equivalent genes in the mouse. There are four CYP2C genes in the human, and numerous equivalent genes in the mouse. In such circumstances, preferably at least one, more preferably two, three, four, five or more or even all of the equivalent murine genes are annulled. CYP3A4 is an example where there is no obvious orthologue in the mouse, but Cyp3a11 could be considered at least one equivalent mouse gene because of its hepatic expression, mode of regulation and sequence homology.

Reference herein to "annulled" is intended to include silencing or deletion or rendering inactive so that the non-human animal's endogenous equivalent gene is unable to express the gene product(s), at least not to any level that is significant to the drug metabolism process. For instance, the expression level of an annulled gene may be less than 20%, preferably less than 10%, more preferably less than 5%, more preferably less than 2%, even more preferably 1% or less of the wild

type expression level. The expression of an annulled gene may preferably be decreased to the point at which it cannot be detected. Although annulling the non-human animal's endogenous equivalent gene(s) is preferred, in some embodiments the non-human animal's endogenous equivalent gene(s) are not annulled. For example, human DNA sequences encoding proteins involved in drug metabolism may be inserted into the mouse Rosa26 locus, which does not itself result in silencing, deletion or rendering inactive of a mouse gene involved in drug metabolism.

In particular, a first aspect of the present invention provides a non-human animal, tissue or cells derived therefrom incorporating at least one human DNA sequence encoding the transcription factor PXR (i.e., the pregnane X receptor), wherein said human DNA sequence comprises least part of intron 6 and/or intron 7 of the human PXR gene.

Partial genomic sequences

In the prior art techniques disclosed to date, researchers have generated transgenic models that incorporate human genes into non-human systems, notably the mouse. However, in devising these systems, little attention has been paid to retaining the context of the human gene as it exists in its natural state.

Generally, cDNA sequences have been used in the prior art rather than respecting the intron/exon structure of the human gene, *e.g.* by incorporating at least one intron of the human gene. This means that any splice variants that might naturally be generated, cannot form. Splice variants are important for a number of reasons. First, they may have a function. Second, they may have a dominant negative effect, for example, by binding to their usual protein partners and altering biological effects of the protein. Third, they may sequester ligand. A system that accurately reflects the *in vivo* situation therefore preferably mirrors the balance of splice variants that exist in any biological system.

Using cDNA also means that mRNA levels are artificially generated and may not reflect the reality of the natural physiological situation.

In contrast, the present invention attempts to mirror the *in vivo* situation by providing the human gene in its entirety where this is possible. This means that the intron-exon junctions are retained as in the natural system so that splicing events can happen exactly as in the natural situation. Where, perhaps because of the length of a gene, it is not simple to transpose the entire human gene into a transgenic system, the invention seeks to use a combination of cDNA and genomic DNA in its constructs so that important intron-exon boundaries, where the majority of splicing events occur, are retained.

Thus, the human DNA sequence encoding a transcription factor, drug-metabolizing enzyme or drug transporter protein can be a partial human genomic gene sequence or a complete human genomic gene sequence.

According to the invention, therefore, where it is known that the majority of splice variants occur as a result of splicing variation within a particular intron, this intron is preferably incorporated as genomic DNA in the construct, while less influential intronic sequences are not retained. This has the result that levels of functional mRNA and functional protein mirror the levels that are found *in vivo* in response to exposure to a particular drug or drug cocktail. This is what is ideally required for a physiologically-relevant model.

Accordingly, whilst cDNA sequences may be used, in preference to these sequences, the invention may use a combination of cDNA and genomic sequences from the gene that is to be humanised. When a combination of cDNA and genomic sequences is used, the human DNA sequence contains some but not all of the introns and exons of the human gene. A number of non-human animal models involving constructs comprising a combination of human cDNA and genomic sequences have been generated by the inventors.For example, in the case of a transgenic animal expressing the human PXR gene, it is disclosed in WO 2006/064197 that due to the large size of more than 35kb of the human PXR gene, the intron-exon structure between exons 4 and 6 is preferably maintained, since most splice variants are observed in this genomic region, since it is located within the ligand-binding domain (*e.g.* see Figure 2). This advantageously retains the sequence where most splice variants are observed and is conveniently located within the ligand-binding domain.

However, it has now been found that in the case of a transgenic animal expressing the human PXR gene, a human DNA sequence encoding the transcription factor PXR preferably comprises least part of intron 6 and/or intron 7 of the human PXR gene. Thus, exons 6 and 8 and/or exons 7 and 8, or preferably exons 6, 7, and 8 are not fused to create a partial cDNA sequence. This avoids the problem of unforeseen cryptic splice sites which may be created when in such a partial cDNA sequence, e.g., at the junction between fused exons 7 and 8. Such cryptic splice sites may lead to the creation of aberrant splice variants, the presence of which may attenuate the expression of the functional human PXR protein in the non-human animal, tissue or cells derived therefrom, or otherwise prevent optimal humanisation of PXR expression. The expression of human DNA sequences comprising at least part of intron 6 and/or intron 7 of the human PXR gene is thus advantageous, because such expression of PXR most accurately reflects the expression levels, including the detailed distribution of splice variants, found in humans.

The data presented in Example 5 (Part A) and in Figure 85 show that mouse expressing a human DNA sequence encoding PXR (i.e., having a huPXR genotype) comprising introns 4, 5, 6 and 7 was yet more sensitive to rifampicin than a mouse expressing a human DNA sequence containing only introns 4 and 5, consistent with the higher sensitivity of humans to rifampicin, compared to mice.

Preferably, a human DNA sequence encoding PXR according to the present invention comprises at least part of introns 4, 5, 6, and/or 7 of the human PXR gene. It is preferred that introns 4, 5, 6 and 7 are all retained. Such a human DNA is exemplified by the huPXR sequence of SEQ ID NO:2.

In other preferred embodiments, the intron-exon structure between exons 4 and 8 of the human PXR gene is maintained (*cf.* SEQ ID NO:2, and see, *e.g.* Figure 62).

In other preferred embodiments, a human DNA sequence encoding PXR according to the present invention further comprises at least part of intron 2, intron 3 and/or intron 8 of the human PXR gene, and/or, moreover, the intron-exon structure between exons 2 and 9 of the human PXR gene is maintained (*e.g.* see Figure 64 and *cf.* SEQ ID NO:3).

In certain preferred embodiments, the invention thus provides non-human animal, tissue or cells according to claim 1, wherein said human DNA sequence comprises a sequence that is at least 50%, preferably 60%, 70%, 80%, 90%, or 95% identical to the sequence of SEQ ID NO:2 or the sequence of SEQ ID NO:3. These sequences are described in more detail herein below.

Another example is provided by the case of the humanization cassette preferably used for CYP3A4: this may contain the 13kb human CYP3A4 promoter, exon 1 and intron 1 as in the normal genomic constitution and a human cDNA consisting of exons 2-13 (*e.g.* see Figure 9).

Another example is provided by the case of the humanization cassette preferably used for CYP2C9: this may contain the 12kb human CYP2C9 promoter, a human cDNA of exons 1-4, intron 4 and a cDNA of exons 5-9 (*e.g.* see Figure 10).

Another example is provided by the case of the humanization cassette preferably used for PPARα: this may maintain the intron-exon structure between exons 3 and 8 of the human PPARα gene, *e.g.* by including at least part of intron 5 and/or intron 6 (see Figure 68).

Complete genomic sequences

Complete genomic DNA sequences may be used. For instance, in the case of a transgenic animal expressing the human CAR gene, the relatively small size of the human CAR, which comprises roughly 7kb from exon 2-9, makes it simple to retain the complete genomic structure in the targeting vector. The construct should preferably retain the intron-exon structure between exons 2

and 9. This advantageously retains the complete genomic structure within the targeting vector and permits coverage of all splice variants of human CAR. Preferably, the genomic human CAR sequence is fused to the translational start site of the mouse CAR gene. The human CAR sequence then contains all genomic sequences of exons 1-9. The 5' and 3'UTRs may be human or may be retained from the mouse genome. All other parts of the coding sequences of the mouse CAR gene can be deleted.

Use of complete genomic gene sequences may conveniently be achieved by use of a cluster of human DNA sequences, as described in more detail elsewhere herein (*e.g.* see Figures 72, 74 and 75).

When a complete genomic DNA sequence is used, the human DNA sequence will contain all of the introns and exons of the human gene, and may optionally further comprise some or all of the regulatory sequences normally associated with the human gene (as described in more detail elsewhere herein).

Preferably, the human DNA sequence encoding PXR according to the invention is inserted into the mouse PXR locus, such that expression of human PXR is under the control of the mouse PXR promoter.

Preferably, the human DNA sequence encoding PXR according to the invention is incorporated in a non-human animal, tissue or cells derived therefrom, which non-human animal, tissue or cells further comprise at least one of any one or more of the following human DNA sequences encoding::

(i) a further transcription factor

(ii) a phase-1 drug-metabolising enzyme;

(iii) a phase-2 drug-metabolising enzyme;

and/or

(iv) a drug transporter protein.

According to the invention, any further transcription factor, phase-1 drug-metabolising enzyme; phase-2 drug-metabolising enzyme; and/or drug transporter protein may be used, depending on the indended use of the non-human animal, tissue or cells. Preferably, the further transcription factor, phase-1 drug-metabolising enzyme; phase-2 drug-metabolising enzyme; and/or drug transporter protein is/are defined as set forth in the claims and herein below.

Transcription factors

Preferably, the human DNA encoding a transcription factor is selected from the group comprising the pregnane X receptor (PXR, also known as the steroid and xenobiotic receptor SXR) and the constitutive androstane receptor (CAR) or multiples thereof or a combination thereof. Animals and cells according to this aspect of the invention are advantageous for the reasons described in detail above. For example, recent evidence further supports the contention that the ligand binding domains of the murine and human CAR proteins are divergent relative to other nuclear hormone receptors, resulting in species-specific differences in xenobiotic responses (Huang *et al.*, 2004, Molecular endocrinology 18(10):2402-2408). Results reported in this paper demonstrate that a single compound can induce opposite xenobiotic responses via orthologous receptors in rodents and humans.

Transgenic mice for human CAR have been created and are described in the examples included herein. Detailed investigations of the induction of drug metabolism pathways in CAR humanised and knock-out mice have been performed. Various different experimental approaches have confirmed that non-human transgenic animals that are humanised with respect to CAR, or which do not express any CAR (knock-out), can readily be obtained using the methods and strategies described herein.

Transgenic mice for human PXR have been created and are described in the examples included herein. Human PXR is found to be expressed in both the liver and GI tract of mice in the predicted manner at levels equivalent to those of the endogenous mouse gene. In this way, typical problems faced by conventional techniques of this type, such as over- or under-expression are avoided. Furthermore, currently available humanised PXR models use the albumin promoter to drive human PXR which has been crossed into a PXR-null background. Therefore, PXR is expressed at very high levels in this model and there is no PXR in any tissue other than the liver. This severely compromises the use of this model to understand the role of hPXR in controlling gene expression in the GI tract or at the blood brain barrier or, indeed, in any other tissue.

In this model, the PXR protein has also been shown to be functional as the mice are responsive to compounds such as rifampicin and TCPOBOP that are known to induce gene expression via this pathway. Strain differences between wild type and the humanised mice have been demonstrated. For example, the humanised mice are shown to be more responsive to compounds such as rifampicin, that are known to be more active to hPXR. Humanised PXR animals thus demonstrated an altered sensitivity to rifampicin relative to the wild type.

Furthermore, there was clearly greater background P450 enzyme activity as measured by 16-beta-hydroxylation of testosterone and 7-benzyloxyquinoline demethylation between wild type and

humanised PXR mice.

In experiments using the inducing agent TCPOBOP, the hepatic microsomal metabolism of testosterone was measured. Again clear differences between the wild type and the humanised PXR animals were observed. In particular, the 7-alpha-hydroxylation of testosterone was constitutively higher in the huPXR animals relative to the wild types.

Consistent with the strain differences in wild type and human PXR, there were marked differences in the sensitivity of the mouse lines to induction by TCPOBOP. In the case of testosterone 16-alpha-hydroxylation, this activity was significantly induced in wild type animals but not in humanised PXR animals. Of particular interest was the observation that the induction of testosterone 16-beta-hydroxylation was much more marked in wild type than in huPXR animals. Indeed, at a dose of 1mg/kg, induction of testosterone 16-beta-hydroxylation was approximately 6-fold in wild type animals but only 1.7-fold in the huPXR animals. This again demonstrates a reduced sensitivity of the humanised mice relative to controls.

Detailed investigations of the induction of drug metabolism pathways in PXR humanised and knock-out mice have been performed. Various different experimental approaches have confirmed that non-human transgenic animals that are humanised with respect to PXR, or which do not express any PXR (knock-out), can readily be obtained using the methods and strategies described herein.

Transgenic animals (such as mice) and cells according to the invention preferably demonstrate the functional properties described above and in the examples herein. For example, such cells and animals preferably do not display induction of Cyp2b10 activity in response to rifampicin. However, such cells and animals do display an induction effect for Cyp3a11, not only with rifampicin but also for TCPOBOP.

It will be appreciated that other human DNA(s) encoding a transcription factor may also be used in the present invention providing that they are capable of regulating a phase-1 drug-metabolising enzyme, a phase-2 drug-metabolising enzyme and/or a drug transporter protein. Examples include PPARs (α, δ and γ), NRF2, the Ah receptor, HNF1 and HNF4. Targeting strategies suitable for knock-in (humanisation) and knock-out of PPARα and the Ah receptor are described in more detail elsewhere herein (see Figures 68 and 69).

Preferably, the human DNA encoding a transcription factor comprises both the pregnane X receptor (PXR) and the constitutive androstane receptor (CAR). In this embodiment of the invention the transgenic animal or tissue or cells derived therefrom may be considered as "double-

humanised" for these transcription factor genes. Such double-humanised models are advantageous over models that only incorporate a single gene (either PXR or CAR) because many drug metabolising enzymes or drug transporters possess elements that are responsive to the binding of both CAR and PXR. Furthermore, the numbers of PXR-responsive elements often differ from the numbers of CAR-responsive elements and so regulation by both transcription factors is generally important. Consequently, models that take account of the effects of both factors are preferable and more closely mirror the physiological situation *in vivo*.

Mice transgenic for both human PXR and human CAR have been created and are described in the examples included herein. Preliminary studies have been performed on the activity of these transcription factors in combination, determined by measuring barbiturate-induced sleeping time. Sleeping time has been known for many years to be directly proportional to the hepatic cytochrome P450 activity and this activity can be at least in part ascribed to the P450 levels in the liver determined by CAR and PXR function. Whereas wild type mice given a narcotic dose of pentobarbitone slept for 21 minutes, the double humanised mice for CAR and PXR slept for 34 minutes. These mice therefore demonstrate a significant difference to their wild type controls indicating that the double humanised mouse has a marked difference in its response to drugs relative to the wild type animals.

Detailed investigations of the induction of drug metabolism pathways in PXR and CAR double-humanised and double-knock-out mice have been performed. Various different experimental approaches have confirmed that non-human transgenic animals that are humanised with respect to both PXR and CAR, or which do not express any PXR or CAR (double-knock-out), can readily be obtained using the methods and strategies described herein.

Transgenic animals (such as mice) and cells according to this aspect of the invention preferably demonstrate the functional properties described above. For example, cells and animals transgenic for human PXR preferably do not display induction of Cyp2b10 activity in response to rifampicin, but do display an induction effect for Cyp3a11, not only with rifampicin but also for TCPOBOP.

The inventors have also noted that the capacity of promoters to induce enzyme expression is different in different tissues. Accordingly, it is of utmost importance for human transcription factors to be used rather than endogenous transcription factors from another animal. Furthermore, the regulatory sequences of the transcription factors and the genes that they regulate should mirror the natural physiological situation as closely as possible. Thus, regulatory sequences are preferably of human origin or non-human animal origin. In more preferred embodiments, the regulatory sequences are of human origin or originate from the target non-human animal (*e.g.* mouse). This

enables the wild-type expression pattern to be retained, as explained elsewhere herein.The use of as many human transcription factors, human drug metabolising enzymes and human drug transporters as possible is important to ensure that this happens.

The ratio of protein levels that are generated by a particular drug are also of significant importance. For example, the action of mouse PXR stimulates expression of different proteins than the action of human PXR and at different levels. The levels of a particular drug and its metabolites depends crucially on which drug metabolising enzymes and transporters are expressed and so, again, it is of utmost importance for human transcription factors to be used rather than endogenous transcription factors from another animal.

The use of human transcription factors is also important from a toxicological standpoint. For example, PXR is naturally regulated by bile acids and other physiological compounds and toxic conditions such as biliary necrosis and biliary cholestasis can result from exposure to a particular drug. It may therefore be that as a result of differences between drug metabolism between human and a test animal, a toxic effect will be noted in that animal that would not be evident in the human.

The regulatory sequences governing expression of the transcription factor(s) may preferably be either of human origin, or may originate from the target animal species e.g. the mouse (as described in more detail elsewhere herein).

The genes that are inserted into the transgenic model are preferably inserted at the point in the genome where the endogenous equivalent gene or gene cluster naturally occurs. This has the advantage that the context of the gene locus is retained, which means that the fidelity of transcription from this site is as close as possible to the level of transcription that occurs in the wild type system.

The inventors have validated aspects of drug metabolism pathways in transgenic animals (*e.g.* huPXR, huCAR and huPXR/huCAR mice) using appropriate assays as described elsewhere herein. The functional properties displayed by the inventors' transgenic mice in these assays reveal that the transgenic animals, tissues and cells of the invention have significant utility in analysis of drug metabolism and toxicity.

In some experiments, the inventors validated aspects of drug metabolism pathways in transgenic animals using inducers of the components of those pathways that are known to act more potently in humans or in mice, as summarised below:

Inducer	**More potent in:**

Rifampicin (Rif)	human
CITCO	human
Phenobarbital (PB)	mouse / human
Dexamethasone (Dex)	mouse
5-Pregnen-3β-ol-20-one-16α-carbonitrile (PCN)	mouse
Clotrimazole	mouse
TCPOBOP	mouse

Species-specific inducers of drug metabolism, such as those listed above, may act primarily via CAR or PXR, or via both PXR and CAR (*e.g.* see Figure 55). The species-specific induction of CAR and PXR can be discriminated using assays that allow a distinction to be made between induction via PXR and incuction via CAR, such as by measuing Cyp3a11 or Cyp2b10 levels or activity (*e.g.* see Figure 48). An overview of the effects of the inducers listed above on induction of Cyp3a11 and Cyp2b10 in mice and humans is provided in Figures 55 and 56.

Accordingly, the invention provides a transgenic mouse, tissue or cells derived therefrom incorporating a human DNA sequence encoding PXR under the control of an endogenous promoter, and optionally having its equivalent endogenous murine PXR genes annulled, which mouse, tissue or cells:

(i) is more sensitive to rifampicin-mediated induction of the expression and/or activity of a cytochrome P450 than the corresponding wild-type mouse, tissue or cells derived therefrom; and/or

(ii) is less sensitive to dexamethasone-mediated or pregnenolone-16α carbonitrile-mediated or clotrimazole-mediated induction of the expression and/or activity of a cytochrome P450 than the corresponding wild-type mouse, tissue or cells derived therefrom.

Preferably, the cytochrome P450 is a Cyp3a enzyme, such as Cyp3a11.

The invention also provides a transgenic mouse, tissue or cells derived therefrom incorporating a human DNA sequence encoding CAR under the control of an endogenous promoter, and optionally having its equivalent endogenous murine CAR genes annulled, which mouse, tissue or cells:

(i) is more sensitive to CITCO-mediated induction of the expression and/or activity of a cytochrome P450 than the corresponding wild-type mouse, tissue or cells derived therefrom; and/or

(ii) is less sensitive to TCPOBOP-mediated induction of the expression and/or activity of a cytochrome P450 than the corresponding wild-type mouse, tissue or cells derived therefrom.

Preferably, the cytochrome P450 is a Cyp2b enzyme, such as Cyp2b10.

Cytochrome P450 expression and activity levels can be determined by appropriate assays, as described elsewhere herein (see Figure 48). For example, cytochrome P450 (*e.g.* Cyp3a11 or Cyp2b10) expression levels can be determined by western blotting. Cytochrome P450 activity can be determined by a 7-benzyloxyquinoline (BQ) activity assay (for Cyp3a) or a pentoxyresorufin-O-deethylation (PROD) activity assay (for Cyp2b).

The invention also provides a transgenic mouse, tissue or cells derived therefrom that possesses a combination of the functional properties mentioned herein. Thus, the invention provides a transgenic mouse, tissue or cells derived therefrom incorporating human DNA sequences encoding PXR and CAR under the control of endogenous promoters, and optionally having its equivalent endogenous PXR and CAR genes annulled, which mouse, tissue or cell:

(i) is more sensitive to rifampicin-mediated induction of the expression and/or activity of a cytochrome P450 than the corresponding wild-type mouse, tissue or cells derived therefrom; or

(ii) is less sensitive to dexamethasone-mediated or pregnenolone-16α carbonitrile-mediated or clotrimazole-mediated induction of the expression and/or activity of a cytochrome P450 than the corresponding wild-type mouse, tissue or cells derived therefrom; and

(iii) is more sensitive to CITCO-mediated induction of the expression and/or activity of a cytochrome P450 than the corresponding wild-type mouse, tissue or cells derived therefrom; or

(iv) is less sensitive to TCPOBOP-mediated induction of the expression and/or activity of a cytochrome P450 than the corresponding wild-type mouse, tissue or cells derived therefrom.

The invention also provides a transgenic mouse, tissue or cells derived therefrom incorporating a human DNA sequence encoding PXR under the control of an endogenous promoter, and optionally having its equivalent endogenous PXR gene annulled, which displays increased dexamethasone-mediated hepatotoxicity relative to the corresponding wild-type mouse. Dexamethasone-mediated hepatotoxicity can be determined by measuring ALT levels using an appropriate assay and an appropriate dose of dexamethasone (*e.g.* at least 20 mg/kg, at least 30 mg/kg, at least 40 mg/kg, at

least 50 mg/kg, or at least 60 mg/kg. Suitable assays are described elsewhere herein.

Preferred transgenic (knock-in and knock-out) mice containing altered transcription factor genes are described in more detail elsewhere herein.

Ma *et al.* (Drug Metab Dispos. 2007 Feb;35(2):194-200) introduced the complete human PXR gene, including 5' and 3' flanking sequences, into PXR knock-out mice by bacterial artificial chromosome (BAC) transgenesis. They observed selective expression of human PXR in the liver and intestine. Treatment of PXR-humanised mice with PXR ligands mimicked the human response, as both hepatic and intestinal Cyp3a11 mRNA and protein were strongly induced by rifampicin, a human-specific PXR ligand, but not by pregnenolone 16α-carbonitrile (PCN), a rodent-specific PXR ligand. In wild-type mice, Cyp3a11 mRNA was strongly induced by PCN, but not by rifampicin. However, Ma *et al.* relates exclusively to PXR humanisation, and furthermore does not disclose or suggest many aspects of the present invention, such as the use of mixed cDNA/genomic constructs, comparison of different transgenic non-human animals, use of reporter constructs (see below) or expression of human sequences under the control of non-human animal regulatory sequences. Another drawback of the model described by Ma *et al.* is that it is unsuitable to combine with modifications of other genes within one animal, because PXR humanisation is achieved by two independent genomic alterations: (i) knock-out of the endogenous PXR gene, and (ii) transgenesis with the human PXR gene at a different genomic location.

Phase-1 drug-metabolising enzymes

The invention provides a non-human animal, tissue or cells according to any one of the aspects of invention described above, wherein the at least one phase-1 drug-metabolising enzyme is a cytochrome P450 isoform, optionally selected from the group comprising CYP1A, CYP3A, CYP2B, CYP2C, CYP2D, and CYP2E isoforms, optionally selected from one or more of CYP1A1, CYP1A2, CYP3A4, CYP3A5, CYP3A9, CYP2C8, CYP2C9, CYP2B6, CYP2B10, CYP2C19, CYP2D6, CYP2E1, and/or the human DNA sequence encoding a phase-1 drug-metabolising enzyme comprises a cytochrome P450 gene cluster, optionally selected from the CYP3A cluster, the CYP2D cluster, the CYP2C cluster, and/or the CYP1A cluster.

Preferably, the human DNA encoding a phase-1 drug-metabolising enzyme is selected from the group comprising the cytochromes P450, including but not limited to CYP1A, CYP3A, CYP2B, CYP2C, CYP2D, and CYP2E isoforms. Preferred isoforms are one or more of CYP1A1, CYP1A2, CYP3A4, CYP3A5, CYP3A9, CYP2C8, CYP2C9, CYP2B6, CYP2B10, CYP2C19, CYP2D6, and CYP2E1.. It will be appreciated that other DNAs encoding a phase-1 drug-metabolising enzyme may also be included in the present invention, providing that they are capable

of modifying a xenobiotic by adding or exposing a polar group on the xenobiotic molecule.

The choice of human P450 isoforms for introduction into P450-humanised non-human animals is predominantly driven by the known relative importance of various P450 isoforms in metabolism in the relevant tissue. Thus, for example, to date the single most significant P450 isoform recognised in the human liver is CYP3A4, and so CYP3A4 is therefore probably the first human P450 isoform of choice for P450 humanisation of liver. The choice of human P450s for the multi-P450-humanised mouse of the present invention is dictated by the need of the user. In this respect it is expected that any one or more of the following human isoforms will be preferred: 3A4, 2D6, 2B6, 2C9, 2C19, 1A1, 1A2, 2C8. However, it will be appreciated that the isoform(s) incorporated into the animal cell is/are dependent on the user's requirement. In this way, the humanised transgenic animal may be "designed" to investigate the role of specific isoforms in the metabolic process.

According to the teaching of the invention, single genes, gene clusters or combinations of single genes or gene clusters may be replaced. Whole gene clusters should preferably be replaced where possible, rather than simply replacing individual genes. This generates a situation in which the ratios of the expression levels of genes in any gene cluster are the same as the ratios in which these genes are expressed *in vivo*. This is a phenomenon that has not received attention in the prior art. The CYP3A, CYP2C, CYP2D and CYP1A clusters are preferred clusters of phase-1 drug-metabolising enzymes for replacement according to the present invention. One, both CYP3A, CYP2C, any other combination, or even all of these gene clusters may be replaced according to the present invention.

According to the invention, therefore, partial, or preferably complete cascades of genes that are implicated in a particular pathway may preferably be replaced. This ensures that the partial redundancy of gene function is retained and so, again, the real physiological situation is mirrored. An example can be provided by the CYP3A P450 cluster. In humans, there are four functional genes in this cluster, that have overlapping substrate specificity. The CYP3A5 protein will, for example, metabolise CYP3A4 substrates. Therefore, if one is attempting to generate a model of drug metabolism, incorporating the CYP3A5 gene in any state other than as part of its entire gene cluster is distancing the generated model from reality.

In contrast, following prior art teachings, were a researcher to decide that it might be a good idea to generate an animal transgenic for the CYP3A5 gene, he would only know to incorporate this gene, and would not think to incorporate the entire gene cluster of which it forms a part. Furthermore, using prior art techniques, it would insert at an arbitrary site and not in its natural contextual position in the genome. Worse still, its expression would be under the direction of a strong

promoter specific for the liver. This strategy would very likely generate a large amount of CYP3A5 protein in the liver, and because of the large amounts of protein provided, this protein would metabolise substrates at a greater rate than those on which it would act *in vivo*. For all these reasons, the method of the invention is advantageous over systems that have been described previously.

Preferably, the clusters of phase 1 drug metabolising enzymes that are used for humanisation are the CYP3A cluster and the CYP2C cluster.

In view of the redundancy in protein function between the human and commonly used target transgenic animals such as the mouse, humanisation for phase 1 drug metabolising enzymes is preferably performed against a deleted background in which only some (for example 1, 2, 3, 4 or 5), and preferably none of the target animal phase 1 drug metabolising enzymes are expressed at significant levels.

Preferred transgenic (knock-in and knock-out) mice containing altered phase-1 drug-metabolising enzyme genes are described in more detail elsewhere herein.

Phase-2 drug-metabolising enzymes

Preferably, the human DNA encoding a phase-2 drug-metabolising enzyme is selected from the group comprising the glucuronyl transferases, for instance, the UGT1A gene or gene cluster, the glutathione transferases, for instance GST (glutathione S-transferases), the sulphonyl transferases and the acetyl transferases. It will be appreciated that other DNAs encoding a phase-2 drug-metabolising enzyme may also be included in the present invention providing that they are capable of conjugating a product of phase-1 metabolism.

Preferably, a cluster of phase 2 drug metabolising enzymes that is used for humanisation is the UGT1A gene cluster.

Preferred transgenic (knock-in and knock-out) mice containing altered phase-2 drug-metabolising enzyme genes are described in more detail elsewhere herein.

Drug transporters

Preferably, the human DNA encoding a drug transporter protein is selected from the group comprising the ATP-binding cassette proteins which include but are not limited to the multi-drug resistance proteins, for instance MDR-1 and multi-drug resistance-associated proteins (MRPs), for example, MRP1 and/or MRP2, or from the organic anion transporting polypeptides (OATPs). It will be appreciated that other DNAs encoding a drug transporter protein may also be included in the present invention providing that they are capable of facilitating the transport of drugs and other

xenobiotic molecules across plasma membranes.

Preferably, the multidrug resistance protein is MDR1.

Preferably, the multi-drug resistance-associated protein is MRP2.

Preferred transgenic (knock-in and knock-out) mice containing altered drug transporter protein genes are described in more detail elsewhere herein.

Regulatory sequences

The present invention resides in part in the humanisation of transgenic non-human animal cell, tissue or animals especially for transcriptional factor(s), wherein the transcriptional factor transgenes are driven by transcriptional factor promoters, that is to say they are "knocked–in" rather than utilising heterologous albumin/tissue specific promoters. Thus the animals of the present invention are able to express the human proteins at not only the appropriate physiological levels but in all tissues, rather than just the liver as is known from the prior art.

It will be appreciated that any human DNA sequences include coding sequences for proteins selected from the group of classes of: human phase-1 metabolism enzymes; human phase-2 metabolism enzymes; human drug transporters; human transcription factors, may ideally be operatively linked to human regulatory DNA sequences. However, the use of human regulatory sequences is not essential, and other endogenous regulatory sequences can be used, *e.g.* mouse sequences.

Preferably, these human DNA sequences of the above-described transcription factors and other proteins are whole genes or are DNA constructs comprising regulatory sequences that may either be derived from humans or animals. In cases where the regulatory sequences are not of human origin, the regulatory sequences may be derived from the target animal, for example, the mouse. By regulatory sequences is meant to include any promoter or enhancer sequences, 5' or 3' UTRs, poly-A termination sequences or other DNA sequences, that are necessary for transcription of the gene of interest or which modulate expression of the gene of interest. Transcripts used for insertion of human sequences are preferably terminated by a poly A motif.

Heterologous promoters have generally been used in the prior art, and those used (such as the albumin promoter) are generally strong promoters, are ligand independent in their action and are constitutively switched on. In normal development, albumin is only expressed neo-natally. This divorces the expression of the protein encoded by the gene from the natural situation in reality, in that the regulatory signals that direct transcription of the gene and the subsequent translation of the mRNA product are not retained in the transgenic system. Researchers in the prior art turned to the

use of such promoters for a variety of reasons. Partly, it was felt necessary to do so because the transcription signals provided by the endogenous promoters were not deemed to be strong enough. Furthermore, it was thought necessary to use promoters that had been shown to be effective in the mouse.

In contrast, the invention preferably incorporates the endogenous promoter with the human gene so that the fidelity of wild type human expression is retained, developmentally, temporally and in a tissue-specific manner.

By "endogenous promoter" is meant a promoter that naturally directs expression of the gene of interest. An endogenous promoter may thus be a human promoter, or may alternatively be the promoter that is endogenous to that introduced gene in the transgenic animal subject. For example, in the case of transgenic mice, the expression of the human gene may be directed by the endogenous mouse promoter for that gene. Thus, it is not essential to insert a human regulatory sequence, *e.g.* a human promoter. By incorporation of all of the 5' upstream sequence that is necessary for promoter activity, preferably including any enhancers, it has been found that it is not in fact necessary to use strong, constitutive promoters; as in the natural situation, the endogenous promoter, in its entirety, is perfectly capable of directing expression of the relevant protein in a physiologically relevant manner. An example is provided by the CYP3A4 gene, which possesses strong enhancer elements up to 13kb upstream of the transcription initiation point. Whilst incorporation of all this sequence allows the appropriate mechanism of transcription to occur, omission of these upstream sequences leads to a system in which incomplete or insufficient regulatory sequences are present to allow the fidelity of gene expression to be retained.

In some embodiments of the invention, use of the non-human animal's endogenous regulatory sequences may be preferred. Such embodiments allow expression of human DNA sequences under the control of the non-human animal's endogenous regulatory sequences, such that the components of the non-human animal's gene expression pathway (*e.g.* regulatory sequences, transcription factors) can interact. Such embodiments may more closely mirror the *in vivo* situation in humans in some cases, *e.g.* where the relevant human regulatory sequence is not capable of interacting with the non-human animal's transcription factors, or said interaction does not provide a relevant level of expression of the human DNA sequence. There are disclosed herein embodiments in which a human DNA sequence is expressed under the control of mouse promoter. Thus, the invention also provides a transgenic non-human animal (*e.g.* mouse), tissue or cells derived therefrom incorporating a human DNA sequence encoding a protein involved in drug metabolism, wherein said human DNA sequence is operatively linked to an endogenous regulatory sequence of the non-

human animal, and the endogenous equivalent gene in the non-human animal is optionally annulled.

One result of using promoters such as the albumin promoter that were exclusively used in the prior art is that the effects of expression of a gene can only be monitored in a particular tissue – in the case of the albumin promoter, this is the liver. Prior art workers were not discouraged by this limitation, because the liver was generally viewed as being the only important tissue for studying drug metabolism, and therefore only expression in the liver was desired. Expression elsewhere in tissues other than the liver was seen as artefactual and therefore a hindrance to an effective model rather than being in any way advantageous. Another drawback of the prior art systems based on the use of the albumen promoter is that for that system to work, a murine PXR null background is required. This means that PXR is not expressed anywhere other than from the transgene in the liver, which has very wide-ranging effects on drug metabolism; such a mouse no longer reflects the natural tissue distribution of a natural mouse.

The inventors are of the view that the liver is not the only important tissue for drug metabolism. Accordingly, what the prior art workers perceived as an advantage, i.e. that exclusive liver-specific expression enabled an accurate assessment of the real physiological situation, the inventors see as a distinct disadvantage because other potentially important tissues are ignored. The invention allows a global, holistic snapshot to be obtained of the drug metabolism process.

Use of the endogenous promoter also carries other advantages with it. In particular, the fidelity of developmental expression is retained. Whereas prior art systems have used liver-specific promoters that sponsor liver expression exclusively, the use of the natural endogenous promoter ensures that the protein is expressed in the tissues in which it naturally occurs, and not only in the adult animal, but also at each developmental stage. This also carries with it the advantage that the transgenic animals are more likely to be viable and thus useful as drug screens and in the development of downstream crosses. It also allows the animals to be used to screen for teratogenic effects of a test compound, as placental expression of transcription factors and drug metabolising enzymes is retained.

Furthermore, the inventors have also noted the existence of a potential "repression" effect whereby a particular drug compound reduces the level of a particular drug transporter or metabolising enzyme and so alters the rate or pathway of disposition. For example, were the levels of human CYP3A4 to decrease, for example as a result of repression of mouse PXR rather than the conventional human transcription factor partner, then an alternative pathway of disposition may be exaggerated. This would give a misleading impression of the enzyme levels that are induced by a

particular drug in an organism. It would also give a misleading impression of the rate and type of metabolism that would operate in the human on exposure to that particular drug.

The inventors have also noted that the duration of induced expression by a particular drug is of great importance. For example, some drugs that are candidates for use in humans may not be metabolised efficiently in the mouse. This means that such a drug remains present at a systemically high concentration for a significant period. This means that transcription factors such as PXR will remain activated for this period, being constantly activated for this period. Associated levels of drug metabolising enzymes, drug transporters and other such enzymes will as a result also be highly expressed during the entire period that the drug remains in the animal. This clearly is misleading and in contrast to the equivalent situation in the human where the metabolism of the drug may be significantly more efficient.

Using the human promoter rather than the mouse promoter may be preferable to drive the expression of transcription factors, phase 1 drug metabolising enzymes, phase 2 drug metabolising enzymes, and/or drug transporters, as it allows the idiosyncrasies of the human expression system to be retained. Again using the example of the mouse CYP3A genes, these have a different number of PXR and CAR response elements to the number that is present in the human CYP3A4 gene. Were the equivalent mouse promoter to be used, then the response to transcriptional activation of PXR by exposure of the animal to drug would be correspondingly different to the response that is evident in a human system. It is also true that in many cases, the benefit of using the human promoter is that a true mouse orthologue does not exist. This would then lead to greater or lesser production of the drug metabolising protein in response to a particular drug, so potentially exaggerating or diminishing the role of that protein in the metabolism of that drug. For example, in a particular mouse system known in the prior art, it may be that because of inappropriately high levels of expression of CYP3A4, driven by an inappropriate promoter, the tested drug is metabolised too quickly for any potentially beneficial effect to be evident. This is of course misleading.

In contrast, using the natural human promoter means that, following this example, the appropriate amount of CYP3A4 would be produced in response to drug activation, and furthermore, would be produced in the correct tissue. The natural physiological response to a particular drug will then be mimicked, in terms of the amount of CYP3A4 that is produced, not only in the liver, but also, say, in the gastro-intestinal tract. If the drug is in fact a substrate for CYP3A4, then it will be metabolised at a rate and in a manner that mirrors the situation in the human. In contrast, a prior art method that uses an albumin promoter to direct expression of inappropriately large amounts of

CYP3A4 will distort the role of this protein and give misleading results. For instance, the drug might in fact only be a weak substrate for CYP3A4, but will nevertheless be metabolised aggressively if high amounts of the protein are present.

Thus, regulatory sequences are preferably of human origin or non-human animal origin. In more preferred embodiments, the regulatory sequences are of human origin or originate from the target non-human animal (*e.g.* mouse). This enables the wild-type expression pattern to be retained (developmentally, temporally and spatially).

As a result, the human protein is produced in the correct place at the correct time. This imparts a realism to the model that is simply not possessed in the prior art systems.

Preferred constructs

In some circumstances, the target gene and the human incorporated gene may share a leader sequence. This may be achieved by retaining at least one intron from the target non-human animal gene in the construct, which usually results in a better expression. This strategy also ensures that the gene product will be guided to the right intracellular location. The human leader sequence might be able to fulfil that function as well, but it is often safer to use the mouse leader instead. For instance, in the case of MRP2, the "leader sequence" of the mouse protein, which is encoded by exon 1, may be retained. The human cDNA without sequences from exon 1 is then introduced into exon 2 of the mouse genomic sequence. The original splice sites for mouse intron 1 will be retained, so that this construct encodes a fusion protein of amino acids from mouse exon 1 and human exons 2-32. This construct ensures a high level of expression and also that the MRP2 is guided to its correct location, the plasma membrane.

In other cases, the incorporated human gene may be brought under control of the promoter of the appropriate target animal gene. For example, in the case of MDR1, the cDNA of human MDR1 may be fused to the translational start site of the corresponding mouse genes (*Mdr1a* or *Mdr1b*). In the example of PXR, a hybrid of human PXR cDNA and genomic sequences may be fused to the translational start site of the mouse PXR gene, whereby the mouse Start-ATG is retained. In the case of CAR, the human sequence may be fused to the translational start site of the mouse CAR gene.

As explained above, the use of a partial or complete human genomic DNA sequence provides significant advantages, *e.g.* by permitting production of splice variants.

Thus, in a further aspect the invention provides a transgenic non-human animal, tissue or cells derived therefrom incorporating a human DNA sequence encoding a protein involved in drug

metabolism under the control of an endogenous regulatory sequence, wherein the human DNA sequence comprises at least one intron, such that at least one splice variant is produced when the human DNA sequence is transcribed in the transgenic non-human animal, tissue or cells derived therefrom. In this aspect of the invention, the endogenous equivalent gene encoding the protein in the non-human animal is optionally annulled.

In some embodiments of this aspect of the invention, the protein involved in drug metabolism is a transcription factor, a phase-1 drug-metabolising enzyme, a phase-2 drug-metabolising enzyme and/or a drug transporter protein as described elsewhere herein. In other embodiments of this aspect of the invention, the protein involved in drug metabolism is a phase-1 or phase-2 drug-metabolizing enzyme, as described elsewhere herein. In other embodiments of this aspect of the invention, the protein involved in drug metabolism is a drug transporter protein as described elsewhere herein.

As explained above, whole gene clusters should preferably be replaced where possible, rather than simply replacing individual genes, because the resulting drug metabolism pathways more closely resemble the *in vivo* situation in humans.

Thus, in a further aspect the invention provides a transgenic non-human animal, tissue or cells derived therefrom incorporating a human DNA sequence encoding a cluster of proteins involved in drug metabolism under the control of endogenous regulatory sequences. In this aspect of the invention, a corresponding cluster of endogenous equivalent genes is optionally annulled. In preferred embodiments of this aspect of the invention, the intron-exon structure of the human DNA sequence is maintained (*i.e.* genomic sequences rather than cDNA sequences are used, as described elsewhere herein).

The invention provides non-human animals, tissue or cells derived therefrom into which a human DNA sequence encoding a protein involved in drug metabolism has been inserted, and in which an endogenous gene encoding a protein involved in drug metabolism is optionally annulled. However, non-human animals, tissues or cells derived therefrom in which an endogenous gene for a protein involved in drug metabolism has been annulled, and which do not further comprise a human DNA sequence encoding a protein involved in drug metabolism are also useful in the invention. Such "knock-out" non-human animals, tissues or cells are particularly useful in parallel with the "knock-in" (humanised) non-human animals, tissues or cells of the invention, because comparison of experimental data generated using knock-in and knock-out mice can reveal further useful information regarding pathways of drug metabolism.

Thus, in a further aspect the invention provides a method for investigating xenobiotic metabolism

or toxicity, comprising the use of:

(i) a non-human animal, tissue or cells derived therefrom incorporating a human DNA sequence encoding a protein involved in drug metabolism under the control of an endogenous regulatory sequence and optionally having its endogenous equivalent gene annulled;

(ii) a corresponding non-human animal, tissue or cells derived therefrom whose endogenous equivalent gene has been annulled but which does not incorporate a human DNA sequence encoding the protein involved in drug metabolism; and

(iii) optionally a corresponding wild-type non-human animal, tissue or cells derived therefrom.

Preferred non-human animals of type (i) include those specified elsewhere herein (*e.g.* those having the genotype huPXR, huCAR, huPPARα, huAhR, huCYP3A4, huCYP3A cluster, huCYP2C9, huCYP2C cluster, huCYP2D6, huCYP1A1/1A2, huUGT, huMDR1/mdr1a$^{-/-}$, huMDR1/mdr1b$^{-/-}$, huMDR1/mdr1a$^{-/-}$/mdr1b$^{-/-}$, or huMRP2).

Preferred non-human animals of type (ii) include those specified elsewhere herein (*e.g.* those having the genotype koPXR, koCAR, koPPARα, koAhR, koCyp3a11, koCyp3a cluster, koCyp2c cluster, koCyp1a1/Cyp1a2, koCyp2d cluster, or koUGT).

Thus, such methods may involve *e.g.,* comparing drug metabolism in (i) a non-human animal with the genotype huPXR, and (ii) a non-human animal with the genotype koPXR (and optionally (iii) a wild-type non-human animal).

Preferably, such methods comprise the use of:

(i) two or more, three or more, four or more, or five or more non-human animals, tissues or cells derived therefrom incorporating respectively two or more, three or more, four or more, or five or more human DNA sequences encoding different proteins involved in drug metabolism under the control of endogenous regulatory sequences, each non-human animal, tissue or cells optionally having its endogenous equivalent gene annulled;

(ii) two or more, three or more, four or more, or five or more corresponding non-human animals, tissues or cells derived therefrom whose endogenous equivalent genes have been annulled but which do not incorporate human DNA sequences encoding the relevant drug metabolism proteins; and

(iii) optionally a corresponding wild-type non-human animal, tissue or cells derived therefrom.

Such methods may involve *e.g.,* comparing drug metabolism in (i) non-human animals with the

genotypes huPXR, huCAR, huCYP2C cluster, huUGT, and (ii) non-human animals with the genotypes koPXR, koCAR, koCyp2c cluster, koUGT (and optionally (iii) a wild-type non-human animal).

In other methods of the invention wherein different transgenic non-human animals are compared, the effect(s) of different regulatory sequences are investigated.

Thus, in a further aspect the invention provides a method for investigating xenobiotic metabolism or toxicity, comprising the use of:

(i) a first non-human animal, tissue or cells derived therefrom incorporating a human DNA sequence encoding a protein involved in drug metabolism under the control of an endogenous human regulatory sequence, the non-human animal, tissue or cells optionally having its endogenous equivalent gene annulled;

(ii) a second non-human animal, tissue or cells derived incorporating a human DNA sequence encoding a protein involved in drug metabolism under the control of an endogenous non-human animal regulatory sequence, the non-human animal, tissue or cells optionally having its endogenous equivalent gene annulled;

(iii) optionally a corresponding non-human animal, tissue or cells derived therefrom whose endogenous equivalent gene has been annulled but which does not incorporate a human DNA sequence encoding the protein involved in drug metabolism; and

(iv) optionally a corresponding wild-type non-human animal, tissue or cells derived therefrom.

For example, drug metabolism can be compared between (i) a first non-human animal with the genotype huPXR, wherein the human DNA sequence encoding PXR is under the control of an endogenous human regulatory sequence, and (ii) a second non-human animal with the genotype huPXR, wherein the human DNA sequence encoding PXR is under the control of an endogenous non-human animal regulatory sequence (and optionally (iii) knock-out and/or (iv) wild-type non-human animals). Such methods enable subtle differences in gene regulation and expression levels between humans and model organisms (*e.g.* mice) to be elucidated.

Other comparative methods are possible. The skilled person will readily be able to select an appropriate 'panel' of non-human animals, tissues or cells derived therefrom as disclosed herein. The type and number of non-human animals, tissues or cells required for comparison will depend on the type of analysis required (*e.g.* depending on the drug metabolism pathway of interest and/or the drug of interest). The invention provides transgenic non-human animals, tissues or cells derived therefrom of various genotypes (see elsewhere herein). The invention provides a tool kit from

which the skilled person can select the tools required for the desired analysis. Thus, the invention provides a method for investigating xenobiotic metabolism or toxicity, comprising the use of at least two, at least three, at least four, at least five, at least six, at least seven, at least eight, at least nine, or at least ten types of non-human animal, tissue or cells derived therefrom, wherein each type of non-human animal, tissue or cells comprises a different genetic modification (*e.g.* knock-in, knock-out, altered regulatory sequence) affecting the amino acid sequence or expression of a protein involved in drug metabolism. The 'holistic' methods disclosed herein, in which multiple different types of transgenic non-human animal, tissue or cells are compared, enable a more rigorous analysis of drug metabolism and toxicity than the prior art methods that involve use of fewer types of transgenic non-human animal, tissue or cell.

The methods of the invention wherein different transgenic non-human animals are compared may involve administering the same drug at the same dose to the different types of non-human animal (*e.g.* "knock-in", "knock-out" and wild-type, human or non-human regulatory sequences) and comparing the metabolism or toxicity of that drug between the different animals.

Transgenic animals

In preferred embodiments of the present invention as described above and below, the transgenic non-human animal and tissues or cells derived therefrom is preferably a mouse but may be another mammalian species, for example another rodent, for instance a rat, hamster or a guinea pig, or another species such as a monkey, pig, rabbit, or a canine or feline, or an ungulate species such as ovine, caprine, equine, bovine, or a non-mammalian animal species. More preferably, the transgenic non-human animal or mammal and tissues or cells are derived from a rodent, more preferably, a mouse.

Although the use of transgenic animals poses questions of an ethical nature, the benefit to man from studies of the types described herein is considered vastly to outweigh any suffering that might be imposed in the creation and testing of transgenic animals. As will be evident to those of skill in the art, drug therapies require animal testing before clinical trials can commence in humans and under current regulations and with currently available model systems, animal testing cannot be dispensed with. Any new drug must be tested on at least two different species of live mammal, one of which must be a large non-rodent. Experts consider that new classes of drugs now in development that act in very specific ways in the body may lead to more animals being used in future years, and to the use of more primates. For example, as science seeks to tackle the neurological diseases afflicting a 'greying population', it is considered that we will need a steady supply of monkeys on which to test the safety and effectiveness of the next-generation pills.

Accordingly, the benefit to man from transgenic models such as those described herein is not in any limited to mice, or to rodents generally, but encompasses other mammals including primates. The specific way in which these novel drugs will work means that primates may be the only animals suitable for experimentation because their brain architecture is very similar to our own.

The invention aims to reduce the extent of attrition in drug discovery. Whenever a drug fails at a late stage in testing, all of the animal experiments will in a sense have been wasted. Stopping drugs failing therefore saves test animals' lives. Therefore, although the present invention relates to transgenic animals, the use of such animals should reduce the number of animals that must be used in drug testing programmes.

An advantage of the present invention is that it avoids problems of species divergence between the human and other mammals that have conventionally been used as test models. One example is provided by the family of peroxisome proliferator activated receptors (PPARs), to which various drugs were in the past developed as hypolipidaemia agents. The development of these drugs was stopped, as they were identified in mouse models to be epigenetic carcinogens. It eventually turned out that that the difference in toxicity between species could be attributed to differences in levels of PPARα in the liver. The phenomenon apparent in the mouse does not occur in humans, because of lower levels of PPARα protein that are present in the liver. There are very clear advantages to models that exhibit *bona fide* levels of protein expression that reflect those present in the human body.

Preferably, the human DNA sequences are each independently linked to human or non-human animal regulatory DNA sequences (e.g. the endogenous human or non-human promoter). This linkage is distinct from the prior art and provides the advantage of improvement over prior art models as this further advances the mirroring of an *in vivo* human situation.

A particular advantage of the humanised transgenic animals, cells and tissues, of the present invention is that they combine the benefits of normal experimental animal models with those of human cell or tissue culture in a single system. This system or humanised transgenic animal will provide the pharmaceutical industry with an improved alternative for use in all pre-clinical metabolism, toxicity and drug disposition studies.

Cells

Another aspect of the invention relates to cells, modified so as to possess properties according to any one of the above-described aspects of the invention. Hepatocytes and neuronal cells are preferred cell types according to the present invention. The cells may be animal cells, including

mammalian cells, such as non-human cells or rodent cells, more specifically, mouse cells.

Cells according to this aspect of the invention may be created from transgenic animals according to the invention using standard techniques, as will be clear to the skilled reader, imbued with knowledge of the present invention. Suitable methods are described in many standard laboratory manuals, such as Davis et al., Basic Methods in Molecular Biology (1986); Sambrook Molecular Cloning; A Laboratory Manual, Third Edition (2000); Ausubel et al., 1991 [supra]; Spector, Goldman & Leinwald, 1998).

In one aspect, such cells may be non-human animal cells, such as mouse cells, generated according to any one of the above-described aspects of the invention. One preferred method of generating such cells is to cross a humanised mouse, as described above, with SV40 immortalised mouse (for example, the immorta-mouse (Taconix). Cells may subsequently be isolated from such animals according to well known techniques in the art. In contrast to prior art transgenic systems, which used the albumin promoter that is only active in the liver and thus only able to generate hepatocytes, cells from transgenic animals generated according to the present invention may be of a diverse selection of different cell types, including cells of significant importance to pharmacokinetics analyses, such as hepatocytes and neuronal cells.

Stem cells isolated from transgenic animals according to the invention, with properties as described above are also useful aspects of the present invention. Such cells may be pluripotent, or partially differentiated. Stem cells may be adult stem cells or embryonic stem cells. More generally, stem cells employed may be from a post-embryonic developmental stage e.g. foetal, neonatal, juvenile, or adult. Stem cells isolated in this manner may be used to generate specific types of cells such as hepatocytes and neuronal cells. Such cells also form an aspect of the present invention.

Preferred selections of replaced genes

According to a further aspect of the invention, there is provided a non-human animal, tissue or cells derived therefrom incorporating:

(i) at least one human DNA sequence encoding a transcription factor; and

(ii) at least one human DNA sequence encoding a drug transporter protein;

and whose endogenous equivalent genes have optionally been annulled.

The inventors are of the view that in the case of models of drug metabolism, it is important to generate animals that are not just transgenic for particular drug metabolising enzymes, but also to incorporate in these models, proteins that are transporters of drugs i.e. drug transporter proteins.

For example, many compounds that activate PXR, the nuclear transcription factor, are also substrates for MDR1 and are thus transported out of cells by this protein. Therefore, in order to create a faithful model of the *in vivo* situation, animals must preferably be transgenic for drug transporter proteins, otherwise a misleading impression will be obtained of the intracellular effects of any particular concentration of drug. Of the drug transporter proteins, MDR1 is preferred. In the case of prior art transgenic mice models, of course, the redundancy of drug transporters that is generated by the presence of both *mdr1a* and *mdr1b* can generate misleading results and so preferably, both these mouse genes should be knocked out and replaced with the human *mdr1* gene.

The expression of genes encoding drug transporter proteins such as MDR1 is also activated by the PXR-based signalling system. Accordingly, because the expression of the phase I, phase II and drug transporter genes is linked by PXR, in addition to the fact that the products of these genes have varied effects on the levels and metabolism of drugs and their metabolites, the integrity of co-ordinated regulation that is maintained according to the present invention is extremely advantageous, particularly when compared to prior art systems.

Preferably, when a non-human animal according to the invention incorporates a human DNA sequence encoding the transcription factor PXR, the human DNA sequence encoding PXR comprises at least part of intron 6 and/or intron 7 of the human PXR gene, or is additionally characterised as described in more detail herein above and in the claims.

In addition, MDR is expressed at a significant degree in the gastro-intestinal (GI) tract and in the environment of the blood-brain barrier. Since both the GI tract and the blood brain barrier are significant sources of drug transport into the blood stream, the presence of physiologically-relevant MDR expression levels imparts an important aspect of the drug metabolism process to the drug model and in demonstrating pharmacological activity. The presence of MDR in the GI tract, for example, can render orally-delivered drug not bioavailable. MDR is very important for drug transport both in an out of the brain. MDR also transports drugs from somatic cells in the liver into the bile.

Other preferred combinations of drug metabolism genes are listed as follows.

According to a further aspect of the invention, there is provided a non-human animal, tissue or cells derived therefrom incorporating:

(i) at least one human DNA sequence encoding a transcription factor; and

(ii) at least one human DNA sequence encoding a phase-1 drug-metabolising enzyme;

and/or

(iii) at least one human DNA sequence encoding a drug transporter protein;

and whose endogenous equivalent genes have optionally been annulled.

According to a further aspect of the invention, there is provided a non-human animal, tissue or cells derived therefrom incorporating:

(i) at least one human DNA sequence encoding a transcription factor; and

(ii) at least one human DNA sequence encoding a phase-2 drug-metabolising enzyme; and/or

(iii) at least one human DNA sequence encoding a drug transporter protein;

and whose endogenous equivalent genes have optionally been annulled.

According to a further aspect of the invention, there is provided a non-human animal, tissue or cells derived therefrom incorporating:

(i) at least one human DNA sequence encoding a transcription factor;

(ii) at least one human DNA sequence encoding a phase-1 drug-metabolising enzyme;

(iii) at least one human DNA sequence encoding a phase-2 drug-metabolising enzyme; and

(iv) at least one human DNA sequence encoding a drug transporter protein;

and whose endogenous equivalent genes have optionally been annulled.

Preferably, these aspects include any one or more of the features hereinbefore described. In preferred embodiments of these aspects, one or more of the human DNA sequences is a partial or complete genomic sequence as described elsewhere herein.

According to a yet further aspect of the invention there is provided a non-human animal, tissue or cells derived therefrom incorporating human DNA sequences encoding a PXR and a CAR transcription factor and at least one human DNA sequence encoding a phase-1 drug-metabolising enzyme and at least one human DNA sequence encoding a phase-2 drug-metabolising enzyme and at least one human DNA sequence encoding a drug transporter protein, wherein the endogenous equivalent genes in the non-human animal, tissue or cells have optionally been annulled.According to a yet further aspect of the invention there is provided a non-human animal, tissue or cells derived therefrom incorporating:

(i) a human DNA sequence encoding a PXR and/or a CAR transcription

factor;

(ii) a human DNA sequence encoding the CYP3A4 enzyme; and

(iii) a human DNA sequence encoding the MDR1 protein.

This aspect of the invention is of particular utility in that the CYP3A4 enzyme is of particular importance in the GI tract and therefore an animal humanised for this isoform will be of importance in investigating the role of drug metabolism in drug bioavailability in the gut. In addition, as set out above, the MDR1 protein is known to be expressed at the blood-brain barrier and when incorporated into the above described humanised mouse will provide a truly representative scenario of the uptake of drugs/xenobiotics into and out of the brain in man.

According a yet further aspect of the invention there is provided a non-human animal, tissue or cells derived therefrom incorporating:

(i) a human DNA sequence encoding a PXR and/or a CAR transcription factor;

(ii) a human DNA sequence encoding the CYP3A4 enzyme;

(iii) a human DNA sequence encoding the UGT1A enzyme; and

(iv) a human DNA sequence encoding the MDR1 protein.

According a yet further aspect of the invention, there is provided a non-human animal, tissue or cells derived therefrom incorporating

(i) a human DNA sequence encoding the transcription factor PXR;

(ii) a human DNA sequence encoding the transcription factor CAR;

(iii) a human DNA sequence encoding the transcription factor AhR; and

(iv) a human DNA sequence encoding the transcription factor PPARα..

Non-human animal humanised for PPARα, and preferably additionally humanised for further transcription factors and/or phase-1 and/or phase 2 drug-metabolising enzymes and/or drug transporter proteins are particularly advantageous for use in assessing, monitoring or investigating non-genotoxic carcinogenic (hyperplastic) effects. Preferably, when said non-human animal humanised for PPARα also incorporates a human DNA sequence encoding the transcription factor PXR, the human DNA sequence encoding PXR comprises at least part of intron 6 and/or intron 7 of the human PXR gene, or is additionaly characterised as described in more detail herein above and in the claims.

Other preferred combinations include combinations of a PXR and/or a CAR transcription factor, a human DNA sequence encoding the MDR1 protein and one or more of: CYP2A9, the CYP3A-cluster, the CYP2C-cluster and UGT or the UGT0-cluster.

Particularly preferred transgenic non-human animals, tissues and cells of the invention have a genotype as specified in the following list. The prefix "hu" refers to humanisation of the relevant endogenous non-human animal gene, and the prefix "ko" refers to a knock-out of the relevant endogenous non-human animal gene.

Unless otherwise required by the context, references to genes and genotypes herein that are in capitals (*e.g.* CYP3A4) refer to a human gene or gene cluster (and possibly also to a gene or gene cluster in a non-human animal), whereas references to genes and genotypes not in capitals (*e.g.* Cyp3a11) refer to a gene or gene cluster in a non-human animal (*e.g.* mouse).

Transcription Factors

huPXR;

koPXR;

huCAR;

koCAR;

huPPARα;

koPPARα;

huAhR; and

koAhR.

Drug-Metabolizing Enzymes

huCYP3A4;

koCyp3a11;

huCYP3A cluster;

koCyp3a cluster;

huCYP2C9;

huCYP2C cluster;

koCyp2c cluster;

huCYP2D6;

koCyp2d cluster;

huCyp2d cluster;

huCYP1A1/1A2 (meaning both CYP1A1 and CYP1A2 are humanised);

koCyp1a1/Cyp1a2 (meaning both Cyp1a1 and Cyp1a2 are knocked out);

huUGT; huUGT1A; huUGT1A cluster; huGST and

koUGT.

Drug Transporter Proteins

huMDR1/mdr1a$^{-/-}$;

huMDR1a;

koMDR1a;

huMDR1/mdr1b$^{-/-}$;

huMDR1b;

koMDR1b;

huMDR1/mdr1a$^{-/-}$/mdr1b$^{-/-}$;

huMDR1a/huMDR1b (*i.e.*, huMDR1a/b);

koMDR1a/koMDR1b;

huMRP2;

huBCRP (the transmembrane ABC-type half-transporter ABCG2)

koMRP2.

Particularly preferred transgenic non-human animals, tissues and cells of the invention incorporate a combination of two or more of the genotypes described herein, some examples of which are specified in the following list, wherein the symbol "/" indicates a combination of relevant genotypes.

Complex Genotypes

huPXR/huCAR;

koPXR/huCAR;

huPXR/koCAR;

koPXR/koCAR;

huPXR/huCAR/huAhR/huPPARα;

koPXR/koCAR/koAhR/koPPARα;

huCyp1A cluster/huAhR;

huCYP2C9/huPXR;

koCyp2c cluster/huPXR;

huCYP2D6/huPXR;

huCyp2d cluster/huPXR/huCAR;

huCYP3A4/huPXR;

koCyp3a11/huPXR;

huCYP3A4/koCyp3a11/huPXR;

koCyp3a cluster/huPXR;

huMDR1/mdr1a$^{-/-}$/huPXR;

huMDR1/mdr1b$^{-/-}$/huPXR;

huMDR1/mdr1a$^{-/-}$/mdr1b$^{-/-}$/huPXR;

huPXR/huCAR/huMDR1a/b;

huPXR/huCAR/koMDR1a/b;

huMRP2/huPXR;

huCYP3A cluster/huPXR/huCAR;

huCYP3A4/huPXR/huCAR;

huCYP3A4/koCyp3a11/huPXR/huCAR;

huCYP3A cluster/huMDR1/mdr1a$^{-/-}$/mdr1b$^{-/-}$/huPXR/huCAR;

huCYP3A cluster/huMDR1/mdr1a$^{-/-}$/mdr1b$^{-/-}$/huUGT/huMRP2/huPXR/huCAR;

huCYP3A cluster/huCYP2C cluster/huMDR1/mdr1a$^{-/-}$/mdr1b$^{-/-}$/huPXR/huCAR;

huCYP3A cluster/huCYP2C cluster/huMDR1/mdr1a$^{-/-}$/mdr1b$^{-/-}$/huUGT/huMRP2/huPXR/huCAR;

huCYP2C cluster/huPXR/huCAR;

huCYP2C9/huPXR/huCAR;

huUGT/huPXR/huCAR;

huUGT/huMRP2/huPXR/huCAR;

huMDR1/mdr1a$^{-/-}$/mdr1b$^{-/-}$/huPXR/huCAR; and

huMRP2/huPXR/huCAR.

Transgenic non-human animals, tissues and cells having a combination of two or more genetic manipulations as described herein are particularly preferred, because the drug metabolism pathways in those non-human animals, tissues and cells more closely resemble the *in vivo* situation in humans. Such non-human animals, tissues and cells may incorporate 2 or more, 3 or more, 4, or more 5 or more, 6 or more, 7 or more, 8 or more, 9 or more, or 10 or more of the genetic manipulations as described herein.

Suitable methods and targeting vectors for generating the transgenic non-human animals, tissues or cells of the invention are described elsewhere herein, and specific targeting strategies are described in the Examples herein and schematically illustrated in the Figures. Each of the specific targeting strategies and targeting vectors described and illustrated herein forms a further aspect of the invention. Thus, the invention provides a method of introducing into a non-human animal cell at least one human DNA sequence encoding a protein involved in drug metabolism using a targeting strategy or targeting vector(s) substantially as described herein or substantially as depicted in the Figures herein.

In particular, the invention provides a transgenic mouse, tissue or cells derived therefrom comprising one or more of the following features:

A. Transcription Factors

huPXR and koPXR	Knock-in (insertion) of a DNA sequence encoding human PXR into the mouse PXR locus, enabling expression of human PXR under the control of the mouse PXR promoter. The DNA sequence encoding human PXR preferably comprises at least part of intron 4 and/or intron 5 of the human PXR gene (*e.g.* Figures 2 and 65). The DNA sequence encoding human PXR may further comprise at least part of intron 6 and/or intron 7 of the human PXR gene (*e.g.* Figure 62). The DNA sequence encoding human PXR may further comprise at least part of intron 2, intron 3 and/or intron 8 of the human PXR gene (*e.g.* Figure 64). The targeting vector(s) preferably include sequence elements that enable PXR knock-out (*e.g.* Phi-C31-mediated knock-out) to produce koPXR.
huCAR and koCAR	Knock-in of a DNA sequence encoding human CAR into the mouse CAR locus, enabling expression of human CAR under the control of the mouse CAR promoter. The DNA sequence encoding human CAR preferably comprises at least part of intron 2, intron 3, intron 4, intron 5, intron 6, intron 7 and/or intron 8 of the human CAR gene (*e.g.* Figure 3). The targeting vector(s) preferably include sequence elements that enable CAR knock-out (*e.g.* Phi-C31-mediated knock-out) to produce koCAR.

huPPARα and koPPARα	Knock-in of a DNA sequence encoding human PPARα into the mouse PPARα locus, enabling expression of human PPARα under the control of the mouse PPARα promoter. The DNA sequence encoding human PPARα preferably comprises at least part of intron 5 and/or intron 6 of the human PPARα gene (*e.g.* Figure 68). The targeting vector(s) preferably include sequence elements that enable PPARα knock-out (*e.g.* Cre-mediated knock-out) to produce koPPARα.
huAhR and koAhR	Knock-in of a DNA sequence encoding human AhR into the mouse AhR locus, enabling expression of human AhR under the control of the mouse AhR promoter. The DNA sequence encoding human AhR preferably comprises exons 3-11 of the human AhR gene (*e.g.* Figure 69). The targeting vector(s) preferably include sequence elements that enable AhR knock-out (*e.g.* Cre-mediated knock-out) to produce koAhR.

B. Drug-Metabolizing Enzymes	
huCYP3A4	Knock-in of a DNA sequence encoding human CYP3A4 into the mouse Rosa26 locus, enabling expression of human CYP3A4 under the control of a human CYP3A4 promoter. The DNA sequence encoding human CYP3A4 preferably comprises at least part of intron 1 of the human CYP3A4 gene (*e.g.* Figure 70).
koCyp3a11	Knock-in of ZsGreen reporter gene into the mouse Cyp3a11 locus, enabling expression of ZsGreen under the control of the mouse Cyp3a11 promoter (*e.g.* Figure 71).

huCYP3A cluster and koCyp3a cluster	Insertion of a DNA sequence encoding the human CYP3A cluster into the mouse Cyp3a cluster, enabling expression of the human CYP3A cluster under the control of human CYP3A promoters. The targeting vector(s) preferably include sequence elements that enable Cre-mediated deletion of the mouse Cyp3a cluster, to produce koCyp3a (*e.g.* Figure 72). The targeting vector(s) preferably include sequence elements that enable Cre-mediated insertion of the human CYP3A cluster subsequent to Cre-mediated deletion of the mouse Cyp3a cluster, to produce huCYP3A. The targeting vector(s) preferably include sequence elements that enable deletion of selection cassettes subsequent to insertion of the human CYP3A cluster into the mouse Cyp3a cluster.
huCYP3A4 and koCyp3a cluster	Knock-in of a DNA sequence encoding human CYP3A4 into the mouse Cyp3a cluster, optionally at the mouse Cyp3a25 locus enabling expression of human CYP3A4 under the control of a human CYP3A4 promoter. Mice in which the Cyp3a cluster is deleted may also be generated.
huCYP2C9	Knock-in of a DNA sequence encoding human CYP2C9 into the mouse Rosa26 locus, enabling expression of human CYP2C9 under the control of a human CYP2C9 promoter. The DNA sequence encoding human CYP2C9 preferably comprises at least part of intron 4 of the human CYP2C9 gene (*e.g.* Figure 73).

huCYP2C cluster and koCyp2c cluster	Insertion of a DNA sequence encoding the human CYP2C cluster into the mouse Cyp2c cluster, enabling expression of the human CYP2C cluster under the control of human CYP2C promoters. The targeting vector(s) preferably include sequence elements that enable Cre-mediated deletion of the mouse Cyp2c cluster, to produce koCyp2c (*e.g.* Figure 74). The targeting vector(s) preferably include sequence elements that enable Cre-mediated insertion of the human CYP2C cluster subsequent to Cre-mediated deletion of the mouse Cyp2c cluster, to produce huCYP2C. The targeting vector(s) preferably include sequence elements that enable deletion of selection cassettes subsequent to insertion of the human CYP2C cluster into the mouse Cyp2c cluster.
huCYP2C9 and koCyp2c cluster)	Knock-in of a DNA sequence encoding human CYP2C9 into the mouse Cyp2c cluster, preferably at the Cyp2c55 locus, or into the Rosa26 locus, enabling expression of human CYP2C9 under the control of a human CYP2C9 promoter. Mice in which the Cyp2c cluster is deleted may also be generated.
huCYP2D6 and koCyp2d cluster	Knock-in of a DNA sequence encoding human CYP2D6 into the mouse Cyp2d cluster, enabling expression of human CYP2D6 under the control of a human CYP2D6 promoter. Mice in which the Cyp2d cluster is deleted may also be generated.
huCYP3A4 and koCyp3a11	Knock-in of a DNA sequence encoding human CYP3A4 into the mouse Cyp3a cluster, enabling expression of human CYP3A4 under the control of the mouse Cyp3a11 promoter. Mice in which the Cyp3a cluster is deleted may also be generated.

huCYP1A1/ CYP1A2 and koCYP1A1/ CYP1A2	Knock-in of DNA sequences encoding human CYP1A1 and human CYP1A2 into the mouse Cyp1a cluster, enabling expression of human CYP1A1 and CYP1A2 under the control of human CYP1A1 and CYP1A2 promoters. Mice in which the Cyp1a cluster is deleted may also be generated.
huUGT cluster and koUGT cluster	Insertion of a DNA sequence encoding the human UGT1 cluster into the mouse Ugt1 cluster, enabling expression of the human UGT1 cluster under the control of human UGT1 promoters. The targeting vector(s) preferably include sequence elements that enable Cre-mediated deletion of the mouse Ugt1 cluster, to produce koUGT (*e.g.* Figure 75). The targeting vector(s) preferably include sequence elements that enable Cre-mediated insertion of the human UGT1 cluster subsequent to Cre-mediated deletion of the mouse Ugt1 cluster, to produce huUGT. The targeting vector(s) preferably include sequence elements that enable deletion of selection cassettes subsequent to insertion of the human UGT1 cluster into the mouse Ugt1 cluster.

C. Drug Transporter Proteins	
huMDR1/ mdr1a$^{-/-}$	Knock-in of a DNA sequence encoding human MDR1 into the mouse Mdr1a locus, enabling expression of human MDR1 under the control of the mouse Mdr1a promoter. The DNA sequence encoding human MDR1 is preferably a human MDR1 cDNA sequence (*e.g.* Figure 76).
huMDR1/ mdr1b$^{-/-}$	Knock-in of a DNA sequence encoding human MDR1 into the mouse Mdr1b locus, enabling expression of human MDR1 under the control of the mouse Mdr1b promoter. The DNA sequence encoding human MDR1 is preferably a human MDR1 cDNA sequence (*e.g.* Figure 77).

huMDR1/ mdr1a$^{-/-}$/ mdr1b$^{-/-}$	Knock-in of DNA sequences encoding human MDR1 into the mouse Mdr1a and Mdr1b loci, enabling expression of human MDR1 under the control of the mouse Mdr1a and Mdr1b promoters. The DNA sequences encoding human MDR1 are preferably human MDR1 cDNA sequences (*e.g.* Figures 76 and 77).
huMRP2	Knock-in of a DNA sequence encoding human MPR2 into the mouse Mrp2 locus, enabling expression of human MRP2 under the control of the mouse Mrp2 promoter. The DNA sequence encoding human MPR2 is preferably a human MPR2 cDNA sequence (*e.g.* Figure 78).

By intron 'n' herein is meant the intron between exons 'n' and 'n+1'. Thus, intron 4 is that between exons 4 and 5, and intron 5 is that between exons 5 and 6, *etc.*

As noted elsewhere herein, the skilled person will readily be able to select an appropriate 'panel' of non-human animals, tissues or cells derived therefrom as disclosed herein. The invention provides a tool kit from which the skilled person can select the tools required for the desired analysis. Thus, the invention provides a method for investigating xenobiotic metabolism or toxicity, comprising the use of at least two, at least three, at least four, at least five, at least six, at least seven, at least eight, at least nine, or at least ten types of non-human animal, tissue or cells derived therefrom, wherein each type of non-human animal, tissue or cells comprises a different genetic modification introduced using a targeting strategy or targeting vectors substantially as described herein or substantially as depicted in the Figures herein.

Further details of the preferred targeting strategies and constructs are provided in the Examples and Figures herein. In particular, preferred targeting strategies are illustrated schematically in Figures 1-14 and 62-83.

Ma *et al.* (Drug Metab Dispos. 2007 Feb;35(2):194-200) introduced the complete human PXR gene, including 5' and 3' flanking sequences, into PXR knock-out mice by bacterial artificial chromosome (BAC) transgenesis. They observed selective expression of human PXR in the liver and intestine. Treatment of PXR-humanised mice with PXR ligands mimicked the human response, as both hepatic and intestinal Cyp3a11 mRNA and protein were strongly induced by rifampicin, a human-specific PXR ligand, but not by pregnenolone 16α-carbonitrile (PCN), a rodent-specific PXR ligand. In wild-type mice, Cyp3a11 mRNA was strongly induced by PCN, but

not by rifampicin. However, Ma *et al.* relates exclusively to PXR humanisation, and furthermore does not disclose or suggest many aspects of the present invention, such as the use of mixed cDNA/genomic constructs, comparison of different transgenic non-human animals, use of reporter constructs (see below) or expression of human sequences under the control of non-human animal regulatory sequences. Another drawback of the model described by Ma *et al.* is that it is unsuitable to combine with modifications of other genes within one animal, because PXR humanisation is achieved by two independent genomic alterations: (i) knock-out of the endogenous PXR gene, and (ii) transgenesis with the human PXR gene at a different genomic location.

Preferred sequences

Preferred targeting strategies and constructs are described herein. Preferred nucleic acid sequences for insertion into non-human animals, tissues or cells derived therefrom are recited in SEQ ID NOs:1-4.

SEQ ID NO:1 is a human PXR nucleotide sequence obtainable using a targeting vector of the invention. The first three nucleotides of SEQ ID NO:1 (atg) are the translational start site from the mouse PXR gene; the start site of the human PXR gene (ctg) is not present. SEQ ID NO:1 comprises sequences from introns 4 and 5 of the human PXR gene, and is obtainable using a targeting strategy as illustrated schematically in Figures 2, 7 and 65.

SEQ ID NO:2 is a human PXR nucleotide sequence obtainable using another targeting vector of the invention. The first three nucleotides of SEQ ID NO:2 (atg) are the translational start site from the mouse PXR gene; the start site of the human PXR gene (ctg) is not present. SEQ ID NO:2 comprises sequences from introns 4, 5, 6, and 7 of the human PXR gene, and is obtainable using a targeting strategy as illustrated schematically in Figure 62.

SEQ ID NO:3 is a human PXR nucleotide sequence obtainable using a targeting vector of the invention. The first three nucleotides of SEQ ID NO:3 (atg) are the translational start site from the mouse PXR gene; the start site of the human PXR gene (ctg) is not present. SEQ ID NO:3 comprises sequences from introns 2, 3, 4, 5, 6, 7 and 8 of the human PXR gene, and is obtainable using a targeting strategy as illustrated schematically in Figure 64.

SEQ ID NO:4 is a human CAR nucleotide sequence obtainable using a targeting vector of the invention. SEQ ID NO:4 contains a 53 bp Phi-C31 recognition site (attB53), which was inserted into intron 2 of the human CAR gene. SEQ ID NO:4 comprises sequences from introns 2, 3, 4, 5, 6, 7 and 8 of the human CAR gene, and is obtainable using a strategy as illustrated schematically in Figures 3, 8 and 66.

Accordingly, in one aspect the invention provides a transgenic non-human animal, tissue or cells derived therefrom, that comprises a DNA sequence as recited in SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3 or SEQ ID NO:4. Preferably, the DNA sequence is stably integrated at the locus of the endogenous equivalent gene (*i.e.* within the murine PXR or CAR locus). The invention also provides a nucleic acid targeting vector that comprises a DNA sequence as recited in SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3 or SEQ ID NO:4, and which further comprises 5' and 3' flanking nucleic acid sequences that are homologous to 5' and 3' regions in the locus of the endogenous equivalent gene, and which optionally further comprises nucleic acid sequence elements that permit conditional deletion of the human DNA sequence after its integration in the locus of the endogenous equivalent gene. The invention also provides a non-human animal, tissue or cells derived therefrom containing such a nucleic acid targeting vector.

Preferred non-human animals, tissues or cells and targeting vectors comprise a nucleic acid molecule that is at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98% or at least 99% identical over its entire length to a nucleic acid molecule as recited in SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3 or SEQ ID NO:4, or a DNA sequence that is complementary to such a nucleic acid molecule.

Methods for introducing replacement genes

According to a further aspect of the invention there is provided a method of introducing at least one functional human transcription factor, at least one phase-1 drug metabolising enzyme, at least one phase-2 drug-metabolising enzyme and at least one drug transporter protein into non-human cell(s) whose own endogenous equivalent genes expressing the aforesaid proteins have optionally been rendered inactive, the method comprising introducing DNAs encoding said human transcription factor, phase-1 and 2 drug metabolising enzyme and drug transporter protein such that said human expression products remain functional, even where the cell's own endogenous genes are rendered inactive. Constructs of any one of the above-described aspects of the invention may be used.

Preferably, endogenous non-human genes whose protein products are analogous to the protein products of the introduced human DNA sequences are deleted. This can be done preferably either by direct targeting with their human counterparts or by flanking these genes or gene clusters with recognition sites and subsequent recombinase-mediated deletion (*e.g.* Cre-mediated or Phi-C31-mediated deletion).

According to the present invention, genes that are inserted into the transgenic model are preferably inserted at the point in the genome where the endogenous equivalent gene or gene cluster naturally

occurs. This has the advantage that the context of the gene locus is retained which means that the fidelity of transcription from this site is as close as possible to the level of transcription that occurs in the wild type system.

Furthermore, according to the invention, the endogenous equivalent gene or genes to those that are inserted into the transgenic system optionally have been annulled. "Annulled" is meant to include silencing or deletion or rendering inactive so that the non-human animal's endogenous equivalent gene is unable to express the gene product(s). A preferable way in which to annul the expression of the endogenous equivalent gene or genes and simultaneously to insert the replacement gene at the point at which it naturally occurs is by the process of homologous recombination, described above. According to this methodology, homology arms of sequence complementary to sites in the target genome flank the insertion sequence and these are used to direct insertion of the desired human gene or genes.

For example, in the case of MDR1, the drug transporter protein, different animal species have different MDR1 isotypes and expression profiles. The mouse has two genes encoding active drug transporters (MDR1a and MDR1b), whose tissue expression is mutually exclusive. In contrast, MDR1 is the only functioning drug transporter of the two MDR genes present in the human (MDR1 and MDR3). When creating a mouse transgenic for MDR, therefore, MDR1 should preferably replace both the endogenous MDR genes, MDR1a and MDR1b.

Another example can be provided by the case of CYP2D6, where there are nine genes in the mouse, corresponding to only one functional gene in humans. Replacement of the mouse gene cluster with the human gene is relatively simple though, since the latter spans less than around 40kb of genomic sequence.

DNA sequences may be deleted by, for example, Cre/lox-mediated deletions. This type of deletion is suitable for deleting of large fragments of DNA (200kb to several megabases). The method has been described in the following papers (Li ZW, Stark G, Gotz J, Rulicke T, Gschwind M, Huber G, Muller U, Weissmann C. Generation of mice with a 200-kb amyloid precursor protein gene deletion by Cre recombinase-mediated site-specific recombination in embryonic stem cells Proc Natl Acad Sci U S A. 1996 Jun 11;93(12):6158-62. Erratum in: Proc Natl Acad Sci U S A 1996 Oct 15;93(21):12052 and in Su H, Wang X, Bradley A. Nested chromosomal deletions induced with retroviral vectors in mice. Nat Genet. 2000 Jan;24(1):92-5).

Cre/lox-mediated insertions of large fragments may also be used to insert the human DNA sequences by a method described in Call LM, Moore CS, Stetten G, Gearhart JD. A cre-lox recombination system for the targeted integration of circular yeast artificial chromosomes into

embryonic stem cells. Hum Mol Genet. 2000 Jul 22;9(12):1745-51.

Preferably, the non-human transgenic animal is humanised for a drug transporter protein by the hitherto undisclosed "knock-in" approach, as shown in the schematic representation of accompanying Figures 1, 2 and 3.

Preferably, the non-human transgenic animal of the present invention is humanised for both PXR and CAR alone or in combination, more preferably in combination.

Preferably, the human genes are at least partially conserved within a construct.

Preferably, in the case of a transgenic animal expressing the human PXR gene, the PXR construct retains the intron-exon structure between exons 4 and 6. This advantageously retains the sequence where most splice variants are observed and is conveniently located within the ligand-binding domain. The preferred human PXR sequence therefore contains the cDNA of exons 1-4, the genomic sequences of intron 4, exon 5 and intron 5 and the cDNA for exons 6-9.

It will be appreciated that alternative splice products between these exons, which might account for proteins with distinct ligand-binding capacity, will be covered by the constructs of the present invention.

Preferably, the CAR construct retains the intron-exon structure between exons 2 and 9. This advantageously retains complete genomic structure within the targeting vector and permits coverage of all splice variants of human CAR.

Other preferred constructs are described above.

In one embodiment of the invention the transgenic animals are produced *de novo* so as to include all the aforementioned features by methods wherein, for example, cre/lox mediated deletion of large fragments of DNA (200kb to several megabase) are achieved (Li ZW, et al. Generation of mice with a 200-kb amyloid precursor protein gene deletion by Cre recombinase-mediated site-specific recombination in embryonic stem cells Proc Natl Acad Sci U S A. 1996 Jun 11;93(12):6158-62. Erratum in: Proc Natl Acad Sci U S A 1996 Oct 15;93(21):12052 and in Su H et al Nested chromosomal deletions induced with retroviral vectors in mice. Nat Genet. 2000 Jan;24(1):92-5), and where cre/lox-mediated insertions of large fragments are achieved as described in Call et al. A cre-lox recombination system for the targeted integration of circular yeast artificial chromosomes into embryonic stem cells. Hum Mol Genet. 2000 Jul 22;9(12):1745-51.

In another embodiment of the invention the transgenic non-human animal of the present invention is produced by crossing. For example, the animal of WO2004/007708 might be crossed with those transgenic animals that have been humanised with PXR and /or CAR and include further

modifications with respect to phase-2 drug metabolising enzymes and drug transporter protein in either transgenic strain of the non-human animal.

In a further embodiment of the invention the transgenic non-human animal is produced *de novo* so as to include all of the aforementioned features, by the methods as hereinafter disclosed.

It will be appreciated that ideally all of the introduced human DNAs are substantially under the control of human promoters.

Model systems

Advantageously, the present invention provides a non-human transgenic animal that mimics the human mechanisms of metabolism, disposition or toxicity of drugs or other xenobiotic compounds on a non-human animal cell by introducing into a non-human animal cell one or more human DNA sequences comprising coding and regulatory sequences necessary to reproduce the regulation and function of one or more proteins responsible for human metabolism, disposition or toxicity of drugs or other xenobiotic compounds where the said non-human animal cell has undergone deletion of endogenous genes encoding proteins whose functions are analogous to those encoded by the introduced human DNA sequences so that the non-human animal cell can be used as a model system for determining the metabolism, disposition or toxicity of drugs or other xenobiotic compounds in a homologous human cell.

It is possible, according to the invention, to "personalise" a particular transgenic system to suit a phenomenon that is worthy of investigation. For example, CYP3A4 gene expression levels may vary as much as 60 times between individuals, and in any individual may also vary over time. This is partly because of inherent genetic differences, but more importantly due to variability in exposure to drugs, toxins, food products and other environmental variables. The system is an adaptive response system which will only keep high enzyme levels for as long as they are needed. Any other implementation would be wasteful. This means that it is not generally appropriate, in any test system, merely to test the effect of a particular drug concentration at one level of CYP3A4. The effects of the drug must ideally be assessed at high CYP3A4 levels and also at low CYP3A4 levels so that response is tested in both a high and a low P450 environment. Of course, systems according to the prior art, using the albumin promoter, simply cannot reflect the real situation found *in vivo*.

Another example is provided by the CYP2D6 protein, which plays a major role in the metabolism of neuroleptic drugs (e.g. anti-depressants and drugs used for treatment of schizophrenia), and is thus of significant importance. Pharmaceutical companies are reluctant to back a drug that is

metabolised by CYP2D6 and therefore need to know as soon as possible during development of any drug, whether or not it has a CYP2D6 liability. This gene shows variation between individuals, and in fact expression is absent in around 6% of Caucasian individuals. It would be of immense benefit to be able to study the metabolism of drugs, particularly of anti-depressants, in environments of both high and low CYP2D6 levels.

These advantages also allow carcinogenicity testing to be performed as well as the acute pharmacological tests described above. For example, according to the invention, P450 levels, either as single genes or a multiple genes and preferably as clusters of genes, may be raised to artificially high levels in order to test for potentially carcinogenic effects of metabolites. At physiologically normal levels, the effects of such metabolites might not be evident. The marked species difference in carcinogenicity of compounds between rodents and man result in the main from the different rates of generation of toxic or mutagenic metabolites, along with other differences in pharmacokinetics and distribution. The ability to increase gene levels in entire clusters is important as it retains the substrate crosstalk between the different proteins expressed by the genes in the cluster.

Bespoke systems of this type may also be exploited to investigate disease. An example is provided by Gilbert syndrome, a phenomenon caused by a polymorphism in the UGT1A1 gene implicated in drug metabolism. According to the invention, a transgenic model animal may incorporate the polymorphism-containing gene in order to allow this syndrome to be evaluated.

According to a yet further aspect of the invention there is provided a host cell transfected with a nucleic acid construct(s) according to any one of the previous aspects of the invention. The cell type is preferably of human or non-human mammalian origin but may also be of other animal, plant, yeast or bacterial origin.

According to a yet further aspect of the invention, there is provided a transgenic non-human animal in which the cells of the non-human animal express the protein(s) encoded by the nucleic acid construct(s) according to any one of the previous aspects of the invention. The transgenic animal is preferably a mouse, because of currently available technology, but may be another mammalian species, for example another rodent, for instance a rat or a guinea pig, or another species such as rabbit, pig, or a canine or feline, or an ungulate species such as ovine, equine, bovine, or a non-mammalian animal species.

In embodiments of the invention relating to the preparation of a transgenic host cell or a transgenic non-human animal comprising the use of a nucleic acid construct as previously described, the cell or non-human animal may be subjected to further transgenesis, in which the transgenesis is the

introduction of an additional gene or genes or protein-encoding nucleic acid sequence or sequences. The transgenesis may be transient or stable transfection of a cell or a cell line, an episomal expression system in a cell or a cell line, or preparation of a transgenic non-human animal by pronuclear microinjection, through recombination events in non-embryonic stem (ES) cells, random transgenesis in non-human embryonic stems (ES) cells or by transfection of a cell whose nucleus is to be used as a donor nucleus in a nuclear transfer cloning procedure.

Methods of preparing a transgenic cell or cell line, or a transgenic non-human animal, in which the method comprises transient or stable transfection of a cell or a cell line, expression of an episomal expression system in a cell or cell line, or pronuclear microinjection, recombination events in ES cells, or other cell line or by transfection of a cell line which may be differentiated down different developmental pathways and whose nucleus is to be used as the donor for nuclear transfer; wherein expression of an additional nucleic acid sequence or construct is used to screen for transfection or transgenesis in accordance with the previous aspects of the invention. Examples include use of selectable markers conferring resistance to antibiotics added to the growth medium of cells, for instance neomycin resistance marker conferring resistance to G418. Further examples involve detection using nucleic acid sequences that are of complementary sequence and which will hybridise with, or a component of, the nucleic acid sequence in accordance with the previous aspects of the invention. Examples would include Southern blot analysis, northern blot analysis and PCR.

Non-human animal cell or transgenic non-human animals produced by the method of the invention can be used as model systems for determining the metabolism of drugs or other xenobiotic compounds in a human.

According to a yet further aspect of the invention there is provided use of a transgenic animal, tissues and/or cells derived therefrom as hereinbefore described that have been modified to contain and express DNA encoding at least one functional human transcription factor, at least one phase-1 drug metabolising enzyme, at least one phase-2 drug-metabolising enzyme and at least one drug transporter protein so as to investigate xenobiotic metabolism or toxicity in said a transgenic animal, tissues and/or cells derived therefrom or other properties or functions of the introduced human proteins such as metabolism and/or biosynthesis of endogenous compounds.

The system of the present invention allows function and regulation of human mechanisms of xenobiotic metabolism, disposition and toxicity to be studied in any tissue or cell type, for instance gastrointestinal tract, blood-brain barrier, liver, kidney in a single animal, tissue or cell derived therefrom.

The system of the present invention may be applied to study effects of human metabolism, disposition or toxicity on anti-tumour effects of a drug in an animal xenograft experiment by expressing humanised metabolic pathways in a non-human grafted tumour cell line and/or in the host animal.

Reporters

The present invention also, advantageously provides non-human animal cells and transgenic non-human animals incorporating introduced reporter genes so that such cells or animals can be used to determine indications of pathways of metabolism of drugs or other xenobiotic compounds in a human cell by convenient assay of the products of reporter gene expression.

Where reporter genes have been incorporated in non-human animal cell or transgenic non-human animals produced by the method of the invention (see below), the cells or animals can be used to determine regulation of genes and also give indications of the likely mechanism and metabolism of drugs or other xenobiotic compounds in an homologous human cell by assaying expression of the reporter gene DNA sequence. The cells or animals can also be used to give indications of the extent of metabolism of drugs or other xenobiotic compounds. For example, analysis of the distribution of reporter gene expression within any particular tissue allows the extent of induction of gene expression to be monitored in response to a particular drug compound.

According to a further aspect of the invention there is provided a non-human animal, tissue or cells derived therefrom incorporating a promoter linked transcriptionally to a human DNA sequence encoding:

(i) a transcription factor; and

(ii) a DNA sequence encoding a phase-1 drug-metabolising enzyme; and/or

(iii) a DNA sequence encoding a phase-2 drug-metabolising enzyme; and/or

(iv) a DNA sequence encoding a drug transporter protein.

The promoter of the transcription factor and/or phase 1 and/or phase 2 drug-metabolising enzyme and/or drug transporter protein may thus be linked to a reporter which allows monitoring for the relative regulation of at least one enzyme involved in drug/xenobiotic disposition. Such an embodiment advantageously allows not only for relative regulation of the enzymes but regulation in both a tissue-specific manner in the transgenic animal or in the whole animal itself in a non-invasive manner as well as the extent and potency of gene induction. Reporters may be linked to the promoters of two or more of (i), (ii), (iii) or (iv) listed above.

Reporter genes are nucleic acid sequences encoding directly or indirectly assayable proteins. They are used to replace other coding regions whose protein products are unsuitable or not amenable to the assay envisaged. Suitable reporter genes that are known in the art and may be used in the present invention are selected from those genes encoding proteins including but not limited to: chloramphenicol-acetyltransferase, β-galactosidase, β-glucuronidase, luciferase, beta-galactosidase, green fluorescent protein, secreted alkaline phosphatase (SEAP), major urinary protein (MUP) or human chorionic gonadotrophin (hCG). It will be understood that the above list of suitable reporter genes is not exhaustive or exclusive and is not intended to limit the scope of the application. The skilled artisan may select another reporter system which will equally be applicable to the present invention.

According to the invention, the promoters that are preferred targets for linkage to reporter genes are PXR, CAR, CYP3A4, Cyp3a11, CYP2C9, CYP2C19, CYP2B6, CYP2D6, UGT1A, MRP2 and MDR1.

The reporter embodiments of the invention can be used in comparative methods. The skilled person will readily be able to select an appropriate 'panel' of reporter non-human animals, tissues or cells derived therefrom as disclosed herein. The type and number of non-human animals, tissues or cells required for comparison will depend on the type of analysis required (*e.g.* depending on the drug metabolism pathway of interest and/or the drug of interest). The invention provides transgenic non-human animals, tissues or cells derived therefrom of various reporter genotypes (see elsewhere herein). The invention provides a tool kit from which the skilled person can select the tools required for the desired analysis.

Thus, the invention provides a method for investigating xenobiotic metabolism or toxicity, comprising the use of:

(i) a first non-human animal, tissue or cells derived therefrom comprising a first endogenous regulatory sequence (*e.g.* a mouse or human promoter) operatively linked to a DNA sequence whose expression can conveniently be measured by assay of transcription and/or translation products; and

(ii) a second non-human animal, tissue or cells derived therefrom comprising a second endogenous regulatory sequence (*e.g.* a mouse or human promoter) different to said first endogenous regulatory sequence operatively linked to a DNA sequence whose expression can conveniently be measured by assay of transcription and/or translation products

wherein the first and second endogenous regulatory sequences are regulatory sequences normally

associated with genes encoding proteins involved in drug metabolism.

The invention also provides a method for investigating xenobiotic metabolism or toxicity, comprising the use of at least two, at least three, at least four, at least five, at least six, at least seven, at least eight, at least nine, or at least ten types of non-human animal, tissue or cells derived therefrom, wherein each type of non-human animal, tissue or cells comprises a different endogenous regulatory sequence (*e.g.* a mouse or human promoter) operatively linked to a DNA sequence whose expression can conveniently be measured by assay of transcription and/or translation products (*i.e.* a reporter sequence), and wherein each endogenous regulatory sequence is a regulatory sequence normally associated with a gene encoding a protein involved in drug metabolism.

In some embodiments, the DNA sequence whose expression can conveniently be measured by assay of transcription and/or translation products will be the same in the different non-human animals, tissues or cells, but in other embodiments different reporter sequences are used.

These methods of the invention wherein different transgenic non-human animals are compared may involve administering the same drug at the same dose to the different types of non-human animal (*i.e.* to different types of reporter) and comparing the metabolism or toxicity of that drug between the different animals.

Particularly preferred transgenic non-human animals, tissues and cells of the invention have a genotype as specified in the following list. The prefix "hu" refers to humanisation of the relevant endogenous non-human animal gene. The prefix "r" denotes that an endogenous regulatory sequence (*e.g.* a mouse or human promoter) for the relevant gene is operatively linked to a DNA sequence whose expression can conveniently be measured by assay of transcription or translation products (*i.e.* a reporter sequence).

Reporter constructs

rCYP3A4;

rCYP2D6;

rCYP2B6;

rCyp3a11;

rMDR1;

Particularly preferred transgenic non-human animals, tissues and cells of the invention incorporate a combination of two or more of the genotypes described herein above, some examples of which

are specified in the following list, wherein the symbol "/" indicates a combination of genetic modifications.

In the following complex genotypes, only a single reporter is present. However, use of multiple reporters within a single non-human animal, tissue or cells derived therefrom is also envisaged.

Complex reporter constructs

rCYP2B6/huCAR;

rCyp3a11/huPXR;

rCyp3a11/huPXR;

rCYP3A4/huPXR;

rCYP2C9/huPXR;

rCYP2D6/huPXR;

rCYP2C19/huPXR;

rMDR1/huPXR;

rCYP3A4/huPXR/huCAR;

rCYP2C9/huPXR/huCAR;

rCYP2D6/huPXR/huCAR;

rCYP2C19/huPXR/huCAR;

rCYP2B6/huPXR/huCAR;

rCYP3A4/huCYP2B6/huPXR/huCAR;

rMDR1/huPXR/huCAR;

rCYP3A4/huCYP2C9/huPXR/huCAR;

rCYP2D6/huCYP2C19/huPXR/huCAR;

rCYP2B6/huMDR1/mdr1a$^{-/-}$/mdr1b$^{-/-}$/huPXR/huCAR;

huPXR/huCAR/rCYP2B6/rCYP2D6/rCYP3A4/rMDR1.

rCYP3A4/huCYP2C9/huCYP2D6/huCYP2C19/huPXR/huCAR;

rCYP3A4/huCYP2D6/huPXR/huCAR;

rCYP3A4/huCYP2C9/huCYP2B6/huMDR1/mdr1a$^{-/-}$/mdr1b$^{-/-}$/huPXR/huCAR;

rCYP3A4/huCYP2B6/MDR1/huPXR/huCAR;

rCYP3A4/huCYP2C9/huCYP2D6/huCYP2C19/huCYP2B6/MDR1/huPXR/huCAR; and

rCYP3A4/huCYP2D6/huCYP2B6/huMDR1/mdr1a$^{-/-}$/mdr1b$^{-/-}$/huPXR/huCAR.

Transgenic non-human animals, tissues and cells having a complex genotype as listed above are particularly preferred, because the drug metabolism pathways in those non-human animals, tissues and cells more closely resemble the *in vivo* situation in humans.

Suitable methods and constructs for generating the transgenic non-human animals, tissues or cells of the invention are described elsewhere herein, and specific targeting strategies are described in the Examples herein and schematically illustrated in the Figures. Each of the specific targeting strategies and constructs described and illustrated herein forms a further aspect of the invention. Thus, the invention provides a method of generating a non-human animal cell comprising a reporter construct as described herein using a targeting strategy or vector substantially as described herein or substantially as depicted in the Figures herein.

In particular, the invention provides a transgenic mouse, tissue or cells derived therefrom comprising one or more of the following genetic manipulations:

rCYP2B6	Expression of a reporter gene under the control of a human CYP2B6 promoter sequence. Preferably, a reporter gene and a human promoter sequence are inserted into the mouse Cyp2b6 locus. Preferably, the reporter gene is a LacZ reporter gene (*e.g.* Figure 79).
rCYP2D6	Expression of a reporter gene under the control of a human CYP2D6 promoter sequence. Preferably, a reporter gene and a human promoter sequence are inserted into the mouse Cyp2d6 locus. Preferably, the reporter gene is a ZsYellow reporter gene (*e.g.* Figure 80).
rCYP3A4	Expression of a reporter gene under the control of a human CYP3A4 promoter sequence. The reporter gene and a human promoter sequence may be randomly inserted / integrated into the mouse genome. Preferably, the reporter gene is a hCG-ZsGreen reporter gene (*e.g.* Figure 81).

rCyp3a11	Expression of a reporter gene under the control of a mouse Cyp3a11 promoter sequence. Preferably, a reporter gene is inserted into the mouse Cyp3a11 locus. Preferably, the reporter gene is a Firefly luciferase (*e.g.* Figure 82) or a ZsGreen (*e.g.* Figure 71) reporter gene.
rMDR1	Expression of a reporter gene under the control of a human MDR1 promoter sequence. Preferably, the human MDR1 promoter sequence comprises both upstream and downstream promoter sequences. Preferably, a reporter gene and a human promoter sequence are inserted into the mouse Rosa26 locus. Preferably, the reporter gene is a Firefly luciferase reporter gene (*e.g.* Figure 83).

Further details of the preferred reporter strategies and constructs are provided in the Examples and Figures herein.

Animals, tissues and cells according to these aspects of the invention allow very useful elements of the process of gene regulation in drug metabolism to be elucidated. For example, although PXR is known to be of great significance in the transcriptional control of the CYP3A4 gene, there are likely to be other important active mechanisms. Exposing a CYP3A4 reporter animal or cell to a drug compound in either a humanised PXR or a PXR null background would be useful in exploring other ways of regulating CYP3A4 than by way of PXR. Another case of interest would be to expose an *mdr1* reporter animal or cell to a drug compound in both a humanised PXR and PXR null background to detect aspects of *mdr1* expression that are independent of PXR regulation.

Accordingly, animals, tissues and cells according to this aspect of the invention may comprise a promoter linked transcriptionally to a human DNA sequence encoding a phase-1 drug-metabolising enzyme; a DNA sequence encoding a phase-2 drug-metabolising enzyme; and/or a DNA sequence encoding a drug transporter protein in a null background for PXR, CAR or any other transcription factor. By "null background" is meant that the gene or genes have been annulled, according to the definition provided above. Such animals, cells, or tissues might be compared under similar conditions (for example, in the presence and absence of a drug or drugs) to in a humanised PXR or CAR background. Accordingly, animals, tissues and cells according to this aspect of the invention may comprise a promoter linked transcriptionally to a human DNA sequence encoding a phase-1 drug-metabolising enzyme; a DNA sequence encoding a phase-2 drug-metabolising enzyme; and/or a DNA sequence encoding a drug transporter protein in a humanised background for a transcription factor. Such a transcription factor may be PXR alone, CAR alone, PXR and CAR or

any other single transcription factor or combination of transcription factors described herein.

By "linked transcriptionally" is meant that the activity of the promoter dictates the expression level of the reporter protein. Preferably, a reporter gene is fused to the translational start site of the corresponding human gene whose promoter is to be investigated. In the case of the CYP3A4, CYP2C9 and CYP2C19 promoters, the transcript of the reporter gene may not be terminated by a polyA motif, but the constructs are designed such that the endogenous polyA motif is potentially used. These constructs are therefore dependent on a correct splicing of the exons 3' to the reporter (see Figure 12). In the cases of CYP2D6 and CYP2B6, the transcript of the reporter gene is preferably terminated by a polyA motif linked to the reporter gene with a synthetic intron (see Figure 13). In case of MDR1, the transcript of the reporter gene is preferably terminated by a polyA motif without an additional intron (see Figure 14).

According to a further aspect of the invention there is provided a non-human animal, tissue or cells derived therefrom incorporating at least one human DNA sequence encoding at least one transcription factor under control of a transcription factor promoter and whose endogenous equivalent genes have optionally been annulled, the non-human animal, tissue or cells further incorporating a promoter linked transcriptionally to a human DNA sequence encoding:

(i) a DNA sequence encoding a phase-1 drug-metabolising enzyme; and/or

(ii) a DNA sequence encoding a phase-2 drug-metabolising enzyme; and/or

(iii) a DNA sequence encoding a drug transporter protein.

The non-human animal, and tissues or cells of this aspect of the invention may incorporate a promoter linked transcriptionally to a DNA sequence encoding a phase-1 drug-metabolising enzyme and a DNA sequence encoding a phase-2 drug-metabolising enzyme. The non-human animal, and tissues or cells may incorporate a promoter linked transcriptionally to a DNA sequence encoding a phase-1 drug-metabolising enzyme and a DNA sequence encoding a drug transporter protein. The non-human animal, and tissues or cells may incorporate a promoter linked transcriptionally to a DNA sequence encoding a phase-2 drug-metabolising enzyme and a DNA sequence encoding a drug transporter protein. Examples of suitable phase-1 drug-metabolising enzymes, phase-2 drug-metabolising enzymes and drug transporter proteins are described herein.

In another aspect, for example, the promoter activity of the *mdr1* gene may be investigated. In this scenario, animals, tissues and cells may comprise a promoter linked transcriptionally to a human DNA sequence encoding a transcription factor, a promoter linked transcriptionally to a human DNA sequence encoding a phase-1 drug-metabolising enzyme; and/or a promoter linked

transcriptionally to a DNA sequence encoding a phase-2 drug-metabolising enzyme in a humanised or null background for *mdr1*.

According to this aspect of the invention there is provided a non-human animal, tissue or cells derived therefrom incorporating at least one human DNA sequence encoding at least one drug transporter protein under control of a drug transporter promoter and whose endogenous equivalent genes have optionally been annulled, the non-human animal, tissue or cells further incorporating a promoter linked transcriptionally to a human DNA sequence encoding:

(i) a DNA sequence encoding a transcription factor; and/or

(ii) a DNA sequence encoding a phase-1 drug-metabolising enzyme; and/or

(iii) a DNA sequence encoding a phase-2 drug-metabolising enzyme.

The non-human animal, and tissues or cells of this aspect of the invention may incorporate a promoter linked transcriptionally to a DNA sequence encoding a phase-1 drug-metabolising enzyme and a DNA sequence encoding a phase-2 drug-metabolising enzyme. The non-human animal, and tissues or cells may incorporate a promoter linked transcriptionally to a DNA sequence encoding a phase-1 drug-metabolising enzyme and a DNA sequence encoding a transcription factor. The non-human animal, and tissues or cells may incorporate a promoter linked transcriptionally to a DNA sequence encoding a phase-2 drug-metabolising enzyme and a DNA sequence encoding a transcription factor. Examples of suitable phase-1 drug-metabolising enzymes, phase-2 drug-metabolising enzymes and transcription factor proteins are described herein.

According to a yet further aspect of the invention, there is provided a nucleic acid construct comprising a targeting vector substantially as depicted in any of Figures 1-14 and 62-83.

Preferably, the construct further includes for the humanisation and corresponding knock-out of at least one phase-1 drug metabolising enzyme, at least one phase-2 drug-metabolising enzyme and at least one drug transporter protein in either the same construct or further independent constructs.

Cells

In another aspect of the invention, an animal cell is produced by any one of the above-described aspects of the invention. In preferred aspects, at least one human regulatory DNA sequence associated with the gene encoding a protein responsible for determining the human metabolism, disposition, distribution or toxicity of drugs or other xenobiotic compounds is operatively linked to a DNA sequence whose expression can conveniently be measured by assay of transcription or translation products to produce a reporter gene DNA sequence which is introduced into the non-

human animal cell. This embodiment provides linkage with one or more reporter sequences such as human chorionic gonadotrophin (hCG).

Assays

The animals, tissues and cells of the present invention may be used to determine how a drug compound is metabolised by a human. In particular, it is possible to examine whether a drug compound modulates the activity or expression levels of a transcription factor, a drug metabolising enzyme or a drug transporter protein. It is possible to examine the ratio of the levels of activation or expression of a transcription factor, a drug metabolising enzyme or a drug transporter protein induced by a drug compound. It is possible to examine whether a drug compound influences the disposition or distribution of a transcription factor, a drug metabolising enzyme or a drug transporter protein within the tissues of the body. It is possible to examine whether a drug compound influences the duration of expression of a transcription factor, a drug metabolising enzyme or a drug transporter protein.

It is possible to measure a phenotypic change in the animal, such as a physiological effect. Such a physiological effect may be, for example, a disease condition (such as biliary necrosis) or a toxic side-effect.

It is possible to examine the rate of metabolism of a drug compound. The rate of metabolism may be determined by measuring the toxicity or activity mediated by the administration of the compound, measuring the half-life of the compound, or measuring the level of a drug metabolising enzyme. For example, the rate of metabolism of the compound may be measured as the rate of formation of the oxidized product or the formation of a subsequent product generated from the oxidized intermediate. Alternatively, the rate of metabolism may be represented as the half-life or rate of disappearance of the initial compound or as the change in toxicity or activity of the initial compound or a metabolite generated from the initial compound. The half-life may be measured by determining the amount of the drug compound present in samples taken at various time points. The amount of the drug compound may be quantified using standard methods such as high-performance liquid chromatography, mass spectrometry, western blot analysis using compound specific antibodies, or any other appropriate method.

It is also possible to examine whether a drug compound is metabolised to a toxic or carcinogenic metabolite, for example, by measuring its covalent binding to tissues, proteins or DNA or by measuring glutathione depletion.

Preferably, measurements of the type described above are performed at more than 1, 3, 5, 10 or

more time points after administration of the drug compound.

Accordingly, further aspects of the invention relate to screening methods that are provided to determine the effect of a drug compound on the activity or expression level of a transcription factor, a drug metabolising enzyme or a drug transporter protein. Such methods involve administering a drug compound to a transgenic animal according to any one of the aspects of the invention described above, or a tissue or cell derived therefrom. . .

The screening step may involve measuring the induction of a gene coding for a transcription factor, a drug metabolising enzyme or a drug transporter protein. The screening step may involve measuring the level of expression of a transcription factor, a drug metabolising enzyme or a drug transporter protein or the duration of such expression. The screening step may involve measuring the distribution of expression of a transcription factor, a drug metabolising enzyme or a drug transporter protein.

The assay can be performed in the presence and absence of the drug compound to ascertain differences in distribution, metabolism and toxicity. The effects of the drug compound in the presence and absence of a particular gene or genes can be ascertained by evaluating the effects of the drug compound on different transgenic animals, cells or tissues. For example, the effects of the drug compound could be evaluated between an animal with a null background and an animal humanised for the gene or genes of interest (e.g. PXR, CAR, MDR1, a phase I metabolising enzyme or a phase 2 metabolising enzyme).

Thus, in a further aspect the invention provides methods for investigating xenobiotic metabolism or toxicity as described herein, comprising administering a drug compound to 2 or more, 3 or more, 4 or more, 5 or more, 6 or more, 7 or more, 8 or more, 9 or more, or 10 or more of the non-human animals, tissues or cells described herein. Preferably, such methods further include a step of comparing the experimental results obtained for different non-human animals, tissues or cells.

More than one drug compound may be administered. For example, a drug compound is determined to activate the CAR transcription factor if the compound mediates induction of the CAR gene. A CAR receptor inverse agonist such as clotrimazole can also administered to an animal expressing the human CAR receptor as a control.

Assays according to further aspects of the invention may provide a screening method for determining whether the metabolism of a first drug compound is modulated by a second drug compound. This method involves administering the first compound in the presence and absence of the second compound to a transgenic animal according to any one of the above-described aspects

of the invention, or a tissue or cell derived therefrom, and monitoring for a phenotypic effect. Alternatively, as above, the screening step may involve measuring the induction of a gene, the level, duration or distribution of expression, of a transcription factor, a drug metabolising enzyme or a drug transporter protein. The second compound is determined to modulate the metabolism of the first compound if the second compound effects a change in any one of these tested factors. For example, a physiological effect may be assayed by measuring the toxicity or activity mediated by the administration of the first compound or measuring the half-life of the first drug compound.

In this manner, assays may be used to facilitate the identification of analogs of a drug compound that have reduced or undetectable ability to activate or induce expression of a particular protein, and thus are expected to have fewer side-effects or a longer half-life *in vivo*.

The invention will now be described by way of example only with reference to the following figures wherein:

Figure 1 shows a schematic representation of humanisation and knock out of PXR/CAR;

Figure 2 shows a schematic representation of a composite cDNA with genomic sequences for the PXR humanisation strategy;

Figure 3 shows a schematic representation of a composite cDNA with genomic sequences for the CAR humanisation strategy;

Figure 4 shows a possible mouse *mdr1a* targeting vector;

Figure 5 shows a possible mouse *mdr1b* targeting vector;

Figure 6 shows a possible construct for MRP2 humanisation;

Figure 7 shows a possible targeting strategy for PXR humanisation;

Figure 8 shows a possible targeting strategy for CAR humanisation;

Figure 9 shows a possible strategy for CYP3A4 humanisation;

Figure 10 shows a possible strategy for CYP2C9 humanisation;

Figure 11 shows a possible overall strategy for cluster exchange;

Figure 12 shows a possible overall strategy for reporter constructs;

Figure 13 shows a possible reporter project strategy for CYP2D6 and CYP2B6;

Figure 14 shows a possible reporter project strategy for MDR1;

Figure 15 shows an example of a PXR typing PCR;

Figure 16 shows Taqman typing of wild type and transgenic mice. All probes and primers used were 'pre-optimised TaqMan® Genomic Assay kits, which were purchased from Applied Biosystems. All assays used were inventoried by Applied Biosystems. Shown are the results from TaqMan analysis of liver (A, B) and small intestine (C, D) of nine wild-type and four hPXR mice, using assays specific for the mouse PXR mRNA transcript (A, C) or the human PXR mRNA transcript (B, D);

Figure 17 shows RT-PCR of huPXR transcripts in transgenic mice;

Figure 18 shows Western blotting of PXR protein from wild type and hPXR mice. Proteins from the livers of treated wild-type and huPXR mice were probed with an antibody specific for the PXR protein. The standard (+ve) was a his-tagged murine PXR;

Figure 19 shows Western blotting of Cyp3a and Cyp2b induction by rifampicin. Microsomal proteins from the livers of rifampicin treated wild-type and huPXR mice were probed with antibodies for the Cyp 3a11 protein (top panel) and Cyp 2b10 (bottom panel);

Figure 20 shows enzyme activity assays of Cyp3a and Cyp2b10 induction by rifampicin (100mg/kg). Shown are the results from enzyme activity assays on liver microsomes using activity of pentoxyresorufin-O-deethylation (PROD) which is attributed to expression level of Cyp 2b10 and the activity of 7-benzyloxyquinoline (BQ) which is attributed to expression level of Cyp 3a;

Figure 21 shows enzyme activity assays in liver microsomes following induction by rifampicin (3mg/kg and 10mg/kg) - 7-benzyloxyquinoline. Shown are the results from enzyme activity assays on liver microsomes using the activity 7-benzyloxyquinoline;

Figure 22 shows enzyme activity assays in liver microsomes following induction by rifampicin (3mg/kg and 10mg/kg) - 6-beta-hydroxylation of testosterone ($p<0.01$);

Figure. 23 shows enzyme activity assays in liver microsomes following induction by rifampicin (3mg/kg and 10mg/kg) - 16-beta-hydroxylation of testosterone ($p<0.05$);

Figure 24 shows enzyme activity assays in liver microsomes following induction by TCPOBOB (0.3mg/kg and 1.0mg/kg) - 7-alpha-hydroxylation of testosterone which was constitutively higher in the huPXR animals relative to the wild types;

Figure 25 shows enzyme activity assays in liver microsomes following induction by TCPOBOB (0.3mg/kg and 1.0mg/kg) - 6-beta-hydroxylation of testosterone;

Figure 26 shows enzyme activity assays in liver microsomes following induction by TCPOBOB (0.3mg/kg and 1.0mg/kg) - 16-alpha-hydroxylation of testosterone. These demonstrate that this

activity was significantly induced in wild type animals ($p<0.05$) but not in huPXR animals;

Figure 27 shows enzyme activity assays in liver microsomes following induction by TCPOBOB (0.3mg/kg and 1.0mg/kg) - 16-beta-hydroxylation of testosterone. These demonstrate that this activity was much more marked in wild type than in huPXR animals;

Figure 28 shows PCR confirmation in two double homozygous PXR/CAR humanised mice that the murine PXR gene has been exchanged for the human counterpart.

Figure 29 shows a Cyp3a11 induction profile in response to rifampicin treatment in wild-type and huPXR mice.

Figure 30 shows a Cyp3a11 induction profile in response to dexamethasone treatment in wild-type and huPXR mice.

Figure 31 shows the results of TaqMan® analysis of PXR mRNA in the livers of wild-type and koPXR mice.

Figure 32 shows effects of rifampicin administration on Cyp3a11 expression and activity in wild-type, huPXR or koPXR mice.

Figure 33 shows that rifampicin does not induce Cyp2b10 expression in wild-type and huPXR mice.

Figure 34 shows effects of dexamethasone administration on the expression and activity of Cyp3a11 and Cyp2b10 in wild-type, huPXR and koPXR mice.

Figure 35 shows effects of dexamethasone administration on Cyp3a11 expression and activity in wild-type, koPXR and huPXR mice.

Figure 36 shows species-specific differences in dexamethasone mediated hepatotoxicity in huPXR and koPXR mice.

Figure 37 shows the liver/body weight ratios of wild-type, huPXR and koPXR mice following administration of either 60 mg/kg rifampicin or dexamethasone.

Figure 38 shows an overview of the effects of rifampicin and dexamethasone on Cyp3a11 and Cyp2b10 expression in wild-type, huPXR and koPXR mice. A medium increase compared to vehicle-treated mice is denoted by '++', a strong increase compared to vehicle-treated mice is

denoted by '+++', and no change compared to vehicle-treated mice is denoted by 'NC'.

Figure 39 shows the results of TaqMan® analysis of CAR mRNA in the livers and intestines of untreated wild-type and koCAR mice, demonstrating that basal expression of CAR mRNA was completely lost in the koCAR mouse.

Figure 40 shows hepatotoxicity data for wild-type, koCAR and koPXR mice, after treatment with dexamethasone or phenobarbital.

Figure 41 shows effects of dexamethasone and phenobarbital administration on the livers of koCAR and koPXR mice.

Figure 42 shows effects of dexamethasone and phenobarbital administration on the guts and livers of koCAR and koPXR mice.

Figure 43 shows densitometric quantification of Western blot bands obtained from TCPOBOP-treated livers of wild-type and koCAR mice.

Figure 44 shows that human CAR mRNA, but not mouse CAR mRNA, is expressed in the livers of huCAR mice (TaqMan® analysis).

Figure 45 shows further results of CAR mRNA analysis in wild-type and huCAR mice by TaqMan® analysis, for liver and small intestine.

Figure 46 shows CITCO hepatotoxicity data for wild-type and huCAR mice.

Figure 47 shows effects of CITCO in wild-type and huCAR mice.

Figure 48 is a schematic illustration of the rationale for screening assays for PXR and CAR activity.

Figure 49 shows effects of TCPOBOP on Cyp2b10 expression and activity in wild-type and huCAR mice.

Figure 50 shows a comparison of the effects of CITCO and TCPOBOP in wild-type and huCAR mice.

Figure 51 shows effects of phenobarbital treatment on Cyp2b10 expression and activity in wild-type and huCAR mice.

Figure 52 shows effects of dexamethasone treatment on Cyp2b10 expression and activity in wild-type and huCAR mice.

Figure 53 shows effects of treatment with CITCO or TCPOBOP on Cyp2b10 expression and activity in wild-type, huCAR, koCAR, huPXR and koPXR mice.

Figure 54 shows effects of TCPOBOP treatment on Cyp3a11 expression and activity in wild-type and huCAR mice.

Figure 55 provides an overview of the effects of selected drug-metabolism inducers on the expression of Cyp3a11 and Cyp2b10 in mouse and human.

Figure 56 shows an overview of the effects of various inducing agents on PXR and CAR target genes in the livers of wild-type, huPXR, koPXR and huCAR mice. A slight increase in expression compared to vehicle-treated mice of the same strain is denoted by a '+', a medium increase in expression compared to vehicle-treated mice of the same strain is denoted by a '++', a strong increase in expression compared to vehicle-treated mice of the same strain is denoted by a '+++', and no change compared to vehicle-treated mice of the same strain is denoted by 'NC'.

Figure 57 illustrates species-specific differences in the hyperplastic response to CAR activators between wild-type and huCAR mice.

Figure 58 shows PXR and CAR mRNA levels in huPXR/huCAR double-humanised mice and in wild-type, huPXR and huCAR mice. Human PXR and CAR mRNA expression is maintained in double humanised mice.

Figure 59 shows effects of rifampicin and phenobarbital treatment in double-humanised huPXR/huCAR, wild-type, huPXR and huCAR mice, and basal levels of Cyp2b10 and Cyp3all protein in huPXR/huCAR, wild-type, huPXR and huCAR mice.

Figure 60 shows tissue samples from wild-type and rCyp2B6/huCAR reporter mice, illustrating the spatial expression pattern for Cyp2B6 in liver microsomes.

Figure 61 shows tissue samples from wild-type and rCyp2D6/huPXR reporter mice, illustrating the spatial expression pattern for Cyp2D6 in liver microsomes.

Figure 62 shows a further possible targeting strategy for PXR humanisation (to produce mice of

genotype huPXR).

Figure 63 shows a schematic map of a targeting vector useful for PXR humanisation.

Figure 64 shows a further possible targeting strategy for PXR humanisation (to produce mice of genotype huPXR).

Figure 65 shows a possible targeting strategy for PXR humanisation and knock-out (to produce mice of genotype huPXR and koPXR).

Figure 66 shows a possible targeting strategy for CAR humanisation and knock-out (to produce mice of genotype huCAR and koCAR).

Figure 67 shows a schematic map of a targeting vector useful for CAR humanisation and knock-out.

Figure 68 shows a possible targeting strategy for the PPARα humanisation and knock-out in mice (to produce mice of genotype huPPARα and koPPARα).

Figure 69 shows a possible targeting strategy for AhR humanisation and knock-out in mice (to produce mice of genotype huAhR and koAhR).

Figure 70 shows a possible targeting strategy for CYP3A4 humanisation in mice (to produce mice of genotype huCYP3A4).

Figure 71 shows a possible targeting strategy for Cyp3a11 knock-out in mice (to produce mice of genotype koCyp3a11), which at the same time produces a reporter construct (of the genotype rCyp3a11).

Figure 72 panels A-C show a possible targeting strategy for the generation of mice humanised with respect to the CYP3A cluster or wherein the CYP3A cluster is knocked out (to produce mice of genotype huCYP3A cluster and koCyp3A cluster). Figure 72A shows a strategy for 5' targeting of the mouse Cyp3a cluster. Figure 72B shows a strategy for 3' targeting of the mouse Cyp3a cluster (at the Cyp3a25 locus). Figure 72C shows an overview of a cluster exchange strategy.

Figure 73 shows a possible targeting strategy for CYP2C9 humanisation in mice (to produce mice of genotype huCYP2C9).

Figure 74 panels A-C show a possible targeting strategy for the generation of mice humanised with

respect to the CYP2C cluster or wherein the CYP2C cluster is knocked out (to produce mice of genotypes huCYP2C cluster and koCyp2C cluster). Figure 74A shows a strategy for 5' targeting of the mouse Cyp2c cluster. Figure 74B shows a strategy for 3' targeting of the mouse Cyp2c cluster. Figure 74C shows an overview of a cluster exchange strategy.

Figure 75 panels A-C show a possible targeting strategy for the generation of mice humanised with respect to the Ugt1 cluster or wherein the Ugt1 cluster is knocked out (to produce mice of genotypes huUGT cluster and koUGT cluster). Figure 75A shows a strategy for 5' targeting of the mouse UGT cluster. Figure 75B shows a strategy for 3' targeting of the mouse UGT cluster. Figure 75C shows an overview of a cluster exchange strategy.

Figure 76 shows a possible targeting strategy for MDR1 humanisation at the mouse Mdr1a locus (to produce mice of genotype huMDR1/mdr1a$^{-/-}$).

Figure 77 shows a possible targeting strategy for MDR1 humanisation at the mouse Mdr1b locus (to produce mice of genotype huMDR1/mdr1b$^{-/-}$).

Figure 78 shows a possible targeting strategy for MRP2 humanisation (to produce mice of genotype huMRP2).

Figure 79 shows a possible targeting strategy for the generation of a CYP2B6 reporter system in mice (to produce mice of genotype rCYP2B6).

Figure 80 shows a possible targeting strategy for the generation of a CYP2D6 reporter system in mice (to produce mice of genotype rCYP2D6).

Figure 81 shows a possible targeting strategy for the generation of a CYP3A4 reporter system in mice (to produce mice of genotype rCYP3A4 mice).

Figure 82 shows a possible targeting strategy for the generation of a Cyp3a11 reporter system in mice (to produce mice of genotype rCyp3a11).

Figure 83 shows a possible targeting strategy for the generation of a MDR1 reporter system in mice (to produce mice of genotype rMDR1).

Figure 84 compares induction of Cyp3a11 protein expression in wild type and huPXR mice upon administration of increasing amounts of dexamethasone.

Figure 85 shows a comparison of huPXR mice prepared using SEQ ID 1 (huPXR) and SEQ ID 3 (huPXR.1)

Figure 86 shows that the huPXR/huCAR doubly humanised mouse maintains expression the of human PXR and CAR mRNAs

Figure 87 shows a comparative phenobarbital induction study in wild type, huPXR, koPXR, huCAR, koCAR, huPXR/huCAR, koPXR/koCAR huPXR/koCAR and koPXR/huCAR mice.

Figure 88 shows a comparative dexamethasone induction study in wild type, huPXR, koPXR, huCAR, koCAR, huPXR/huCAR, koPXR/koCAR huPXR/koCAR and koPXR/huCAR mice.

Figure 89 shows the results of a comparative study of the PXR-dependence of dexamethasone-mediated hepatotoxicity (ALT levels and liver weight) in wild type, huPXR, koPXR, huCAR, koCAR, huPXR/huCAR, koPXR/koCAR huPXR/koCAR and koPXR/huCAR mice.

Figure 90 schematically summarises the relationship of metabolic pathways, nuclear receptors and non-genotoxic carcinogenesis.

Figure 91 schematically summarises the phenomenon of phenobarbital-induced carcinogenesis in the murine liver.

Figure 92 summarises the results of a study of murine *vs.* human species-specific differences in the hyperplastic response to phenobarbital, using wild type, huPXR/huCAR double-humanised and in koPXR/koCAR double-knockout mice.

Figure 93 shows the measurement of species-specific differences (pharmacokinetics) in rifampicin-mediated drug-drug interaction.

Figure 94 shows the loss of Cyp3a11 mRNA expression in the koCyp3a11 mouse, both in the absence of a xenobiotic agent and upon administration of dexamethasone.

Figure 95 shows the alteration of midazolam metabolism in male koCyp3a11 compared to wild type mice.

Figure 96 shows quantitative PCR (Q-PCR) data obtained from the huCYP3A4/huPXR mouse using CYP3A4-specific primers.

Figure 97 shows quantitative PCR (Q-PCR) data obtained from human liver samples using

CYP3A4-specific primers.

Figure 98 shows the induction by four different xenobiotic agents of Cyp3A4 protein expression in huCYP3A4/huPXR/koCyp3a11 mice.

Figure 99 shows results of the validation of the koCyp3A cluster mouse.

Figure 100 summarises the targeting strategy for the preparation of the huCYP3A cluster exchange mice.

Figure 101 schematically outlines steps in the preparation of the huCYP3A cluster exchange mice, and provides genotyping data confirming germline transmission of the Cyp3a cluster exchange ES cell clones.

Figure 102 summarises the targeting strategy for insertion of a human CYP2D6 expression cassette at the site of the mouse Cyp2d cluster.

Figure 103 shows the expression of human CYP2D6 protein as measured in the liver of a heterozygous huCYP2D6 mouse.

Figure 104 summarises the targeting strategy for deletion of the loxP-flanked mouse Cyp2c cluster.

Figure 105 summarises the targeting strategy for insertion of a human CYP2C9 expression cassette at the mouse Cyp2c cluster ("CYP2C9 in Cyp2c55 humanised" mouse)

Figure 106 shows the characterisation by RT-PCR of huMRP2 mice.

Figure 107 shows the measurement of bilirubin levels (total and direct bilirubin) in the urine of wild type *vs.* huPXR/huMRP2 mice.

Figure 108 shows the validation of the rCYP2B6 reporter mouse

Figure 109 shows the validation of the rCYP2D6 reporter mouse

Figure 110 shows the validation of the rCYP3A4 reporter mouse

Figure 111 : Method for the production of a transgenic mouse. A transgenic mouse is produced by the insertion of one or more altered embryonic stem cells into a developing blastocyst. The blastocyst is then implanted into a pseudo-pregnant mouse and allowed to develop, producing a chimera. Crossing the chimera with a deleter strain effects the excision of the mouse target gene

and produces mice which are heterozygous for the humanised gene. Crossing two of said heterozygotes produces mice homozygous for the humanisation event.

Figure 112 : Method of humanisation of CYP3A4 using the corresponding human promoter A target strategy for the generation of mice humanised for CYP3A4 wherein the mouse CYP3A cluster is deleted and the human expression cassette can be knocked out subsequently.

Figure 113 : Method of humanisation of CYP2C9 using the corresponding human promoter A target strategy for the generation of mice humanised for CYP2C9 wherein the mouse CYP2C cluster is deleted and the human expression cassette can be knocked out subsequently.

Figure 114 : Method of humanisation of CYP2D6 using the corresponding human promoter A target strategy for the generation of mice humanised for CYP2D6 wherein the CYP2D cluster is deleted and the human expression cassette can be knocked out subsequently.

Figure 115 : PCR analysis demonstrates successful generation of heterozygous CYP2D humanised mouse. Use of specific primers allows analysis of the mouse chromosome by PCR. The results demonstrate successful introduction of the desired human replacement CYP2D gene sequence and the deletion of the mouse CYP2D cluster.

Figure 116 : Method of humanisation of huCYP3A4 using the mouse Cyp3a11 promoter A target strategy for the generation of mice humanised for CYP3A4 using the Cyp3a11 promoter wherein the mouse CYP3A cluster is deleted.

Figure 117 : Strategy for the deletion of the mouse Cyp3a Cluster. (A) Schematic representation of the chromosomal organisation and orientation of functional genes within the mouse Cyp3a Cluster (adapted from Nelson et al., 2004, Pharmacogenomics 14(1): 1). Pseudogenes are not listed. (B) Exon/Intron structure of Cyp3a57 and Cyp3a59. Exons are represented as black bars and the ATGs mark the translational start sites of both genes. The positions of the targeting arms for homologous recombination are highlighted as light grey (Cyp3a57) and in dark grey (Cyp3a59) lines, respectively. (C) Vectors used for targeting of Cyp3a57 (left) and Cyp3a59 (right) by homologous recombination. LoxP , lox5171, frt and f3 sites are represented as white, striped, dotted or black triangles, respectively. (D) Genomic organisation of the Cyp3a Cluster in double targeted ES cells after homologous recombination on the same allele at the Cyp3a57 and Cyp3a59 locus. (E) Deletion of the mouse Cyp3a Cluster after Cre-mediated recombination at the loxP sites. All exons and introns from Cyp3a57, Cyp3a16, Cyp3a41, Cyp3a11 and Cyp3a25 are completely deleted and

Exons 1 to 4 and the promoter of Cyp3a59. Therefore, the only functional Cyp3a gene that remains after Cre-mediated deletion is Cyp3a13, which is separated from the rest of the Cluster by >800 kb genomic DNA and a number of functional Cyp-unrelated genes. Primers used to demonstrate successful deletion of the mouse Cyp3a Cluster are depicted as arrows. For the sake of clarity sequences are not drawn to scale. TK = Thymidine Kinase expression cassette, Hygro = Hygromycine expression cassette, ZsGreen = ZsGreen expression cassette, P = Promoter that drives the expression of Neomycin, 5'Δ Neo = ATG-deficient Neomycin.

Figure 118 depicts the methodology for the Cyp2c cluster exchange. A) 5' targeting of mouse Cyp2c cluster; B) 3' targeting of mouse Cyp2c cluster; C) Overview: Cyp2c cluster knockout.

Figure 119 depicts the methodology for the Cyp2d cluster exchange. A) '3' targeting of mouse Cyp2d26; B) 5' targeting of mouse Cyp2d cluster; C) Overview: Cyp2d knockout.

Figure 120 depicts the methodology for the CYP1A knockout. A) 5' targeting of mouse Cyp1a; B) 3' targeting of mouse Cyp1a; C) Overview: Cyp1a knockout.

Figure 121 shows PXR and CAR mRNA levels in huPXR/huCAR double-humanised mice and in wild-type, huPXR and huCAR mice. Human PXR and CAR mRNA expression is maintained in double humanised mice.

Figure 122 shows effects of rifampicin and phenobarbital treatment in double-humanised huPXR/huCAR, wild-type, huPXR and huCAR mice, and basal levels of Cyp2b10 and Cyp3all protein in huPXR/huCAR, wild-type, huPXR and huCAR mice.

Figure 123 shows a possible targeting strategy for the PPARα humanisation and knock-out in mice (to produce mice of genotype huPPARα and koPPARα).

Figure 124 shows a possible targeting strategy for AhR humanisation and knock-out in mice (to produce mice of genotype huAhR and koAhR).

Figure 125 shows the results of an experiment in which humanised, knockout and wild type mice are exposed to PB treatment. Physiological effects that are monitored include hepatomegaly, P450 induction and hepatocellular proliferation.

Figure 126 shows the liver/body weight ratios in WT, huPXR/huCAR and PXRKO/CARKO mice. The values are expressed as Mean ±SD (% mean own control ± %SD; n=3. A Student's t-test (2-sided) was performed on the results; * and ** indicate that the difference is statistically

significant from control mice at $p<0.05$ and $p<0.01$, respectively.

Figure 127 shows hepatic S-phase Labelling Indices in PB-treated mice. Osmotic pumps containing BrdU (15mg/ml/PBS) were implanted into WT, huPXR/huCAR and PXRKO/CARKO mice prior to PB treatment (80mg/kg/4days/IP). Livers sections were labelled using a Brdu antibody (Dako). All microscopic images were captured at a magnification of 40x. Data represents random sampling of 10 images per lobes (2) counting approximately 180,000 cells/animal group, as according to Pat-0013-0014. Values are expressed as Mean ± SD, n=9-10 for control mice, n=8-9 for PB-treated mice. A Student's t-test (2-sided) was performed on the results with *** indicating that the difference is statistically significant from control mice at $p<0.01$.

Figure 128 shows Hepatic apotopic indices in PB-treated mice. Livers sections were labelled using a TUNEL in situ cell detection kit (Roche). All microscopic images were captured at a magnification of 40x. Data represents random sampling of 20 images per lobes (2) counting approximately 380,000 cells/animal group. Values are expressed as Mean ± SD, n=9-10 for control mice, n=8-9 for PB-treated mice. A Student's t-test (2-sided) was performed on the results with no statistical significance found.

Figure 129 shows H&E staining on liver sections taken from control and PB-treated WT, huPXR/huCAR and PXRKO/CARKO mice. Portal vein (P) and central vein (C) are labelled. A 20x objective lens was used to capture the images.

Figure 130 shows Enzyme activity measurements a) MROD, b) EROD, c) BQ, d) PROD and e) BROD assays. Each assay was performed on individual liver microsomes. Values are expressed as Mean ± SD (n=9/10). A Student's t-test (2-sided) was performed on the results; with *, ** and *** statistically different from own control mice at $p<0.05$, $p<0.01$ and p0.00l, respectively.

Figure 131 shows the effect of PB on hepatic Cyp2bl0 and Cyp3all protein expression. Liver microsomes (0.3ug) from each animal were pooled (n=9/10) for wild type, huPXR/huCAR and PXRKO/CARKO mice and characterised for Cyp2bl0 and Cyp3all by immunoblotting using rabbit polyclonal CH4 (1:2000 dilution) and CH32 antibodies (1:2000 dilution), respectively (C. Henderson, University of Dundee, UK); +ve, control was either purified recombinant his-tagged Cyp3all membranes (0.1pmol/ µl) or purified recombinant his-tagged Cyp2bl0 membranes (0.01pmol/µ1). Blots were developed using ECL and exposed for 30secs.

Examples

Example 1: The Humanised PXR/CAR Mouse

The method provides for the humanisation of mice for PXR and CAR alone or as a double humanised form for each of the genes (see Figure 1). We have combined these different humanisations by a step-wise transformation of embryo stem cells (ES cells) rather than by conventional breeding and thus are able to generate PXR/CAR double humanised ES cells, which are usable for subsequent modifications with human DNA sequence encoding a phase-1 or 2 drug-metabolising enzyme human DNA sequences encoding a drug transporter protein.

The application of PhiC31 recombination sites enabled us to knock out both genes by crossing the humanised animal to a Phi31 deleter strain.

In contrast to existing models, we utilise a "knock-in" approach to replace the endogenous genes by their human orthologues (Figures 2 and 3). Therefore the PXR and CAR genes will be kept under their normal genomic context and expression levels and transcript distribution is advantageously physiologically regulated or controlled. As a result of this, the model of the present invention will resemble the human situation more closely than prior art attempts.

Additionally, genomic structures of human genes are at least partially conserved within the construct of the present invention. In the example of PXR, we have used a composite construct of cDNA and genomic sequences (see Figure 2). Due to the large size of more than 35kb of the human PXR gene, we kept the intron-exon structure solely between 4 and 6, since most splice variants are observed in this genomic region since it is located within the ligand-binding domain. The relatively small size of the human CAR, which comprises roughly 7kb from exon 2-9, enabled us to retain the complete genomic structure in our targeting vector (Figure 3).

The ES cells comprising humanised PXR and/or CAR can be further modified with human genes that regulate drug metabolising enzymes (phase1 and 2) and/or drug transporter proteins. It will be possible to cross the animals cells with humanised PXR/CAR with the HRN mouse below or to create a de novo non-human transgenic animal with all of the aforementioned criteria.

Example 2: The HRN™ Mouse

All P450s require reducing electrons supplied by the enzyme cytochrome P450 reductase (CPR). Deletion of CPR therefore simultaneously inactivates all P450s. While CPR deletion is lethal in the

embryo, HRN™ mice use a developmentally regulated conditional CPR deletion targeted to post-natal liver cells. HRN™ mice therefore survive to adulthood and can breed while nevertheless completely lacking hepatic P450-mediated metabolism (Henderson CJ *et al.* J Biol Chem. 278:13480-6, 2003). They therefore provide a suitable background on which to express human P450 activities in order to achieve P450 humanisation.

Transgenic Mouse Production

An adenoviral vector may be used to introduce the human P450/CPR combination to HRN™ cells. Alternatively, germ line transgenic animals incorporating the same transgenes can be produced. This is achieved by first generating transgenic mice incorporating the selected CYP3A4/CPR humanisation transgenes and then crossbreeding these with HRN™ mice to produce CYP3A4-humanised animals. Production of CYP3A4/CPR transgenic mice is achieved by using targeted transfection of embryonic stem cells and subsequent blastocyst injection. Crossbreeding of CYP3A4/CPR transgenics with HRN™ to produce P450-humanised animals may be used for the production of multi-P450-humanised mice. Alternatively, embryonic stem cells may be produced where the CPR gene is flanked by *lox*P sites and where expression sequences for targeted human P450(s) and CPR or for human P450-CPR fusion protein(s) have been introduced. Animals derived from such embryonic stem cells may then be crossbred with various animal strains in which cre recombinase is expressed under the control of different promoters to produce offspring P450-humanised in different tissues or under different induction conditions, depending on the tissue specificity or inducibility of the promoter controlling cre recombinase expression.

Humanisation Strategies

In order to establish the optimal method of expressing functional human P450 activities in HRN™ cells, experimental transgenes encoding cytosolic fusion proteins, targeted fusion proteins, targeted CPR with separate cytosolic P450s are compared and evaluated. In each case, the ability of the P450 to interact with the CPR component is determined by expressing these alternatives in appropriate cell culture systems and then testing them *in vivo* by adenovirus transfection of HRN™ mice.

Example 3: Humanization projects

Type 1: Expression of human cDNA from the corresponding mouse promoter

Projects: MDR1-humanization

Methods: The targeting vectors are constructed with standard molecular cloning procedures. These vectors are designed in such a way, that the cDNA of human MDR1 is fused to the translational start site of the corresponding mouse genes (*Mdr1a* and *Mdr1b*). The Mdr1a targeting vector carries an FRT-flanked hygromycin resistance cassette, the Mdr1b targeting vector an F3-flanked neomycin resistance cassette. In both cases the transcripts are terminated by a polyA motif. In case of *Mdr1a* the targeting event removes the 3'part of exon2, in case of Mdr1b the 3'part of exon2 to exon4 are deleted. See Figures 4 and 5.

In two consecutive rounds of standard electroporation in C57BL/6N mouse ES cells both mouse genes are modified due to homologous recombination. Clones from each round of transfection are selected with G418 and hygromycin, respectively, and positive clones are identified by Southern blot analysis. Selected clones are expanded, injected into *BALBc*-blastocysts and transferred into foster mothers according to standard operation procedures. Litters from these fosters are visually inspected and chimerism is determined by hair colour. Highly chimeric animals are used for further breeding in a C57BL/6N genetic background. Selection markers are removed *in vivo* by crossing to an FLP-deleter strain.

Type 2: Expression of a fusion of mouse gene and human cDNA from the corresponding mouse promoter

Projects: MRP2-humanization

Methods: The targeting vector is constructed with standard molecular cloning procedures. The vector is designed in such a way, that the "Leader sequence" of the mouse protein, which is encoded by exon1, will be retained. The human cDNA without sequences from exon1 is introduced into exon2. The original splice sites for mouse intron1 will be retained, so that this construct potentially encodes a fusion protein of amino acids from mouse exon1 and human exon2-32. The transcript is terminated by a polyA motif. The targeting vector carries an FRT-flanked neomycin resistance cassette (see Figure 6).

The targeting vector is transfected by standard electroporation into C57BL/6N mouse ES cells. Clones are selected with G418 and positive clones are identified by Southern blot analysis. Selected clones are expanded, injected into *BALBc*-blastocysts and transferred into foster mothers according to standard operation procedures. Litters from these fosters are visually inspected and

chimerism is determined by hair colour. Highly chimeric animals are used for further breeding in a C57BL/6N genetic background. Selection markers are removed *in vivo* by crossing to an FLP-deleter strain.

Type 3: Expression of a hybrid of human cDNA and genomic sequences from the corresponding mouse promoter

Projects: PXR humanization

Methods: The targeting vector is constructed with standard molecular cloning procedures. The vector is designed in such a way, that a hybrid of human PXR cDNA and genomic sequences is fused to the translational start site of the mouse PXR gene, whereby the mouse Start-ATG is retained. The human PXR sequence contains the cDNA of exon1-4, genomic sequences of intron4, exon5 and intron5 and cDNA of exon6-9. This human PXR sequence is provided herein as SEQ ID NO:1. The transcript is terminated by a polyA motif. The targeting vector carries an FRT-flanked hygromycin resistance cassette and a splice acceptor polyA motif 3' to the selection cassette. Furthermore, att sites have been inserted into mouse intron1 and 3' to the splice acceptor polyA motif, which allow the generation of a PXR knock out by removal of the intermediate sequences with the site-specific Phi-C31 recombinase (see Figure 7).

The targeting vector is transfected by standard electroporation into C57BL/6N mouse ES cells. Clones are selected with hygromycin and positive clones are identified by Southerm blot analysis. Selected clones are expanded, injected into *BALBc*-blastocysts and transferred into foster mothers according to standard operation procedures. Litters from these fosters are visually inspected and chimerism is determined by hair colour. Highly chimeric animals are used for further breeding in a C57BL/6N genetic background. Selection markers are removed *in vivo* by crossing to an FLP-deleter strain.

Confirmation that the murine PXR gene has been exchanged for the human counterpart

Humanised mice for PXR have been generated using the above strategy and are phenotypically normal following visual inspection. They have been typed using PCR (see Figure 15). The mice live to at least 3 months age. Examples include male "30643": DOB 12.12.2004. Remained alive at 19.04.05. Female "30792": DOB 19.12.04. Remained alive at 19.04.05. These mice can be successfully bred:

- 30643 x 30792 set up at 01.02.2004
- Litter born 22.02.2005 – 6 pups – weaned 30.03.2005
- Litter born at 06.04.2005 – 7 pups – to be weaned

Further typing has been performed using Taqman® analysis (see Figure 16). This analysis clearly demonstrated the presence of a murine PXR transcript only in the wild type animals and the presence of the human transcript only in the transgenic mouse lines demonstrated that gene exchange had taken place and that the gene construct was being actively transcribed in the correct tissues.

RT-PCR was then performed to confirm the presence of a full-length human PXR transcript in the humanised mice (see Figure 17). This analysis demonstrated the presence of two transcripts of size 1.3 and 1.1 kilo-bases, indicating that the entire humanised PXR gene had been transcribed. The size of the fragments obtained indicated that the correct, as well as alternative spliced variants were present.

Sequence analysis of the full length transcript confirmed that an open reading frame for the entire PXR mRNA had been produced. The presence of PXR was subsequently confirmed by Western blot analysis using an antibody raised against the murine PXR.

Demonstration that the human PXR was functional

In order to demonstrate that the human PXR protein was functional, mice were challenged with PXR-activating compounds including pregnenolone-16α carbonitrile (PCN), dexamethasone, TCPOBOP and rifampicin (Rif). While rifampicin is reported to be a more potent PXR inducer in humans, dexamethasone and PCN are reported to be more potent inducers in mice. Thus, these inducers enable the PXR phenotype of the non-human animals, tissues and cells of the invention to be discriminated.

Dosing solutions were prepared on the day of administration by adding corn oil to the requisite quantity of test substance and stirring to obtain a solution or fine suspension. The concentration of PCN was 10mg/ml and Rif 2.5mg/ml of supplied chemical, without any correction for purity. Records of preparation were retained.

Control animals were administered vehicle (corn oil) daily by intraperitoneal injection. The volume of vehicle and solutions of inducing agents administered was 10ml/kg

bodyweight. This route of administration was chosen for consistency with previously published work.

Animals received 4 daily doses and were killed approximately 24 hours after the last dose.

Quantification of Cyp3a11, Cyp2b10 and PXR protein in liver microsomes of all mice samples was carried out by SDS-PAGE and Western blotting. To detect murine and human PXR protein, an antibody (RF8) was employed. For the identification of Cyp3a and Cyp2b, anti-cyp2b antibodies were used, (CH32 and CH4, respectively). Results are shown in Figure 18.

These data demonstrated a protein of the correct molecular weight identified in both the wild type mice and the humanised PXR mice. The molecular weights of the two proteins were shown to be the same, which is consistent with the calculated molecular weight from the predicted amino acid sequence. In addition, it was interesting to note that the level of expression of PXR between the murine and humanised animals was very similar, consistent with the fact that expression is driven off the same promoter.

Analysis for the PXR inducible proteins in the cytochrome P450 Cyp3a gene family demonstrated a marked induction in the humanised PXR mouse (see Figure 19). Interestingly, the cytochrome P450 Cyp2b10 was not markedly induced by this treatment, demonstrating that the induction was via the PXR protein and not by the alternative transcription factor, constitutive androstane receptor (CAR). In this experiment, Cyp3a11 was also induced by rifampicin in wild type animals. Although rifampicin is reported to exhibit species differences in its ability to interact with PXR, the fact that no differences were observed here could be accounted for by the high dose of compound used.

Enzyme activity assays

Using enzyme activity assays on liver microsomes, we were able to confirm the Western blot results. The activity in pentoxyresorufin-O-deethylation (PROD) is attributed to expression level of Cyp 2b10 and the activity of 7-benzyloxyquinoline (BQ) is attributed to expression level of Cyp 3a. Enzyme activity data for Cyp3a and Cyp2b induction by rifampicin are shown in Figure 20.

Induction study in huPXR mice using low doses of rifampicin and TCPOBOP

In order to demonstrate strain differences between wild type and the huPXR mouse, mice were challenged with low doses of rifampicin or TCPOBOP. These compounds were chosen because

rifampicin has been reported to be a more efficient inducer of human PXR than murine transcription factor, and TCPOBOP is reported to be a more efficient inducer of cytochrome P450 gene expression in the mouse than in human systems.

The study consisted of 3 animals per group. Control groups consisted of mice treated with either corn oil daily for 4 days or mice administered a single injection of corn oil. The treated mice were dosed with inducing agents as detailed in Table 3.1.

Table 3.1: Experimental design for the induction study in huPXR mice using low doses of rifampicin and TCPOBOP

Mouse Strain	Inducing agent	Dose (mg/kg)	Vehicle	Regimen
C57BL/6J	Control I	-	Corn oil	4 x ip daily
huPXR	Control I	-	Corn oil	4 x ip daily
C57BL/6J	RIF	3	Corn oil	4 x ip daily
C57BL/6J	RIF	10	Corn oil	4 x ip daily
huPXR	RIF	3	Corn oil	4 x ip daily
huPXR	RIF	10	Corn oil	4 x ip daily
C57BL/6J	Control II	-	Corn oil	Single ip

huPXR	Control II	-	Corn oil	Single ip
C57BL/6 J	TCPOBOP	0.3	Corn oil	Single ip
C57BL/6 J	TCPOBOP	1	Corn oil	Single ip
huPXR	TCPOBOP	0.3	Corn oil	Single ip
huPXR	TCPOBOP	1	Corn oil	Single ip

PXR activity was determined by measuring the metabolism of a number of cytochrome P450 substrates which are metabolised to different extents by different cytochrome P450 enzymes. The following assays were performed:

- 7-benzyloxyquinoline (BQ) activity was determined in liver microsomes.
- Testosterone hydroxylase activity was determined in liver microsomes (LMS HPLC-006).
- The specific activities of these reactions was expressed per mg protein as determined using the Lowry assay (LMS Spec-0001).

In the first experiment, 7-benzyloxyquinoline was taken as substrate. In the rifampicin treated animals, in the first instance, there was clearly a difference between the background CYP3A enzyme activity between wild type and humanised PXR mice (Figure 21). In addition, although this enzyme activity was not inducible by rifampicin, there was a marked difference in enzyme activity between the wild type and humanised PXR animals at a dose of 3 mg/kg.

In subsequent experiments, testosterone hydroxylation was measured in liver microsome fractions from wild type and huPXR mice. It was interesting to note that at a dose of 10 mg/kg there was no induction of 6-beta- hydroxylation of testosterone in wild type animals whereas there was a significant induction in the human PXR animals, demonstrating an altered sensitivity of these mice

to rifampicin relative to the wild type (Figure 22).

There was a similar finding for the measurement of the 16-beta-hydroxylation of testosterone which, similar to the 7-benzyloxyquinoline demethylation, exhibited higher activity in controls (Figure 23). Again, this activity was induced in the huPXR animals but not in the wild type. Indeed, the activity was approximately 3-fold in the huPXR mice.

There was a similar finding in the 2-alpha-hydroxylation of testosterone. In addition, the induction of Cyp3a11 by rifampicin demonstrates that the huPXR is functionally active in the huPXR mice.

In experiments using the inducing agent TCPOBOP the hepatic microsomal metabolism of testosterone was also measured. Again clear differences between the wild type and the huPXR animals were observed. In particular, the 7-alpha-hydroxylation of testosterone was constitutively higher in the huPXR animals relative to the wild types (Figure 24).

The 6-beta-hydroxylation of testosterone was inducible in both strains of mouse. Although there were no significant differences between the wild type and huPXR mice, this does demonstrate again that the human PXR is active (Figure 25).

Consistent with the strain differences in wild type and human PXR, there were marked differences in the sensitivity of the mouse lines to induction by TCPOBOP. In the case of testosterone 16-alpha-hydroxylation, this activity was significantly induced in wild type animals but not in huPXR animals, and of particular interest was the observation that the induction of testosterone 16-beta-hydroxylation was much more marked in wild type than in huPXR animals (see Figures 26 and 27 respectively). Indeed, at a dose of 1mg/kg, induction of testosterone 16-beta-hydroxylation was approximately 6-fold in wild type animals but only 1.7-fold in the huPXR animals. This again demonstrates a reduced sensitivity of the humanised mice relative to controls.

Further investigation of Rifampicin induction profiles in huPXR mice

The response of huPXR mice to administration of rifampicin (Rif) was further compared to wild type mice in an induction study. Increasing amounts of rifampicin (0, 3, 10, 20, 40, 60 mg/kg mouse body weight) were administered to wild-type and huPXR mice (Figure 29). For each dosage, observations were made in triplicate, *i.e.*, each different dose was administered to three animals.

Expression of Cyp3a11 was quantified by SDS-PAGE followed by Western blotting and

densitometric quantification (Figure 29). A clear difference was observed between huPXR and wild-type mice. Induction of the expression of the Cyp3a11 protein clearly began to increase at lower rifampicin doses in huPXR mice than in the wild-type mice.

This result was consistent with measurements of BQ activity (an assay for Cyp3a11) in the rifampicin-treated huPXR and wild-type mice: in the BQ assay the amount of 7-HQ product formed in huPXR mice increased more rapidly with the increasing rifampicin dose than in wild-type mice (Figure 29).

These observations with rifampicin are consistent with successful humanisation of Cyp3a11 induction in the huPXR mice.

Investigation of dexamethasone induction profiles in huPXR mice

The response of huPXR mice to administration of dexamethasone was evaluated in comparison to wild-type mice in an induction study. Increasing amounts of dexamethasone (0, 1, 3, 10 mg/kg mouse body weight) were administered to wild-type and huPXR mice (Figure 30).

Expression of Cyp3a11 was measured by SDS-PAGE followed by Western blotting (Figure 30). A clear difference was observed between huPXR and wild-type mice. Cyp3a11 expression was induced by increasing amounts of dexamethasone only in the wild-type mouse (*i.e.* not in the huPXR mouse).

This result is consistent with measurements of BQ activity (an assay for Cyp3a11) in the dexamethasone-treated huPXR and wild-type mice. In the BQ assay the amount of 7-HQ product formed in huPXR mice increased with the dexamethasone dose only in wild-type mice, not in huPXR mice (Figure 30).

Together with the induction profiles obtained with rifampicin (see above), these results further confirm that PXR humanisation was achieved in the huPXR mice.

This is the first report of a mouse model where the human PXR gene has been exchanged for the endogenous murine protein. The advantages of such a model relative to other PXR humanised mice lie in the fact that:-

- The construct used allows the studies to be undertaken to understand the role of alternative splicing of PXR in controlling its level of expression.

- Use of the endogenous mouse promoter PXR is expressed in all tissues in a similar fashion to those of the endogenous gene.

- The level of PXR expression seems to be very similar to the endogenous gene, therefore problems with potential over/under expression are reduced.

- The currently available humanised PXR models use the albumin promoter to drive human PXR which has been crossed into a PXR null background. Therefore, PXR is expressed at very high levels in this model and there is no PXR in any tissue other than the liver. This severely compromises the use of this model to understand the role of hPXR in controlling gene expression in the GI tract or at the blood brain barrier or, indeed, in any other tissue.

Humanised PXR mice have a number of utilities. For example, such mice can be used to understand the role of human PXR in the control of gene expression in any tissue in the mouse. They can also be used to evaluate whether drugs in development or environmental chemicals have the capacity to modulate human PXR functions in a manner which may be beneficial or deleterious. The model also allows studies into understanding the role of human PXR in mediating the potential toxic effects of drugs or chemicals which may result from perturbations in, for example, bile acid homeostasis and therefore their relevance to man. The model can also be used to evaluate chemicals that may be either antagonistic or agonistic to this signalling pathway.

The koPXR mouse

TaqMan® analysis of PXR mRNA in wild-type and koPXR mice, both in the absence of any PXR-inducing drug and following administration of 10mg/kg dexamethasone, confirmed that expression of PXR mRNA was completely lost in the koPXR mouse (Figure 31).

Further validation of the huPXR and koPXR lines

The inventors have performed numerous further experiments to confirm the functional validation of the huPXR and koPXR mice.

Microarray analysis of gene expression in the huPXR and koPXR lines

The effect of PXR knockout and humanisation on the expression of genes in toxicologically relevant pathways was evaluated by microarray analysis. The majority of genes up-regulated in the liver of koPXR mice were found to be genes that are involved in toxicologically relevant pathways.

For most genes up-regulated in the koPXR mice, the up-regulation was reversed in huPXR mice.

The microarray data is summarised in the following table, wherein an empty white cell indicates that gene expression was unchanged; a grey cell indicates that gene expression was altered less than 1.5-fold; a negative number indicates down-regulated expression; and a positive number indicates up-regulated expression:

Microarry analysis of gene expression in liver: change in gene expression levels in huPXR (= hPXR) and koPXR (= PXRKO) with respect to wild type mouse.

GenBank	Name	CXR hPXR Fold Change	CXR PXRKO Fold Change
Xenobiotic metabolism			
NM_025797	Cyb5		1.7
NM_028089	Cyp2C55		6.5
NM_206537	Cyp2C54		4.0
NM_019823	Cyp2C22		-1.7
NM_010007	Cyp2J5		1.6
NM_007818	Cyp3a11		6.7
X71478	Cyp4a10		1.8
NM_010936	Nr1i2		-30.0
NM_201645	Ugt1a1		3.8
Drug transport			
NM_013454	Abca1		2.0
NM_011920	Abcg2		1.7
Fatty acid/lipid metabolism			
NM_007381	Acadl		1.8
NM_007382	Acadm		2.3
NM_025826	Acadsb		3.7
NM_015729	Acox1		1.7
BC006692	Acsl1		2.1
NM_023737	Ehhadh		2.2
NM_011978	SLC27A2		3.6
Cholesterol biosynthesis			
NM_020010	Cyp51		2.2
NM_010191	Fdft1		1.8
NM_134469	Fdps		2.0
M62766	Hmgcr		1.6
NM_177960	Idi1		
Immune response			
NM_007987	Fas		1.5
NM_010185	Fcer1g		-2.0
V00821	Ighm		-1.9
NM_010798	Mif		-1.7
NM_009246	Serpina1		2.8
Lipid/bile acid/carboxylic acid transport			
NM_007469	Apoc1		2.5
NM_009696	Apoe		3.6
NM_022026	Aqp9		2.8
NM_011387	Slc10a1		2.6
NM_144856	Slc22a7		
NM_011977	Slc27a1		1.5
NM_013797	SlcO1a1		2.2
Nuclear hormone receptors/coactivators			
NM178060	Thra		2.4
NM013464	Ahr		2.3
AK035641	Arnt		1.5

Effects of rifampicin in huPXR, koPXR and wild-type lines

Upon administration of 60 mg rifampicin per kg body weight to wild-type, huPXR or koPXR mice, expression of Cyp3a11 was increased in both wild-type and huPXR, but not in koPXR mice. Experiments were conducted in triplicate. Cyp3a11 expression levels were measured by SDS-PAGE followed by Western blotting. Both pooled and individualized results of the Western blotting experiments are shown in Figure 32.

These observations were consistent with the results of the BQ dealkylation assay, which was carried out following rifampicin treatment in parallel to the Western blotting experiments and showed corresponding marked differences between the wild-type, huPXR and koPXR mice (Figure 32). In the BQ assay, rifampicin administration led to only minimal 7-hydroxyquinoline formation in the koPXR (PXR KO) strain, and intermediate 7-hydroxyquinoline formation in the huPXR. BQ dealkylation was induced more strongly in the wild-type than in huPXR or koPXR. These results illustrate how differences in Cyp3a11 induction profiles between wild-type, huPXR and koPXR mice are more readily identifiable at lower rifampicin doses (see Figure 30).

In contrast to the above observations relating to Cyp3a11, treatment with rifampicin did not induce expression or activity of Cyp2b10 in either wild-type or huPXR mice (Figure 33).

Effects of dexamethasone in huPXR, koPXR and wild-type lines

Upon administration of 60 mg dexamethasone (Dex) per kg body weight to wild-type, huPXR or koPXR mice, expression of Cyp3a11 was substantially increased in wild-type mice, somewhat less increased in huPXR mice, and only marginally increased in koPXR mice. Experiments were conducted in triplicate. Cyp3a11 expression levels were measured by SDS-PAGE followed by Western blotting. Both pooled and individualized results of the Western blotting experiments are shown in Figure 34.

In further experiments, increasing amounts of dexamethasone (0, 1, 3, 10 and 30 mg/kg body weight) were administered to wild type and huPXR mice. The results are shown in Figure 84. Induction of Cyp3a11 was clearly significantly weaker in the PXR humanised mouse.

These observations were consistent with the results of the BQ dealkylation assay, which was carried out following dexamethasone treatment in parallel to the Western blotting experiments and showed corresponding marked differences between the wild-type, huPXR and PXR mice (Figure 34). In the BQ assay, upon dexamethasone administration, no 7-hydroxyquinoline formation was

observed in the koPXR (PXR KO) strain, and intermediate 7-hydroxyquinoline formation was observed in the huPXR strain. Dexamethasone most strongly induced BQ dealkylation in the wild-type. These results were confirmed in an independent repeat experiment (Figure 35).

Western blotting revealed that Cyp2b10 is induced following dexamethasone administration (60 mg/kg) in wild-type, huPXR and koPXR mice (Figure 34, showing both pooled and individualised results), and corresponding results were obtained in the PROD activity assay (Figure 34).

Hepatotoxicity assessment in huPXR, koPXR and wild-type mice

Following administration of either rifampicin or dexamethasone, evaluation of indicators of hepatotoxicity revealed species-specific differences. Following administration of dexamethasone, alanine aminotransferase (ALT) levels in mouse plasma were increased about 22-fold in huPXR mice and about 40-fold in koPXR mice, whereas no increase was observed in wild-type mice (Figure 36). It is possible that these results arise from hepatotoxic effects caused by the absence of murine PXR-mediated metabolism of dexamethasone in koPXR and huPXR. One dexamethasone-treated huPXR mouse died prior to termination of the experiment.

In contrast, rifampicin treatment led to no increase in ALT levels (Figure 36).

In Figure 36, the results of the BQ demethylation activity assay obtained at the same dosage of dexamethasone are shown for comparison (black bars: untreated control; white bars: 60 mg/kg dexamethasone).

Liver/body weight ratios of wild-type, huPXR and koPXR mice were measured following administration of either rifampicin or dexamethasone. In each case, the ratio was greatest for the koPXR strain (Figure 37).

Figure 38 provides a summary of the effects of rifampicin and dexamethasone (each at 60 mg/kg) on Cyp3a11 and Cyp2b10 expression, in wild-type, huPXR and koPXR mice.

Type 4: Expression of a human genomic sequence from the corresponding mouse promoter

Projects: CAR humanization

Methods: The targeting vector is constructed with standard molecular cloning procedures. The vector is designed in such a way, that the genomic human CAR sequence is fused to the translational start site of the mouse CAR gene. The human CAR sequence contains all genomic

sequences of exons 1-9, except the 5' and 3'UTRs, which are retained from the mouse genome. This human CAR sequence is provided herein as SEQ ID NO:4. All other parts of the coding sequences of the mouse CAR gene will be deleted. The transcript is terminated by a polyA motif. The targeting vector carries an FRT-flanked neomycin resistance cassette. Furthermore, att sites have been inserted into human intron2 and 3' to the selection marker, which allow the generation of a CAR knock out by removal of the intermediate sequences with the site-specific Phi-C31 recombinase (see Figure 8).

The targeting vector is transfected by standard electroporation into PXR humanized C57BL/6N mouse ES cells. Clones are selected with hygromycin and positive clones are identified by Southern blot analysis. Selected clones are expanded, injected into *BALBc*-blastocysts and transferred into foster mothers according to standard operating procedures. Litters from these fosters are visually inspected and chimerism is determined by hair colour. Highly chimeric animals are used for further breeding in a C57BL/6N genetic background. Selection markers are removed *in vivo* by crossing to an FLP-deleter strain.

The targeting vector is transfected by standard electroporation into C57BL/6N mouse ES cells. Clones are selected with hygromycin and positive clones are identified by Southern blot analysis. Selected clones are expanded, injected into *BALBc*-blastocysts and transferred into foster mothers according to standard operation procedures. Litters from these fosters are visually inspected and chimerism is determined by hair colour. Highly chimeric animals are used for further breeding in a C57BL/6N genetic background. Selection markers are removed *in vivo* by crossing to an FLP-deleter strain.

The koCAR mouse

As mentioned above, the insertion of att sites into mouse intron1 (attB53) and 3' to the splice acceptor polyA motif (attP50), allows the generation of a CAR knock-out mouse, referred to herein interchangeably as koCAR or CAR KO, *etc.* To generate this knock-out, sequences between the att sites were removed using the site-specific Phi-C31 recombinase (see Figure 66).

TaqMan® analysis of CAR mRNA in the livers and intestines of untreated wild-type and koCAR mice confirmed that basal expression of CAR mRNA was completely lost in the koCAR mouse (Figure 39).

Functional validation of koPXR and koCAR mice

Liver/body weight ratios of wild type, koCAR and koPXR mice are shown in Figure 40, for untreated animals, and animals treated with dexamethasone or phenobarbital. For the same animals, plasma levels of alanine aminotransferase (ALT), alkaline phosphatase (ALP) and aspartate aminotransferase (AST) were also measured (see Figure 40). No hepatotoxicity was detected in the wild-type, koPXR or koCAR mice at the doses of dexamethasone and phenobarbital tested. Both compounds were administered at 40 mg/kg.

Cyp3a11 and Cyp2b10 expression in the liver of wild-type, koCAR and koPXR mice was analysed by Western blotting for untreated animals and animals treated with dexamethasone (40 mg/kg) or phenobarbital (40 mg/kg). Pentoxyresurfin-*O*-deethylation (PROD) activity, which is reflective of Cyp2b10 expression and CAR activity, was analysed in the same animals. The results of these analyses are shown in Figure 41. In koCAR mice, phenobarbital-induced expression of both Cyp3a11 and Cyp2b10 was significantly reduced. However, expression of both Cyp3a11 and Cyp2b10 was still induced by dexamethasone treatment in koCAR mice, as was PROD activity.

Cyp2b10 expression in the gut of wild-type, koCAR and koPXR mice was also analysed by Western blotting for untreated animals and animals treated with dexamethasone (40 mg/kg) or PB (40 mg/kg). These results are compared with the results of the equivalent study in liver in Figure 42. The induction of Cyp210 expression by dexamethasone observed in liver of koCAR mice was not observed in gut tissue.

Cyp2b10 expression in liver was also compared in koCAR and wild type mice after treatment with TCPOBOP (1 mg/kg). The results of densitometric quantification of Western blot bands obtained from liver samples are shown in Figure 43. In contrast to the wild type, no Cyp2b10 was induced at 1 mg/kg TCPOBOP in koCAR mice.

Further validation of the huCAR and koCAR lines

A TaqMan® analysis using mouse and human-specific probes demonstrated that only human CAR mRNA (not mouse CAR mRNA) is expressed in the livers of huCAR mice generated according to the invention (Figure 44).

PCR analysis of CAR transcripts confirmed that the full-length human mRNA and all human splice variants as reported by Auerbach *et al.* (Nucleic Acids Res. 2003 Jun 15;31(12):3194-207) are expressed in the huCAR mice. In addition to the reference splicing isoform of exons 6, 7 and 8, the three further isoforms described in Auerbach et al. were observed, *i.e.* (a) a variant comprising

a 12-base-pair insertion leading to a 5' extension of exon 7, (b) a variant comprising a 15-base-pair insertion leading to a 5' extension of exon 8, and (c) a variant wherein exon 7 is deleted (leading to an in-frame deletion of 39 amino acids).

Further TaqMan® analyses of CAR mRNA in wild-type and huCAR mice, for both liver and small intestine, are shown in Figure 45. Only human CAR mRNA (not also mouse CAR transcripts) were observed in huCAR mice.

Liver/body weight ratios of wild type and huCAR mice are shown in Figure 46 for untreated animals, and animals treated with 1, 10 or 50 mg/kg of the human CAR activator CITCO (6-(4-chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl)oxime; Maglich et al., J Biol Chem. 2003 May 9;278(19):17277-83). Plasma levels of alanine aminotransferase (ALT), alkaline phosphatase (ALP) and aspartate aminotransferase (AST) were also measured (see Figure 46). No hepatotoxicity was detected in either the wild-type or huCAR mice at the CITCO doses tested (1 mg/kg, 10 mg/kg and 50 mg/kg).Cyp3a11 and Cyp2b10 expression in the liver of wild-type and huCAR mice was analysed by Western blotting for untreated animals and animals treated with increasing amounts of the human CAR activator CITCO. Varying doses of CITCO were administered in a single injection (0, 1, 10, 50 mg CITCO per kg mouse body weight) and animals were sacrificed after 48 hours.

The results of this CITCO study are shown in Figure 47. The response of huCAR mice to treatment with CITCO clearly differed from that of wild-type mice. While CITCO did not induce expression of either Cyp2b10 or Cyp3a11 in wild type mice, expression of both proteins was clearly induced by CITCO in huCAR mice (see the bands at 10 and 50 mg CITCO per kg mouse body weight in Figure 47).

As in the validation of the huPXR and koPXR mice described above, induction analyses by Western blotting were supplemented with enzyme activity assays. While PXR induces expression of Cyp3a11, CAR induces the expression of both Cyp3a11 and Cyp2b10. Cyp3a11 and Cyp2b10 expression may each be specifically assessed by means of the 7-benzylquinoline (BQ) assay and the pentoxyresorufin-*O*-deethylation (PROD) assay, respectively. BQ (Cyp3a11) activity in the absence of PROD (Cyp2b10) activity therefore indicates PXR activity, whereas any PROD (Cyp2b10) activity indicates CAR activity (see Figure 48).

The above visualisation by Western blotting of the Cyp2b10 induction profile under the influence of CITCO is consistent with the results obtained in the PROD activity assay, which is reflective of

Cyp2b10 expression and thus CAR activity. In the huCAR mouse, but not in the wild-type, the administration of increasing amounts of the human-specific inducing agent CITCO led to a marked, dose-dependent induction of PROD activity (Figure 47).

Expression of Cyp2b10 was shown by Western blotting and the PROD activity assay to be induced by 1 mg/kg TCPOBOP in wild type mice, but not in huCAR mice (Figure 49; experiments conducted in triplicate). This is consistent with the observation that TCPOBOP induces cytochrome P450 metabolism more strongly in mice than in humans.

The induction profile of Cyp2b10 in wild-type and huCAR mice was analysed further, both in response to treatment with human-specific CITCO and in response to mouse-specific TCPOBOP. The results of Western blotting and the PROD assay obtained from liver microsome samples with increasing amounts of either CITCO or TCPOBOP are compared in Figure 50. While TCPOBOP induced Cyp2b10 more strongly in the wild-type than huCAR mice, the situation was reversed for the human-specific inducer CITCO, which led to a stronger response in huCAR than in the wild-type. These data confirmed that a humanized response to CAR induction was obtained in the huCAR mouse.

In contrast, phenobarbital (at 40 mg/kg), which has been reported to induce cytochrome P450 metabolism to a comparable extent in mouse and in human, induced Cyp2b10 expression both in the wild-type and in huCAR mice. This is demonstrated by the results of both Western blotting analysis and the PROD activity assay in liver (Figure 51).

Dexamethasone, which has been reported to induce cytochrome P450 metabolism more strongly in mice than in humans, induced Cyp2b10 expression in both wild-type and huCAR mice when administered at 10 mg/kg. However, Western blots indicate that induction is less pronounced in huCAR than in wild-type mice (Figure 52), and the PROD activity assay confirmed that Cyp2b10 induction by dexamethasone was weaker in huCAR than in wild type mice. This result further confirms successful humanisation of the CAR-mediated response to cytochrome P450 inducers in huCAR mice.

Cyp2b10 induction by either CITCO or TCPOBOP in wild-type, huCAR, koCAR, huPXR (CITCO only) and koPXR mice is compared in Figure 53. Both Western blots and the PROD activity test (resurofin formation) revealed strong induction of Cyp2b10 by human-specific CITCO (10 mg/kg) in the humanised huCAR mice, consistent with the conclusion that humanisation has been achieved in these mice (Figure 53, left). In comparison, Cyp2b10 was not substantially induced by

CITCOO in wild-type mice. Some induction was also observed in koPXR mice, which is consistent with the dependence of Cyp2b10 on CAR rather than PXR.

As expected, TCPOBOP (1 mg/kg) strongly induced Cyp2b10 protein expression and PROD activity in wild-type mice (Figure 53, right). In koPXR mice, TCPOBOP induced Cyp2b10 protein expression and PROD activity to an extent that was comparable to wild type, confirming that Cyp2b10 induction depends on CAR rather than PXR. However, TCPOBOP did not significantly induce Cyp2b10 protein expression or PROD activity in humanized huCAR mice, consistent with the relative specificity of this agent for mouse rather than human.

Expression of Cyp3a11 was shown by Western blotting and the BQ activity assay to be somewhat induced by 1 mg/kg TCPOBOP in wild type mice, but not in huCAR mice (Figure 54; experiments conducted in triplicate). This is consistent with the observation that TCPOBOP induces cytochrome P450 metabolism more strongly in mouse than in human and further confirms successful humanization of the CAR-mediated response to cytochrome P450 inducers in huCAR mice.

An overview of the effects of selected drug-metabolism inducers on the expression of Cyp3a11 and Cyp2b10 in wild-type mouse and human is provided in Figure 55.

An overview of various inducing agents on PXR and CAR target genes in wild-type, huPXR, koPXR, huCAR and koCAR mice is provided in Figure 56.

Non-genotoxic carcinogenicity study in huCAR mice: demonstration of CAR-dependent species-specific differences in wild-type and huCAR mice

In order to assess the hyperplastic response in the huCAR mice, the fraction of BrdU positive cells in the liver was determined in wild-type and huCAR treated with CAR activators. Animals were treated either with TCPOBOP (a mouse-specific inducer) or CITCO (a human-specific inducer). Animals received a single dose of TCPOBOP (3 mg/kg) or 20mg/kg CITCO 4 times daily. The fraction of BrdU positive cells in the liver of treated mice were determined one day after administration of the single or final dose, and compared to equivalent measurements obtained from untreated animals. As is shown in Figure 57, the mouse-specific inducer TCPOBOP led to elevated counts of BrdU positive liver cells only in wild-type animals, whereas the human-specific inducer CITCO led to elevated counts of BrdU positive liver cells only in huCAR animals, not in the wild-type.

These results further confirm that the huCAR mouse line reproduces species-dependent differences in the response to CAR activators. In particular, these results show that huCAR mice are humanized with respect to their hyperplastic response to non-genotoxic carcinogens.

PCR confirmation in two double homozygous PXR/CAR humanised mice that the murine PXR gene has been exchanged for the human counterpart

Humanised mice for PXR and CAR ("huPXR/huCAR") were generated using mice which contained humanised PXR and crossing these into mice which contained humanised CAR to produce mice containing both humanised PXR and humanised CAR. The mice are phenotypically normal following visual inspection. They have been typed using PCR (see Figure 28) and are homozygously humanised for PXR and CAR. Examples include mice designated "42749" and "42752".

Transcription of PXR and CAR mRNA was quantified by RT-PCR in huPXR/huCAR mice and compared to the relative levels of corresponding mRNA expression in wild-type, huPXR and huCAR mice (Figure 58). It was thereby confirmed that the huPXR/huCAR mice maintain the levels of human PXR and human CAR expression observed in mice humanised with respect to single genes.

In Figure 86, further PCR data are shown, for liver and small intestine, to demonstrate that the huPXR/huCAR mice maintain the levels of human PXR and human CAR expression observed in mice humanised with respect to single genes.

Double-humanised huPXR/huCAR mice, as well as wild-type, huPXR and huCAR mice were treated with the inducers rifampicin and/or phenobarbital. Expression of Cyp2b10 and Cyp3a11 in these inducer-treated mice, as well as in corresponding untreated mice, was visualised and compared by SDS-PAGE followed by Western blotting (Figure 59). The basal levels of Cyp2b10 and Cyp3a11 in huPXR, huCAR and huPXR/huCAR mice are compared to the basal levels observed in wild-type mice in Figure 59. This relative quantification shows that basal Cyp2b10 levels increase in the order huPXR → huCAR → huPXR/huCAR. However, basal Cyp3a11 were less markedly increased in huCAR mice. Cyp3a11 levels were increased to an approximately equal extent (more than 2 fold) in both huPXR and double-humanised huPXR/huCAR mice.

Treatment with the human-specific inducer rifampicin led to an increase in the levels of Cyp3a11 in all mice. Whereas the administration of rifampicin and phenobarbital in combination appeared

to have no additional effect in the wild type, induction of Cyp3a11 was somewhat stronger in huPXR.

Pentobarbitone sleeping test

In this experiment, the activity of these transcription factors in combination was determined by measuring the barbiturate induced sleeping time. Sleeping time has been known for many years to be directly proportional to the hepatic cytochrome P450 activity and this activity can be at least in part ascribed to the P450 levels in the liver determined by CAR and PXR function.

Mice were given a single intraperitoneal dose of Narcoren (sodium pentobarbitone; purchased via a Veterinary Consultant; distributed by Merial GmbH, Germany) at 25mg/kg of body weight. The time taken for the mice to lose, and subsequently to regain, their righting reflex was measured. Results are given in Table 4.1 below:

Table 4.1: Results of pentobarbitone sleeping test

Genotype	Sex	Age (weeks)	ID	Weight (g)	Sleeping time (min)
wt	male	10	42912	21.2	21
PXR/CAR hum	male	10	42749	24.8	34

Whereas wild type mice given a narcotic dose of pentobarbitone slept for 21 minutes, the double humanised mice for CAR and PXR slept for 34 minutes. These mice therefore demonstrate a significant difference to their wild type controls indicating that the double humanised mouse has a marked difference in its response to drugs relative to the wild type animals.

Further comparative validation of various PXR and/or CAR humanised and/or knockout lines

Phenobarbital

The effects of phenobarbital on Cyp3a11 and Cyp2b10 induction were compared in wild type, huPXR, koPXR, huCAR, koCAR, huPXR/huCAR, koPXR/koCAR huPXR/koCAR and koPXR/huCAR mice (see Figure 87). 40 mg/kg body weight phenobarbital were administered, whereupon Cyp3a11 and Cyp2b10 protein expression, benzyloxyquinoline (BQ) activity and

PROD activity were compared to an unexposed mouse of the same genotype (control). Though phenobarbital is described as a PXR activator *in vitro*, the phenobarbital-mediated activation of Cyp3a11 and Cyp2b10 *in vivo* is predominantly CAR-dependent. Little or no induction of Cyp3a11 and Cyp2b10 protein expression or activity in the BQ and PROD assays was observed in mice in which CAR had been knocked out. On the other hand, Cyp3a11 and Cyp2b10 protein expression and activity in the BQ and PROD assays was observed in mice in which PXR had been knocked out, but not CAR.

Dexamethasone

The effects of dexamethasone on Cyp3a11 and Cyp2b10 induction were compared in wild type, huPXR, koPXR, huCAR, koCAR, huPXR/huCAR, koPXR/koCAR huPXR/koCAR and koPXR/huCAR mice (see Figure 88). 30 mg/kg body weight dexamethasone were administered, whereupon Cyp3a11 and Cyp2b10 protein expression, benzyloxyquinoline (BQ) activity and PROD activity were compared to an unexposed mouse of the same genotype (control). Induction of Cyp3a11 by dexamethasone was found to be dependent on murine PXR, was lost in the koPXR mouse and could not be compensated by human PXR. This is consistent with the observation that mice are more sensitive to dexamethasone than humans. On the other hand, Cyp2b10 induction by dexamethasone was partially PXR- and CAR-independent. Some induction by dexamethasone of Cyp2b10 protein expression, as well as PROD activity, was observed in all mice tested in this study, including the koPXR/koCAR double knockout mouse.

Hepatotoxicity

The effects of dexamethasone (30 mg / kg body weight) on plasma ALT levels and on liver weight were compared in wild type, huPXR, koPXR, huCAR, koCAR, huPXR/huCAR, koPXR/koCAR huPXR/koCAR and koPXR/huCAR mice (see Figure 89). The increase in ALT levels and liver weights was clearly more pronounced in mice lacking murine PXR (*i.e.*, huPXR, koPXR, huPXR/huCAR, koPXR/koCAR, huPXR/koCAR, koPXR/huCAR). This study further indicates that hepatotoxic effects are greater in the absence of murine PXR-mediated metabolism of dexamethasone.

Non-genotoxic carcinogenesis

Figure 90 schematically summarises the relationship of metabolic pathways, nuclear receptors and non-genotoxic carcinogenesis. Such carcinogenesis follows upon intermediate stages of

metabolite-induced hypertrophy and hyperplasia.

In mice and rats, phenobarbital causes liver cancer. Cell replication and liver weights increase within 1-2 weeks, and liver tumours are observed after 1-2 years (see Figure 91). However, this effect in not observed in humans.

The inventors therefore studied and compared effects of phenobarbital in the livers of wild type, huPXR/huCAR double-humanised and in koPXR/koCAR double-knockout mice (see Figure 92). Hepatomegaly (liver/body weight ratios), P450 induction (PROD assay / Cyp2b10 activity) and hyperplasia (hepatocellular proliferation; % BrdU-positive cells) were measured and compared to unexposed mice of the same genotype (control). Phenobarbital led to both hepatomegaly and P450 induction in both wild type and double-humanised huPXR/huCAR mice, whereas these effects of phenobarbital were not observed in koPXR/koCAR double-knockout mice. However, phenobarbital led to hepatocellular proliferation only in wild-type mice. No increase in BrdU positive cells was observed in huPXR/huCAR double-humanised or in koPXR/koCAR mice double-knockout.

The hyperplastic response of huPXR/huCAR mice to phenobarbital corresponds better to the situation in humans than the response of wild type mice. Therefore, the huPXR/huCAR double-humanised mice are considered to be of considerable value in assessing the true hazard of non-genotoxic rodent "liver growth carcinogens" to humans. The same applies to other humanised mice of the invention, in particular, *e.g.*, the huPXR/huCAR/huPPARa mice. Such humanised mice are generally also useful tools which allow the complexities of xenobiotic-induced liver growth (see also Figure 90) and species-specific differences in such growth to be unravelled.

The inventors' investigation of non-genotoxic carcinogenic effects are described in further detail below in Example 8.

Species-specific differences in drug-drug interactions

The inventors compared the effect of pre-treatment with rifampicin on the pharmacokinetics of midazolam (MDZ) in wild type and huPXR (= hPXR) mice (see Figure 93). Mice were pre-administered rifampicin at 10 mg / kg body weight per day, for 3 days prior to administration of midazolam and the recording of pharmacokinetic data. In wild type mice, no significant effect was observed. However, in the huPXR mouse, pre-treatment with rifampicin decreased the maximal midazolam concentration (Cmax) by 64 %, and the area under the concentration-time curve

($AUC_{0\text{-}180\ min}$) by 60 %. Thus, significant humanisation-dependent pharmacokinetic effects – here, drug-drug interactions – cannot be assessed in the presence of murine PXR, but become apparent only when murine PXR is replaced by the human PXR gene.

Summary of work on Types 3 and 4 above

A model has been developed where human PXR is expressed in both the liver and GI tract of mice in the predicted fashion at levels equivalent to those of the endogenous gene. The PXR protein has been shown to be functional as the mice are responsive to compounds known to induce gene expression via this pathway. Equivalent humanisation has also been achieved with respect to the CAR gene (huCAR mice). Strain differences between wild type and the humanised mice have been demonstrated and the humanised mice have been shown to be more responsive to compounds known to be more active in humans than in mice, *i.e.,* to human PXR or human CAR rather than murine PXR or murine CAR. The construction of knock-out lines has also been confirmed for both the PXR and the CAR genes (koPXR and koCAR). Moreover, mice which contained humanised PXR have also been crossed into mice which contained humanised CAR to produce mice containing both humanised PXR and humanised CAR.

Type 5: Expression of a hybrid of human cDNA and genomic sequences from the corresponding human promoter by insertion into the ROSA26 locus

Projects: CYP3A4 and CYP2C9 humanization

Methods: The targeting vectors are constructed with standard molecular cloning procedures. The basic ROSA26 targeting vector is designed in a way, that the neomycin gene will be expressed from the endogenous ROSA26 promoter in correctly targeted ES clones. The Neo transcript is terminated by a polyA motif.

In case of CYP3A4 the humanization cassette, 3' to the selection marker, contains the 13kb human CYP3A4 promoter, exon1 and intron1 as in the normal genomic constitution and a human cDNA consisting of exons2-13. The transcript is terminated by a polyA motif (see Figure 9).

In case of CYP2C9 the humanization cassette, 3' to the selection marker, contains the 12kb human CYP2C9 promoter, a human cDNA of exons1-4, intron4 and a cDNA of exons5-9. The transcript is terminated by a polyA motif (see Figure 10).

The targeting vector is transfected by standard electroporation into C57BL/6N mouse ES cells.

Clones are selected with G418 and positive clones are identified by Southern blot analysis. Selected clones are expanded, injected into *BALBc*-blastocysts and transferred into foster mothers according to standard operation procedures. Litters from these fosters are visually inspected and chimerism is determined by hair colour. Highly chimeric animals are used for further breeding in a C57BL/6N genetic background.

The huCYP3A4/huPXR mouse

Mice doubly humanised for Cyp3A4 and PXR (huCYP3A4/huPXR) were prepared, and it was confirmed that the mice expressed full-length CYP3A4 mRNA in liver. Quantitative PCR (Q-PCR) data obtained from three different sample types in wild-type and huCYP3A4/huPXR mice (male liver, and small intestine, and female liver) using CYP3A4-specific primers is shown in Figure 96.

In male liver induced with 60mg/kg rifampicin, amplification of CYP3A4 mRNA was characterised by a CT cycle number of 27, whereas corresponding uninduced samples led to CT values of 31-34. In female liver and male small intestine samples, higher uninduced mRNA levels were observed (lower CT values; CT = 25-27 and 24, respectively). Sex and organ dependent variation of induced CYP3A4 mRNA expression levels was thus observed (male liver < female liver < male intestine). In wild type mouse, amplification of CYP3A4 mRNA was consistently characterised by higher CT cycle numbers (*i.e.*, lower mRNA levels).

An equivalent quantitative PCR experiment on human liver samples (n= 20) shows that the levels of CYP3A4 expression in huCYP3A4/huPXR mice are comparable to those in human liver (compare CT cycle numbers in Figure 97 and Figure 96).

The huCYP3A4/huPXR/koCyp3a11 mouse

Figure 98 shows CYP3A4 protein expression in huCYP3A4/huPXR/koCyp3a11 mice, upon induction with four different xenobiotic agents:

Induction agent	*Dose (mg / kg body weight)*
Phenobarbital	80
Phenytoin	80

TCPOBOP	3
Dexamethasone	60

In each case, male and female animals were treated and compared. In uninduced animals, Cyp3A4 protein was barely detectable.

The huCYP2C9 mouse

Mice humanised for CYP2C9 were prepared, in which a CYP2C9 cDNA/genomic construct was knocked into the Rosa26 locus. Expression of human CYP29C is driven by the human CYP29C promoter. The validation results for these huCYP2C9 mice are equivalent to those obtained for the mice humanised with respect to CYP3A4 (see above).

Type 6: Replacement of large genomic regions (Cluster exchanges)

Projects: CYP3A-cluster exchange, CYP2C-cluster exchange, replacement of the mouse Cyp2D cluster by a human CYP2D6 expression cassette, and UGT-humanization.

Methods: The targeting vectors are constructed with standard molecular cloning procedures. The general principle is that two kinds of targeting vectors are constructed and used for two consecutive rounds of transfection by standard electroporation into C57BL/6N mouse ES cells. The first vector contains a functional TK cassette and a 5' deleted Neo gene interrupted from its tranlational Start-ATG and promoter by a wt-loxP site. The second vector carries functional TK and Hygromycin cassettes, a wt-loxP and a lox511 site. The final targeting vectors for each of the cluster exchanges are designed in such a way, that in correctly targeted ES clones the genomic sequences intermediate to the wt-loxP sites can be removed by Cre-mediated deletion. This results in a knock out of the loxP-flanked gene cluster. Modified Bacterial Artificial Chromosomes (BACs) will be used to introduce human sequences by Cre-mediated insertion into re-derived ES cells. The selection cassettes flanking the humanized clusters can be removed by FLP mediated deletion (see Figure 11).

The koCyp3A cluster mouse

A koCyp3A cluster mouse, which lacks the genes Cyp3a57, Cyp3a16, Cyp3a41, Cyp3a44, Cyp3a11, Cyp3a25 and Cyp3a59. The inventors confirmed that the koCyp3A cluster mouse did not express Cyp3A11 at the protein level (Figure 99). Administration of rifampicin (60 mg / kg body weight) failed to induce any Cyp3A11 protein expression, though in the wild type

control, protein induction was clearly shown (Figure 99). Likewise, no activity in the benzyloxyquinoline (BQ) assay was observed in the koCyp3A cluster mouse, either when untreated or upon administration of rifampicin (60 mg / kg body weight), though the BQ activity was clearly induced in the wild type control (Figure 99).

The koCyp3A cluster mouse exhibited normal plasma clinical chemistry values, and normal liver, small intestine and kidney histology.

The results of a microarray analysis of the change in the expression of phase I (Cytochrome P450; Cyp450) enzymes relative to wild type, in the absence and the presence of 60 mg / kg body weight rifampicin, are shown in Figure 99.

The huCYP3A cluster mouse

A bacterial artificial chromosome (BAC) carrying an approximately 200 kb human CYP3A cluster (comprising CYP3A4, CYP3A7 and optionally CYP3A5) was used to insert said human CYP3A cluster into the loxP-flanked site of the mouse Cyp3a Cluster, according to the strategy shown in Figure 100. CYP3A gene expression in these mice is driven by human promoters. PCR genotyping with respect to the 200kb huCYP3A cluster confirmed germline transmission of the Cyp3a cluster exchange ES cell clones (see Figure 101, mice 1 and 2).

The huCYP2D6 mouse

A human CYP2D6 expression cassette was inserted at the site of the mouse Cyp2d cluster, with Cre-mediated deletion of the loxP-flanked mouse Cyp2d cluster, according to the targeting strategy shown in Figure 102. As shown in that figure, the human gene CYP2D6 may optionally be deleted by FLP-mediated recombination, by crossing with an FLP deleter strain.

Human CYP2D6 protein was shown by the inventors to be expressed in the liver of heterozygous male huCYP2D6 mice. A corresponding corresponding protein band was observed in human liver microsomes, but not in wild type mice (see Figure 103).

The koCyp2c cluster mouse

A mouse from which the endogenous Cyp2c cluster (Cyp2c55, 2c65, 2c66,2c29, 2c38, 2c39, 2c67, 2c68, 2c40, 2c69, 2c37, 2c54, 2c50 and 2c70) has been deleted has been created (heterozygous animals) following the targeting strategy shown in Figure 104.

The "CYP2C in Cyp2c55 humanised" mouse

To create these mice (currently heterozygous), a human CYP2C9 expression cassette was inserted at the mouse Cyp2c Cluster, and the loxP-flanked 1200-kb (approx.) Cyp2c mouse cluster deleted by Cre-mediated recombination, according to the targeting strategy shown in Figure 105. As shown in that Figure, the human CYP2C9 cassette may optionally be deleted by FLP-mediated recombination, by crossing with an FLP deleter strain.

Example 4: Reporter projects

Projects: CYP3A4-, CYP2C9-, CYP2C19-, CYP2B6-, CYP2D6- and MDR1-reporter

Methods: The targeting vectors are constructed with standard molecular cloning procedures. For all reporters human BACs are modified in such a way, that a reporter gene is fused to the translational start site of the corresponding human gene. Modified BACs carry an FRT-flanked Keo cassette, permitting the selection of bacterial colonies with kanamycin and mouse ES cell clones with G418. In case of CYP3A4, CYP2C9 and CYP2C19 the transcript of the reporter gene is not terminated by a polyA motif, but the constructs are designed such, that the endogenous polyA motif is potentially used. These constructs are therefore dependent on a correct splicing of the exons 3' to the reporter (see Figure 12).

In case of CYP2D6 and CYP2B6 the transcript of the reporter gene is terminated by a polyA motif linked to the reporter gene with a synthetic intron (see Figure 13).

In case of MDR1 the transcript of the reporter gene is terminated by a polyA motif without an additional intron (see Figure 14).

The modified BAC are linearized with NotI and transfected into C57BL/6N mouse ES cells either by standard electroporation or lipofection with lipofectamin2000. For the construction of complex genotypes, the linearized modified BAC are transfected into an appropriate genetic background. For example, for construction of lines combining PXR or CAR humanisation with the desired reporter gene expression, the BAC is transfected into PXR or CAR humanized C57BL/6N mouse ES cells. Clones are selected with G418 and positive clones with randomly integrated DNA are identified by PCR analysis. Selected clones are expanded, injected into *BALBc*-blastocysts and transferred into foster mothers according to standard operation procedures. Litters from these fosters are visually inspected and chimerism is determined by hair colour. Highly chimeric animals are used for further breeding in a C57BL/6N genetic background. Selection markers are removed

in vivo by crossing to an FLP-deleter strain.

Validation of reporter strains

The complex-genotype strain rCyp2B6/huCAR was generated according to the methods described herein and the targeting strategy provided herein (see Example 5 below for further details of preferred rCyp2B6 strains) and using standard methods that are well known to persons skilled in the art. Expression of the lacZ reporter gene was placed under the control of the Cyp2B6 promoter in a huCAR background. Figure 60 shows tissue samples from wild-type and rCyp2B6/huCAR reporter mice (liver microsomes). After administration of 40 mg phenobarbital per kg body weight, the lacZ reporter gene allows straightforward localisation of Cyp2B6 promoter activity.

The complex-genotype strain rCyp2D6/huPXR was generated according to the methods described above and the targeting strategy provided herein (see Example 5, below for further details of preferred rCyp2D6 strains) and using standard methods that are well known to persons skilled in the art. Expression of the ZsYellow reporter gene was thus placed under the control of the Cyp2D6 promoter in a huPXR background. Figure 61 shows tissue samples from wild-type and rCyp2D6/huPXR reporter mice (liver microsomes). The ZsYellow reporter gene allowed the clear localisation of Cyp2D6 promoter activity.

Example 5: Additional information regarding targeting strategies

A. Transcription Factors

huPXR and koPXR

Further strategies for PXR humanisation and knock-out have been identified by the inventors. As in the PXR humanisation strategy described above, the vectors are designed such that a hybrid of human PXR cDNA and genomic sequences is fused to the translational start site of the mouse PXR gene, whereby the mouse start site (ATG) in exon2 is retained.

In one strategy, the human PXR sequence contains a cDNA of exon2-4, genomic sequences of intron4, exon5, intron5, exon6, intron6, exon7 and intron7 and a cDNA of exon8-9 (see Figure 62). This human PXR sequence is provided herein as SEQ ID NO:2 (in this sequence, the human "CTG" has been deleted and the initial "ATG" of SEQ ID NO:3 corresponds to the start site for translation in the mouse). This huPXR mouse is designated huPXR.1 (see Figure 85 and below for its characterisation). The transcript is terminated by a polyA motif. The targeting vector carries an

FRT-flanked hygromycin resistance cassette. Furthermore, att sites are inserted into mouse intron1 and at the 5' end of the 3' homology arm, which allows the generation of a PXR knock-out by removal of the intermediate sequences with the site-specific Phi-C31 recombinase (see below). A representation of the complete targeting vector comprising the above features is provided in Figure 63.

This strategy enables expression of human PXR under the control of the mouse promoter, in absence of the mouse PXR expression. Assuming that splicing in mice mimics the human situation, the known human splice variants between Exon4 and Exon8 will be expressed, and aberrant variants created by unforeseen cryptic splice sites will be avoided.

The targeting vector is transfected by standard electroporation into C57BL/6N mouse ES cells. Clones are selected with hygromycin and positive clones are identified by Southern blot analysis. Selected clones are expanded, injected into *BALBc*-blastocysts and transferred into foster mothers according to standard operation procedures. Litters from these fosters are visually inspected and chimerism is determined by hair colour. Highly chimeric animals are used for further breeding in a C57BL/6N genetic background. Selection markers are removed *in vivo* by crossing to an FLP-deleter strain.

Such PXR humanisation strategies, wherein the targeting construct contains PXR intron6 and intron7 provide mouse lines that are humanised with respect to PXR, but might not fully reflect the normal splicing pattern in humans (*e.g.* if a cryptic splice site is created by fusing exon7 and 8). Additional human PXR intron and exon sequences can be included in the targeting vector to improve the splicing pattern, if desired. For example, the targeting vector may retain all of the human PXR genomic sequences downstream of exon 2 (*i.e.*, intron 2, exon 3, intron 3, exon 4, intron 4, exon 5, intron 5, exon 6, intron 6, exon 7, intron 7, exon 8 and intron 8 (see Figure 64). A human PXR sequence including these additional genomic sequences is provided herein as SEQ ID NO:4 (in this sequence, the human "CTG" has been deleted and the initial "ATG" of SEQ ID NO:4 corresponds to the start site for translation in the mouse).

The type of targeting construct shown in Figure 2 is preferred for generating PXR knock-out mice, because that targeting construct contains an additional splice acceptor polyA motif to prevent expression of undeleted mouse PXR exons.

The insertion of att sites into mouse intron1 (attB53) and 3' to the splice acceptor polyA motif (attP50), allows the generation of a PXR knock-out mouse, referred to herein interchangeably as

koPXR or PXR KO, *etc.*. To generate this knock-out, sequences between the att sites were removed using the site-specific Phi-C31 recombinase (see Figure 65).

A huPXR.1 mouse as described above in this Example, wherein the human PXR sequence contains a cDNA of exon2-4, genomic sequences of intron4, exon5, intron5, exon6, intron6, exon7 and intron7 and a cDNA of exon8-9 (see Figure 62 and SEQ ID NO:2), has been prepared and characterised (see Figure 85).

Sequence analysis demonstrated that the huPXR.1 mouse only expresses known human splicing variants of PXR. As is shown in Figure 85, compared to the huPXR mouse containing intron4 and intron5 and otherwise cDNA (the exon/intron structure of SEQ ID NO:1), rifampicin led to a yet stronger induction of Cyp3a11 and of benzyloxyquinoline (BQ) assay activity in the huPXR.1 mouse further containing introns 6 and 7 (the exon/intron structure of SEQ ID NO:2). This further increased sensitivity to rifampicin is consistent with the higher sensitivity of humans to this substance, compared to mice. The inventors take the position that the huPXR.1 mouse particularly accurately reflects the expression and activity of PXR in humans.

Moreover, higher concentrations of rifampicin induced Cyp2b10 expression and PROD activity only in the huPXR.1 mouse, but not in the wild type or in the huPXR mouse containing the exon/intron structure of SEQ ID NO:1 (see Figure 85). Humanised PXR thus acts not only on Cyp3a11, but is also able to induce the expression of Cyp2b10 at high doses of rifampicin. As CAR is also able to activate both the Cyp3a11 and the Cyp2b10 genes, this observation using the huPXR.1 sequence emphasises the importance of cross-talk between receptors, and demonstrates the value of the "panel" approach, in which the sequence and expression of multiple receptors is humanised, preferably at least PXR and CAR, more preferably also further receptors, phase-1 and/or phase-2 drug metabolising enzymes and/or drug transporters, as described above.

huCAR and koCAR

As noted elsewhere above, a preferred human CAR sequence is provided herein as SEQ ID NO:4. This sequence contains a 53 bp Phi-C31 recognition site (attB53) within intron2. A preferred targeting strategy is shown in Figure 66. A preferred targeting vector is shown in Figure 67.

huPPARα and koPPARα

A DNA sequence encoding human PPARα is inserted into the mouse PPARα locus, as shown in Figure 68, enabling expression of human PPARα under the control of the mouse PPARα promoter.

The DNA sequence encoding human PPARα comprises at least part of intron 5 and intron 6 of the human PPARα gene (Figure 68). The targeting vector(s) include sequence elements that enable Cre-mediated PPARα knock-out to produce koPPARα (Figure 68).

huAhR and koAhR

A DNA sequence encoding human AhR is inserted into the mouse AhR locus (knock-in) as shown in Figure 69, enabling expression of human AhR under the control of the mouse AhR promoter. The DNA sequence encoding human AhR comprises exons 3-11 of the human AhR gene (Figure 69). The targeting vector(s) include sequence elements that enable Cre-mediated AhR knock-out to produce koAhR (Figure 69).

B. Drug-Metabolizing Enzymes

huCYP3A4

A DNA sequence encoding human CYP3A4 is inserted into the mouse Rosa26 locus (knock-in), as shown in Figure 70, enabling expression of human CYP3A4 under the control of a human CYP3A4 promoter. The DNA sequence encoding human CYP3A4 preferably comprises intron 1 of the human CYP3A4 gene.

koCyp3a11

The ZsGreen reporter gene is inserted into the mouse Cyp3a11 locus by homologous recombination, as shown in Figure 71, eliminating expression of mouse Cyp3a11, and enabling ZsGreen under the control of the mouse Cyp3a11 promoter.

Loss of Cyp3a11 mRNA expression in koCyp3a11 mice

Figure 94 shows that Cyp3a11 mRNA is not expressed in the liver of koCyp3a11 mice, either in the absence of a xenobiotic agent or upon administration of 60 mg / kg body weight dexamethasone.

Altered midazolam metabolism in koCyp3a11 mice

The also inventors studied the pharmacokinetics of midazolam (MDZ; metabolisation to form 1-hydroxymidazolam) in male koCyp3a11 mice, in comparison to male wild type mice. As is apparent from the data plotted in Figure 95, 1-hydroxymidazolam formation was significantly

reduced in koCyp3a11 (i.e., in this case, rCyp3a11) mice.

huCYP3A cluster and koCyp3A cluster

A DNA sequence encoding the human CYP3A cluster is inserted into the loxP-flanked mouse CYP3A cluster, as shown in Figure 72, enabling expression of the human CYP3A cluster under the control of human CYP3A promoters. The targeting vector includes loxP sequence elements that enable Cre-mediated deletion of the mouse CYP3A cluster, to produce koCyp3A (see Figure 72C). Cre-mediated deletion of the mouse CYP3A cluster is followed by Cre-mediated insertion of the human CYP3A cluster, to produce huCYP3A (Figure 72C). After the insertion of the human CYP3A cluster into the mouse CYP3A cluster, selection cassettes are deleted, using FRT sites that are also present in the targeting vector (Figure 72C).

huCYP3A4 and koCyp3a cluster

A DNA sequence encoding human CYP3A4 is inserted into the mouse Cyp3a cluster at the Cyp3a25 locus, enabling expression of human CYP3A4 under the control of the 13 kb human CYP3A4 promoter. Mice in which the mouse Cyp3a cluster is deleted may also be generated.

huCYP2C9

A DNA sequence encoding human CYP2C9 is inserted into the mouse Rosa26 locus, as shown in Figure 73, enabling expression of human CYP2C9 under the control of the 12 kb human CYP2C9 promoter. The DNA sequence encoding human CYP2C9 comprises a 1,2 kb sequence of human CYP2C9 intron 4 (Figure 73).

huCYP2C cluster and koCyp2C cluster

A DNA sequence encoding the human CYP2C cluster is inserted into the mouse CYP2C cluster, as shown in Figure 74, enabling expression of the human CYP2C cluster under the control of human CYP2C promoters. The targeting vector includes loxP sequence elements that enable Cre-mediated deletion of the mouse CYP2C cluster, to produce koCyp2C (Figure 74C). Cre-mediated deletion of the mouse CYP2C cluster is followed by Cre-mediated insertion of the human CYP2C cluster, to produce huCYP2C (Figure 74C). After the insertion of the human CYP2C cluster into the mouse CYP2C cluster, selection cassettes are deleted, using FRT sites that are also present in the targeting vector (Figure 74C).

huCYP2C9 and koCyp2c cluster

A DNA sequence encoding human CYP2C9 is inserted into the mouse Cyp2c cluster, enabling expression of human CYP2C9 under the control of the 12 kb human CYP2C9 promoter. Mice in which the mouse Cyp2c cluster is deleted may also be generated.

huCYP2D6 and koCyp2D cluster

A DNA sequence encoding human CYP2D6 is inserted into the mouse Cyp2d cluster, enabling expression of human CYP2D6 under the control of the 9kb human CYP2D6 promoter. Mice in which the mouse Cyp2d cluster is deleted may also be generated.

huCYP3A4 and koCyp3a11

A DNA sequence encoding human CYP3A4 is inserted into the mouse Cyp3a cluster at the Cyp3a11 locus, enabling expression of human CYP3A4 under the control of the mouse Cyp3a11 promoter. Mice in which the mouse Cyp3a cluster is deleted may also be generated

huCYP1A1/CYP1A2 and koCyp1A1/CYP1A2

DNA sequences encoding human CYP1A1 and human CYP1A2 are inserted into the mouse Cyp1a cluster, enabling expression of human CYP1A1 and human CYP1A2 under the control of the human CYP1A1 and CYP1A2 promoters. Mice in which the mouse Cyp1a cluster is deleted may also be generated.

huUGT cluster and koUGT cluster

A DNA sequence encoding the human Ugt1 cluster is inserted into the mouse Ugt1 cluster, as shown in Figure 75, enabling expression of the human Ugt1 cluster under the control of human Ugt1 promoters. The targeting vector includes loxP sequence elements that enable Cre-mediated deletion of the mouse Ugt1 cluster, to produce koUGT (Figure 75C). Cre-mediated deletion of the mouse Ugt1 cluster is followed by Cre-mediated insertion of the human Ugt1 cluster, to produce huUGT (Figure 75C). After the insertion of the human Ugt1 cluster into the mouse Ugt1 cluster, selection cassettes are deleted, using FRT sites that are also present in the targeting vector (Figure 75C).

C. Drug Transporter Proteins

huMDR1/mdr1a$^{-/-}$

A DNA sequence encoding human MDR1 is inserted into the mouse Mdr1a locus, as shown in Figure 76, enabling expression of human MDR1 under the control of the mouse Mdr1a promoter. The DNA sequence encoding human MDR1 is the human MDR1 cDNA sequence, starting with the initial ATG.

huMDR1/mdr1b$^{-/-}$

A DNA sequence encoding human MDR1 is inserted into the mouse Mdr1b locus, as shown in Figure 77, enabling expression of human MDR1 under the control of the mouse Mdr1b promoter. The DNA sequence encoding human MDR1 is the human MDR1 cDNA sequence, starting with the initial ATG.

huMDR1/mdr1a$^{-/-}$/mdr1b$^{-/-}$

The cDNA sequence for human MDR1, starting with the initial ATG, is inserted into both the mouse Mdr1a and Mdr1b loci, enabling expression of human MDR1 under the control of the mouse Mdr1a and Mdr1b promoters. The targeting is achieved in the same way as for the Mdr1a and Mdr1b single humanisation, wherein Mdr1b is targeted as shown in Figure 77 in ES cells that have previously been manipulated at the Mdr1a locus according to the strategy provided in Figure 76.

huMRP2

The cDNA sequence encoding human MPR2 is inserted into the mouse Mrp2 locus, as shown in Figure 78, enabling expression of human MRP2 under the control of the mouse Mrp2 promoter. In this strategy, (i) the mouse leader encoded by exon 1 is retained, (ii) the human cDNA will be introduced on exon2, and splice site retained, (iii) the human transcript is terminated by a polyA motif, and (iv) the complete intron 2 sequence is retained.

Figure 106 shows the characterisation by RT-PCR of huMRP2 mice. PCR products of expected sizes arising from the human MRP2 transgene were observed.

The inventors have also created huPXR/huMRP2 mice. The comparison of bilirubin levels in the urine of huPXR/huMRP2 *vs.* wild type mice revealed no significant differences, either with respect to total or direct bilirubin (see Figure 107). In contrast, in $MRP2^{-/-}$ mice, it has previously been reported that direct bilirubin levels were are 6-fold higher than in wild type mice (there being no difference in total bilirubin; see Journal of Pharmacology and Experimental Therapeutics 317:579 (2006)). The lack of any such difference in huPXR/huMRP2 mice indicates that the huMRP2 is functional.

D. Reporter strategies

rCYP2B6

The LacZ reporter gene and the transcriptionally linked human CYP2B6 promoter (up to 70kb, depending on the extent of BAC insertion) are inserted into exon 1 of the mouse Cyp2b6 locus in BL6 ES cells, as shown in Figure 79. The selection marker keo is deleted using the FRP sites and FLP recombinase, as indicated in Figure 79. This strategy allows expression of the LacZ reporter gene in mice under the control of the human CYP2B6 promoter.

The validation of the rCYP2B6 reporter mouse is represented in Figure 108.

rCYP2D6

The ZsYellow reporter gene and the transcriptionally linked human CYP2D6 promoter (up to 50kb, depending on the extent of BAC insertion) are inserted into exon 1 of the mouse Cyp2d6 locus in BL6 ES cells, as shown in Figure 80. The selection marker keo is deleted using the FRP sites and FLP recombinase, as indicated in Figure 80. This strategy allows expression of the ZsYellow reporter gene in mice under the control of the human CYP2D6 promoter.

The validation of the CYP2D6 reporter mouse is represented in Figure 109.

rCYP3A4

The hCG-ZsGreen reporter gene and the transcriptionally linked human CYP3A4 promoter (up to 40kb, depending on the extent of BAC insertion) are inserted into exon 1 of the mouse Cyp3a4 locus in BL6 ES cells, as shown in Figure 81. The selection marker keo is deleted using the FRP sites and FLP recombinase, as indicated in Figure 81. This strategy allows expression of the hCG-ZsGreen reporter gene in mice under the control of the human CYP3A4 promoter.

The validation of the CYP3A4 reporter mouse is represented in Figure 110.

rCyp3a11

The Firefly luciferase reporter gene is inserted into exon 1 of the mouse Cyp3a11 locus, as shown in Figure 82. This strategy allows expression of the hCG-ZsGreen reporter gene in mice under the control of the mouse Cyp3a11 promoter. The procedure is alternatively carried out using the ZsGreen reporter gene (Figure 71).

rMDR1

10kb of the human MDR1 upstream promoter (USP) including exon1 and 15kb of the downstream promoter (DSP) including exons 2 and 3 are fused to the start ATG of the firefly luciferase, as shown in Figure 83. USP and DSP have been described in several publications as essential elements for MDR1 expression (Ueda K, Pastan I and Gottesman MM, 1987. Isolation and sequence of the promoter region of the human multidrug-resistance (P-glycoprotein) gene. J Biol Chem., Dec 25;262(36):17432-6; Chen CJ, Clark D, Ueda K, Pastan I, Gottesman MM and Roninson IB, 1990. Genomic organization of the human multidrug resistance (MDR1) gene and origin of P-glycoproteins. J Biol Chem., Jan 5;265(1):506-14; Raguz S, Tamburo De Bella M, Tripuraneni G, Slade MJ, Higgins CF, Coombes RC and Yague E, 2004. Activation of the MDR1 upstream promoter in breast carcinoma as a surrogate for metastatic invasion. Clin Cancer Res. Apr 15;10(8):2776-83.). The targeting vector is used for targeted transgenesis into the ROSA26 locus by homologous recombination as shown in Figure 83.

These specific targeting strategies are preferred strategies for generating transgenic mice, tissues or cells derived therefrom having the genotypes (including the complex genotypes) described herein.

Example 6: Two-step cluster deletion and humanisation

6.1: CYP3A4 humanisation using the corresponding human promoter and knockout of the mouse CYP3A cluster

The mouse CYP3A cluster was flanked with loxP and FRT sites and a CYP3A4 expression cassette was inserted at one end of the mouse cluster, as shown in Figure 111, enabling expression

of human CYP3A4 under the control of the 13 kb human CYP3A4 promoter.

The loxP sequence elements included in the targeting vectors enable Cre-mediated deletion of the mouse CYP3A cluster, to allow the expression of CYP3A4 in the absence of the corresponding mouse genes (see Figure 111).

Subsequently, the FRT sequence elements enable Flp-mediated deletion of the human expression cassette, resulting in a complete knockout at the mouse CYP3A locus (Figure 111).

6.2: CYP2C9 humanisation using the corresponding human promoter and knockout of the mouse CYP2C cluster

The mouse CYP2C cluster was flanked with loxP and FRT sites and a CYP2C9 expression cassette was inserted at one end of the mouse cluster, as shown in Figure 112, enabling expression of human CYP2C9 under the control of the 12 kb human CYP2C9 promoter.

The loxP sequence elements included in the targeting vectors enable Cre-mediated deletion of the mouse CYP2C cluster, to allow the expression of CYP2C9 in the absence of the corresponding mouse genes (Figure 112).

Subsequently, the FRT sequence elements enable Flp-mediated deletion of the human expression cassette, resulting in a complete knockout at the mouse CYP2C locus (Figure 112).

6.3: CYP2D6 humanisation using the corresponding human promoter and knockout of the mouse CYP2D cluster

The mouse CYP2D cluster was flanked with loxP and FRT sites and a CYP2D6 expression cassette was inserted at one end of the mouse cluster, as shown in Figure 113, enabling expression of human CYP2D6 under the control of the 9kb human CYP2D6 promoter.

The loxP sequence elements included in the targeting vectors enable Cre-mediated deletion of the mouse Cyp2d cluster, to allow the expression of CYP2D6 in the absence of the corresponding mouse genes.

Use of specific primers has allowed analysis of the mouse chromosome by PCR. The results demonstrate successful introduction of the desired human replacement CYP2D gene sequence and the deletion of the mouse Cyp2d Cluster (see Figure 114).

Subsequently, the FRT sequence elements enable Flp-mediated deletion of the human expression cassette, resulting in a complete knockout at the mouse CYP2D locus (Figure 113).

6.4: CYP3A4 humanisation using the mouse Cyp3a11 promoter and knockout of the mouse CYP3A cluster

A DNA sequence encoding human CYP3A4 is inserted into the mouse Cyp3a cluster at the Cyp3a11 locus, as shown in Figure 10, enabling expression of human CYP3A4 under the control of the mouse Cyp3a11 promoter. This targeting strategy is designed to allow the expression of CYP3A4 in male mice, which may not be possible using the CYP3A4 promoter.

The DNA sequence encoding human CYP3A4 comprises at least part of intron 1 of the human CYP3A4 gene (see Figure 115).

Due to the inclusion of FRT sites in the targeting vector as shown in Figure 10, the selection marker (neo^R in Figure 115) can be deleted by crossing with an FLP deleter strain, subsequent to insertion of human CYP3A4 into the mouse Cyp3a11 locus of the wild type.

Due to the inclusion of both loxP and FRT sites in the targeting vector as shown in the Figure, most of mouse Cyp3a11 cluster can be deleted by crossing with an FLP deleter strain or a Cre deleter strain, if the human CYP3A4 is inserted into Cyp3a cluster 5′ targeted cells.

Example 7: Multiple cluster knockout and/or replacement

7.1: Cyp3a cluster exchange

The 5' end of the mouse Cyp3a cluster was targeted by homologous recombination, so that a frt, lox5171 and wildtype loxP site are inserted downstream of Exon1 of the Cyp3a57 gene as depicted in Figure 117a. Furthermore, a hygromycin resistance and a thymidine kinase (TK) expression cassette were co-introduced as depicted.

Correctly-targeted ES cells were further modified by a subsequent step of homologous recombination at the 3' end of the mouse Cyp3a cluster, so that a wildtype loxP and a f3 site are inserted downstream of Exon4 of the Cyp3a59 gene. A 5' deficient neomycin resistance and a TK expression cassette were co-introduced as depicted in Figure 117b. The translational start ATG is separated in frame from the 5'deficient neomycin resistance cassette by the loxP site, so that additional amino_acids encoded by the loxP site are attached to the N-terminus of the neomycin

protein.

To allow the deletion of the mouse Cyp3a cluster by Cre-mediated recombination, the targeting at both ends of the cluster has to be on the same allele. This was tested *in vitro* by transfection of double targeted ES cells with Cre and subsequent selection with Gancyclovir. Only in cells with targeting on the same allele will the TK genes be deleted at both ends of the cluster as depicted in Figure 117e, so that the TK-mediated conversion of the nucleoside analog ganciclovir to a toxic triphospate analog is prevented. Therefore, only ES cells with targeting on the same allele survive the ganciclovir selection.

Surviving ES cell clones were further analysed by PCR to confirm that the mouse Cyp3a cluster was deleted and PCR-positive clones were injected into blastocysts to generate chimeric mice. By subsequent crosses mice with a homozygous deletion of the Cyp3a cluster were finally generated.

The remaining wildtype loxP and the lox5171 sites in the ES cells can in due course be used subsequently to insert replacement genes, e.g. the human CYP3A cluster, at the former position of the mouse cluster by Cre-mediated insertion. The remaining promoter, the ATG and in frame wildtype loxP site can be utilized to insert again a 5'deficient selection cassette, e.g. 5'deficient neomycin, to select for clones with correct insertion with high efficiency. The frt and f3 sites at both ends can be used to delete the selection cassettes introduced with the replacement genes by Flp-mediated deletion, provided that additional frt and f3 sites are co-introduced in the right orientation with the replacement cassette.

The Cyp3a13 gene which is located 800bp upstream of Cyp3a57 and which is separated from the murine Cyp3a cluster by a number of Cyp-unrelated genes is not affected by the deletion of the mouse Cyp3a cluster described above.

7.2: Cyp2c cluster exchange

The 5' end of the mouse Cyp2c cluster was targeted by homologous recombination, so that a wildtype loxP and a f3 site are inserted downstream of Exon1 of the Cyp2c55 gene. A 5' deficient neomycin resistance and a TK expression cassette were co-introduced as depicted in Figure 2a. The translational start ATG is separated in frame from the 5'deficient neomycin resistance cassette by the loxP site, so that additional amino acids encoded by the loxP site are attached to the N-terminus of neomycin protein.

Correctly targeted ES cells were further modified by a subsequent step of homologous

recombination at the 3' end of the mouse Cyp2c cluster, so that a frt, lox5171 and wildtype loxP site are inserted downstream of Exon5 of the Cyp2c70 gene as depicted in Figure 2b. Furthermore, a hygromycin resistance and a thymidine kinase (TK) expression cassette were co-introduced as depicted.

To allow the deletion of the mouse Cyp2c cluster by Cre-mediated recombination, the targeting at both ends of the cluster has to be on the same allele. This was tested *in vitro* by transfection of double targeted ES cells with Cre and subsequent selection with ganciclovir. Only in cells with targeting on the same allele are the TK genes at both ends of the cluster deleted as depicted in Figure 118c, so that the TK-mediated conversion of the nucleoside analog ganciclovir to a toxic triphospate analog is prevented. Therefore, only ES cells with targeting on the same allele survive the ganciclovir selection. Surviving ES cell clones were further analysed by PCR to confirm that the mouse Cyp2c cluster was deleted and PCR-positive clones were injected into blastocysts to generate chimeric mice. By subsequent crosses mice with a homozygous deletion of the Cyp2c cluster will finally be generated.

The remaining wildtype loxP and the lox5171 sites in the ES cells can be used subsequently to insert replacement genes, e.g. the human CYP2C cluster, at the former position of the mouse cluster by Cre-mediated insertion. The remaining promoter, the ATG and in frame wildtype loxP site can be utilized to insert again a 5'deficient selection cassette, e.g. 5'deficient neomycin, to select for clones with correct insertion with high efficiency. The frt and f3 sites at both ends can be used to delete the selection cassettes introduced with the replacement genes by Flp-mediated deletion, provided that additional frt and f3 sites are co-introduced in the right orientation with the replacement cassette.

7.3: Cyp2d cluster deletion

The 3' end of the mouse Cyp2d cluster was targeted by homologous recombination, so that a frt, lox5171 and wildtype loxP site are inserted downstream of Exon1 of the Cyp2d26 gene as depicted in Figure 119a. Furthermore, a hygromycin resistance cassette was co-introduced as depicted. As the subsequent deletion of the mouse cluster will leave behind an intact Cyp2d26 promoter and exon1 of the Cyp2d26 gene, which might interfere with the expression of human CYP2D6 to be inserted at the 5' end of the cluster as described below, a splice acceptor polyadenylation motif (sApA) was included into the 3' targeting vector, which terminates the expression from the Cyp2d26 promoter.

Correctly targeted ES cells were further modified by a subsequent step of homologous recombination at the 5' end of the mouse Cyp2d cluster, so that a human CYP2D6 expression cassette flanked by an frt and a wildtype loxP site and a neomycin resistance cassette 3' to the loxP site are inserted downstream of Exon3 of the Cyp2d22 gene as depicted in Figure 119b.

Deletion of the mouse Cyp2d cluster while maintaining the human expression cassette can be achieved by Cre-mediated recombination at the loxP sites and simultaneous deletion of the mouse cluster and the human expression cassette, resulting in a complete knockout of the Cyp2d locus (as depicted in Figure 3c), is the result of Flp-mediated recombination at the frt sites.

To allow the deletion of the mouse Cyp2d cluster by Cre- or Flp-mediated recombination, the targeting at both ends of the cluster has to be on the same allele. Therefore, double targeted ES clones were further analyzed by *in vitro* deletion with Cre, followed by a PCR analysis with a primer pair that allows the detection of the deletion of the cluster. PCR analysis was performed on a pool of deleted and undeleted clones, as *in vitro* Cre-deletion occurs only in a fraction of transfected cells and there is no possibility to select for individual cells in which the deletion was successful. PCR-positive undeleted parental clones were injected into blastocysts to generate chimeric mice. By crosses with either Cre- or Flp-deleter strains or strains with tissue specific expression of the respective recombinase and subsequent intercrosses homozygous Cyp2d cluster deleted mice either with deletion in the whole body or in specific tissues and that contain or do not contain a human expression cassette will be generated.

Example 8: Investigation of non-genotoxic carcinogenic effects

8.1: PCR confirmation in double homozygous PXR/CAR humanised mice that the murine PXR gene has been exchanged for the human counterpart

Humanised mice for PXR and CAR ("huPXR/huCAR") were generated using mice which contained humanised PXR and crossing these into mice which contained humanised CAR to produce mice containing both humanised PXR and humanised CAR. The mice are phenotypically normal following visual inspection. They have been typed using PCR (see Figure 28) and are homozygously humanised for PXR and CAR. Examples include mice designated "42749" and "42752".

Transcription of PXR and CAR mRNA was quantified by RT-PCR in huPXR/huCAR mice and compared to the relative levels of corresponding mRNA expression in wild-type, huPXR and

huCAR mice (Figure 121). It was thereby confirmed that the huPXR/huCAR mice maintain the levels of human PXR and human CAR expression observed in mice humanised with respect to single genes.

Double-humanised huPXR/huCAR mice, as well as wild-type, huPXR and huCAR mice were treated with the inducers rifampicin and/or phenobarbital. Expression of Cyp2b10 and Cyp3a11 in these inducer-treated mice, as well as in corresponding untreated mice, was visualised and compared by SDS-PAGE followed by Western blotting (Figure 122). The basal levels of Cyp2b10 and Cyp3a11 in huPXR, huCAR and huPXR/huCAR mice are compared to the basal levels observed in wild-type mice in Figure 122. This relative quantification shows that basal Cyp2b10 levels increase in the order huPXR → huCAR → huPXR/huCAR. However, basal Cyp3a11 were less markedly increased in huCAR mice. Cyp3a11 levels were increased to an approximately equal extent (more than 2 fold) in both huPXR and double-humanised huPXR/huCAR mice.

Treatment with the human-specific inducer rifampicin led to an increase in the levels of Cyp3a11 in all mice. Whereas the administration of rifampicin and phenobarbital in combination appeared to have no additional effect in the wild type, induction of Cyp3a11 was somewhat stronger in huPXR.

8.2: Pentobarbitone sleeping test in double homozygous PXR/CAR humanised mice

In this experiment, the activity of these transcription factors in combination was determined by measuring the barbiturate induced sleeping time. Sleeping time has been known for many years to be directly proportional to the hepatic cytochrome P450 activity and this activity can be at least in part ascribed to the P450 levels in the liver determined by CAR and PXR function.

Mice were given a single intraperitoneal dose of Narcoren (sodium pentobarbitone; purchased via a Veterinary Consultant; distributed by Merial GmbH, Germany) at 25mg/kg of body weight. The time taken for the mice to lose, and subsequently to regain, their righting reflex was measured. Results are given in Table 1 below:

Table 1: Results of pentobarbitone sleeping test

Genotype	Sex	Age (weeks)	ID	Weight (g)	Sleeping time (min)

Wt	male	10	42912	21.2	21
PXR/CAR hum	male	10	42749	24.8	34

Whereas wild type mice given a narcotic dose of pentobarbitone slept for 21 minutes, the double humanised mice for CAR and PXR slept for 34 minutes. These mice therefore demonstrate a significant difference to their wild type controls indicating that the double humanised mouse has a marked difference in its response to drugs relative to the wild type animals.

Summary of work in 8.1 and 8.2 above

A model has been developed where human PXR is expressed in both the liver and GI tract of mice in the predicted fashion at levels equivalent to those of the endogenous gene. The PXR protein has been shown to be functional as the mice are responsive to compounds known to induce gene expression via this pathway.

Equivalent humanisation has also been achieved with respect to the CAR gene (huCAR mice). Strain differences between wild type and the humanised mice have been demonstrated and the humanised mice have been shown to be more responsive to compounds known to be more active in humans than in mice, *i.e.*, to human PXR or human CAR rather than murine PXR or murine CAR. The construction of knock-out lines has also been confirmed for both the PXR and the CAR genes (koPXR and koCAR).

Moreover, mice which contained humanised PXR have also been crossed into mice which contained humanised CAR to produce mice containing both humanised PXR and humanised CAR.

8.3: huPPARα and koPPARα

A DNA sequence encoding human PPARα has been inserted into the mouse PPARα locus, as shown in Figure 123, enabling expression of human PPARα under the control of the mouse PPARα promoter. The DNA sequence encoding human PPARα comprises at least part of intron 5 and intron 6 of the human PPARα gene (Figure 123). The targeting vector(s) include sequence elements that enable Cre-mediated PPARα knock-out to produce koPPARα (Figure 123).

8.4: huAhR and koAhR

A DNA sequence encoding human AhR is inserted into the mouse AhR locus (knock-in) as shown

in Figure 124, enabling expression of human AhR under the control of the mouse AhR promoter. The DNA sequence encoding human AhR comprises exons 3-11 of the human AhR gene (Figure 124). The targeting vector(s) include(s) sequence elements that enable Cre-mediated AhR knock-out to produce koAhR (Figure 124).

8.5: Proof of concept: the effect of the non-genotoxic carcinogen, PB, in rodents

Phenobarbital (hereafter PB) is known to cause liver cancer in mice and rats but does not do the same in humans. Following long term treatment to PB, rodents develop liver tumours. Initially, PB causes a hyperplastic response and cell replication and liver weight increases for the first two weeks of treatment. However, after approximately two years, liver tumours become evident in treated animals. Under this type of analysis, PB would be deemed unsafe for use in humans. However, PB is indeed safe, having been sold for many years with no record of liver tumour incidence in treated patients. This illustrates the shortcomings of current animal models to test for drug safety in humans, and means that there is unnecessary drug attrition occurring at this stage of the safety testing process. The question which drug companies need to answer, at as early a stage of testing as possible, is whether hyperplastic responses to chemicals observed in animals are actually relevant to man?

The transcription factor CAR is known to be essential for responses to PB-like inducers. Wei et al, 2000 (Nature) showed that in wild type mice, CAR activators increased liver mass, reflective of cellular hypertrophy and hyperplastic response. In contrast, CAR KO mice showed no increased liver mass after treatment with CAR activators. Furthermore, induction of DNA synthesis as determined by increased incorporation of BrdU in wild type mice was also absent in CAR KO mice. Similarly, Cheung et al, 2004 showed that humanisation of the mice for PPARα decreased the increase in liver weight elicited by treatment with various drugs in wild type animals. The humanised mice also showed a lack of increased replicative DNA synthesis, as seen in the wild type animals.

In order to assess whether humanised PXR/CAR mice mimic the response to PB in humans, the huPXR/huCAR and PXRKO/CARKO mouse models were used. The mutant mouse strains were obtained from Artemis. The WT mouse strain C57BL/6J was obtained from Harlan (UK). All animals were between 10 and 16 weeks of age.The following parameters were studied:

- Liver/body weight ratios

- BrdU incorporation analysed as a measure of cell proliferation
- Haematoxylin and eosin (H&E) liver histopathology
- Expression and activity of P450s by SDS-PAGE and Western blotting in liver microsomes
- Apoptotic indices as analysed by TUNEL assay in the liver

The study consisted of 6 groups with 10 WT mice per group, 9-10 PXRKO/CARKO mice per group and 9 huPXR/huCAR mice per group (Table 2). All animals were implanted with osmotic pumps (Alzet 2001) containing bromodeoxyuridine (BrdU, 15mg/ml in phosphate buffered saline [PBS], pH7.4) 5 days before termination for all mice (Day -1). Post operation all animals had no abnormalities detected. On Day 1 all animals were dosed with either 80mg/kg PB/saline or saline alone by intraperitoneal injection for 4 days as detailed in Table 2.

Table 2: Experimental design

Grp	Mouse #	Number mice/ group	Mouse Strain	Inducing agent	Dose (mg/ kg)	Regimen	Implant minipumps with Brdu	First dose date	Termination Date
1	1-10	10	WT	Control	-	4 x daily	17-Oct-07	18-Oct-07	22-Oct-07
2	11-19	9	huPXR/ huCAR	Control	-	4 x daily	17-Oct-07	18-Oct-07	22-Oct-07
3	20-28	9	PXRKO/ CARKO	Control	-	4 x daily	17-Oct-07	18-Oct-07	22-Oct-07
4	29-38	10	WT	PB	80	4 x daily	17-Oct-07	18-Oct-07	22-Oct-07
5	39-47	9	huPXR/ huCAR	PB	80	4 x daily	17-Oct-07	18-Oct-07	22-Oct-07
6	48-57	10	PXRKO/ CARKO	PB	80	4 x daily	17-Oct-07	18-Oct-07	22-Oct-07

PXR/CAR-dependent hepatomegaly by PB

Following treatment with PB or the vehicle, the mice were sacrificed and their livers were removed and weighed. Hepatomegaly was observed in both the WT and "humanised" mice, but not in the PXRKO/CARKO in response to PB treatment, as shown by increases in liver body weight ratios if 118% and 122%, respectively (Table 3, Figure 126).

Table 3: Liver/body weight ratios. Values are expressed as Mean ± SD (% mean own control ± % SD); n=3. A Student's t-test (2-sided) was performed on the results; * and ** statistically different from control mice at $p<0.05$ and $p<0.01$, respectively.

Mouse line	Treatment	Body weight (g) - Dayl	Body weight (g) - Day 5	Liver weight (g)	Liver/Body weight Ratio
WT	Control	27.9 ±2.1 (100 + 8)	27.7 ±2.4 (100 ± 8.7)	1.44 ±0.22 (100 ± 15)	5.2 ±0.7 (100 ± 13.5)
	PB	26.4 ± 1.8 (106 + 6)	23.2 ± 1.3* (84 ± 5)	1.44 ±0.09 (101 ±6)	6.2 ±0.2** (118 ±3)
huPXR/huCAR	Control	25.4 ± 1.9 (100 ±7)	25.6 ± 1.9 (100 ± 8)	1.40+0.14 (100 ±10)	5.5 ±0.4 (100 ±8)
	PB	26.3 ± 1.2 (104 ± 5)	25.4 ± 1.5 (99 ± 6)	1.69 ±0.16** (121 ±11)	6.7 ±0.6** (122 ± 12)
PXRKO/CARKO	Control	27.2+1.9 (100 ±7)	27.3 ± 2.4 (100 ±9)	1.45 ±0.22 (100 ± 15)	5.3 ±0.5 (100 ±10)
	PB	27.2 + 2.2 (100 ±8)	26.6 + 2.2 (98 ±8)	1.42 + 0.23 (98 ± 16)	5.3 ±0.4 (100 ±9)

Hepatic cell proliferation

All mouse liver and duodenum sections were analysed for BrdU incorporation as a measure of cell proliferation. The method used was an indirect BrdU labelling assay. PB increased the hepatocellular labelling index (S-phase) in the WT mice by approximately 5-fold and appeared to have no effect on cell proliferation in the huPXR/huCAR or PXRKO/CARKO (Figure 127, Table 4).

Liver in situ cell death

A 50% inhibition of hepatocellular apoptosis by non-genotoxic carcinogens has been previously demonstrated with consistency in rats. However, no such consistency has been observed in mice. An indirect TUNEL labelling assay was used to analyse hepatic in situ death (Figure 128 / Table 4). The present study has shown a marker variation in apoptotic indices in mouse liver. Thus, small (e.g. 50%) compound-induced decreases, upon a low background, were not readily demonstrable.

H&E analysis

Two samples of the liver (one from the lobe, one from the median lobe) and one sample of the small intestine were taken and preserved in 4% neutral buffered formaldehyde (NBF). The preserved liver samples of all mice of all groups were trimmed, processed and embedded in

paraffin. The paraffin-embedded samples were sent to Progenix, Inverkeithing, UK where they were sectioned at a nominal thickness of about 5 æm and then stained with haematoxylin and eosin (H&E). One section of each organ sample was examined by Dr. Ortwin Vogel, Consultant Pathologist, Kiel, Germany. Subsequent to his histopatholoical analysis of all H&E stained mouse livers and small intestines, Dr. Vogel reported the following finding;

Microscopically, a slight to moderate centrilobular hepatocellular hyperthrophy was noted, in PB treated huPXR/huCAR and WT animals (Figure 129). This finding is considered to be related to the treatment with PB. In contrast, no unequivocal evidence of a hepatocellular hypertrophy was noted in PXRKO/CARKO mice following treatment with PB. In addition, mitotic figures indicating hepatocellular proliferation were noted mainly in PB-treated WT mice but also, with a lower incidence, in the "humanised" and null mouse lines.

Table 4: Hepatic S-phase Labelling Indices in PB-treated mice. Values are expressed as Mean ± SD. A Student's t-test (2-sided) was performed on the results; with *** statistically different from control mice at pO.OOl. NS, no sample available for analysis

% BrDu Positive Mouse #	Mouse line	Treatment	Cells	Mean	SD	%mean	%SD	T-Test
1	WT	Control	4.39	1.75	1.10	100	63	
2	WT	Control	2.14					
3	WT	Control	2.18					
4	WT	Control	2.35					
5	WT	Control	1.39					
6	WT	Control	1.31					
7	WT	Control	0.74					
8	WT	Control	0.99					
9	WT	Control	0.77					
10	WT	Control	1.24					
11	huPXR/huCAR	Control	1.07	1.93	0.67	100	35	
12	huPXR/huCAR	Control	1.65					
13	huPXR/huCAR	Control	2.02					
14	huPXR/huCAR	Control	1.53					
15	huPXR/huCAR	Control	1.18					
16	huPXR/huCAR	Control	2.02					
17	huPXR/huCAR	Control	3.05					
18	huPXR/huCAR	Control	2.79					
19	huPXR/huCAR	Control	2.03					
20	PXRKO/CARKO	Control	2.75	1.96	1.04	100	53	
21	PXRKO/CARKO	Control	3.86					
22	PXRKO/CARKO	Control	0.74					
23	PXRKO/CARKO	Control	1.8G					
24	PXRKO/CARKO	Control	1.28					
25	PXRKO/CARKO	Control	1.09					
26	PXRKO/CARKO	Control	1.10					
27	PXRKO/CARKO	Control	2.02					
28	PXRKO/CARKO	Control	2.94					
29	WT	PB	9.45	9.30	3.64	531	208	0.0002
30	WT	PB	5.54					
31	WT	PB	16.69					
32	WT	PB	NS					
33	WT	PB	6.S1					
34	WT	PB	6.32					
35	WT	PB	9.50					
36	WT	PB	8.09					
37	WT	PB	7.64					
38	WT	PB	13.60					
39	huPXR/huCAR	PB	0.37	2.00	1.18	104	61	0.87
40	huPXR/huCAR	PB	0.-18					
41	huPXR/huCAR	PB	1.19					
42	huPXR/huCAR	PB	1.86					
43	huPXR/huCAR	PB	3.GO					
44	huPXR/huCAR	PB	1.98					
45	huPXR/huCAR	PB	3.18					
46	huPXR/huCAR	PB	3.24					
47	huPXR/huCAR	PB	2.13					
48	PXRKO/CARKO	PB	2.16	2.83	1.07	144	55	0.09
49	PXRKO/CARKO	PB	1.98					
50	PXRKO/CARKO	PB	1.82					
51	PXRKO/CARKO	PB	2.30					
52	PXRKO/CARKO	PB	3.77					
53	PXRKO/CARKO	PB	5.10					
54	PXRKO/CARKO	PB	3.71					
55	PXRKO/CARKO	PB	1.88					
56	PXRKO/CARKO	PB	3.06					
57	PXRKO/CARKO	PB	2.51					

All other microscopic findings recorded in the liver did not significantly distinguish PB-treated mice from control mice or the differences were regarded as random events. All these findings are considered to be spontaneous in nature and within the normal background pathology commonly seen in mice. No microscopic findings were recorded in the small intestine. Under the conditions of this study, PB (80mg/kg/4days/IP) produced pathological evidence of a hepatocellular hypertrophy/hyperplasia in mice of the WT strain as well as in mice of the huPXR/huCAR strain. Evidence of hepatocellular hyperplasia occurred in PB-treated mice of the PXRKO/CARKO strain.

Hepatic P450 induction

P450 catalytic activities in the liver microsomal fractions were quantified. For the quantification of mouse Cyp2b10 and Cyp3a11 activities, the dealkylation of pentoxyresofurin (PROD) and the debenzylation of benzyloxyquinoline (BQ) activities, respectively. In addition, benzyloxyresorufin-O-demethylase (BROD), methoxyresorufin -O-demethylase (MROD) and 7-ethoxyresorufin-)-deethylase (EROD) activities were also measured and evaluated (see Figure 130).

EROD activity is indicative of Cyp1a1/1a2 and Cyp1b1 in the mouse, whereas MROD is a substrate for Cyp1a2. However, other isoforms may be contributing to the measured activities. The results from the two enzyme assays are in reasonably good agreement with each other (Figure 130a-b) As the Cyp1a2 gene is constitutively expressed, this may explain the basal levels of activity observed in both activity assays. Cyp1a1 is only expressed following induction in mice (Ikeya et al, 1989) and Phenobarbital has been reported not to induce this P450 in C57BL/6J mice (Sakuma et al, 1999). The MROD and EROD data demonstrated that enzyme activities were increased by PB treatment in WT and huPXR/huCAR mice. Both assays revealed no increase due to PB treatment in the PXRKO/CARKO line. Earlier data using the PXR/CAR mouse panel demonstrated PB, at 40mg/kg/4days, could activate Cyo1a2 via the CAR mediated pathway, although other data are consistent with these earlier findings.

A 5-fold increase in BQ activity was seen in the PB-treated huPXR/huCAR mice, whereas a marginal increase was detected in the WT animals (Figure 130c). These data demonstrate a clear species difference between the mouse lines, indicating that the human receptors have a greater sensitivity to PB than their murine counterparts. Furthermore, this mechanism appears to be

dependent on the presence of the receptors, as verified by the absence of Cyp3a11 induction in the PB-treated PXRKO/CARKO mice.

BROD and PROD are markers of Cyp2b10 activity in the mouse. In both the WT and huPXR/huCAR mouse lines, marker Cyp2b10 induction was observed, at similar levels following treatment with PB (Figure 130d-e). However, PROD or BROD activities were not altered in the PXRKO/CARKO mice upon exposure to PB. Overall, PB induced P450 catalytic activities in the WT and huPXR/huCAR mouse lines, but not in the animals which were devoid of these receptors. This clearly indicates that PB-mediated P450 induction is CAR/PXR-dependent.

In accordance with P450 activity data, quantification of Cyp2b10 and Cyp3a11 protein in pooled mouse liver microsomes by Western blotting revealed that both P450s were induced by PB in WT and humanised mouse lines but not in the PXRKO/CARKO animals (Figure 131). Furthermore, the species difference in Cyp3a11 induction is confirmed at the protein levels, suggesting that PB has a greater sensitivity for the human receptors over its murine equivalents.

In conclusion it can be said that hepatomegaly occurred only in WT mice and in huPXR/huCAR mice. The KO PXR/KO CAR showed no effect. The same pattern was mirrored for P450 induction. However, the most striking result was found when the mice were tested for hepatocellular proliferation (as determined by incorporation of the DNA precursor BrdU), where it was found that only the WT mice displayed a proliferation of hepatocytes. Both KO and humanised animals showed no proliferative effect whatsoever.

Important conclusions, therefore, are that:

- PB induced hypertrophy and hyperplasia in WT mice.
- PB did not induce hepatomegaly (neither hypertrophy or hyperplasia) in PXRKO/CARKO mice.
- PB induced hypertrophy but not hyperplasia in huPXR/huCAR mice.

These mice and huPXR/huCAR/huPPARa will be of considerable value in assessing the true hazard of non-genotoxic rodent "liver growth carcinogens" to humans. They will also provide useful tools to unravel the complexities of xenobiotic-induced liver growth and species differences in such growth.

References

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CLAIMS

1. A non-human animal, tissue or cells derived therefrom incorporating at least one human DNA sequence encoding the transcription factor PXR (i.e., the pregnane X receptor), wherein said human DNA sequence comprises least part of intron 6 and/or intron 7 of the human PXR gene.

2. A non-human animal, tissue or cells according to claim 1, wherein said human DNA sequence comprises at least part of introns 4, 5, 6, and/or 7 of the human PXR gene.

3. A non-human animal, tissue or cells according to any preceding claim, wherein in said human DNA sequence the intron-exon structure between exons 4 and 8 of the human PXR gene is maintained.

4. A non-human animal, tissue or cells according to any preceding claim, wherein said human DNA sequence further comprises at least part of intron 2, intron 3 and/or intron 8 of the human PXR gene.

5. A non-human animal, tissue or cells according to any preceding claim, wherein in said human DNA sequence the intron-exon structure between exons 2 and 9 of the human PXR gene is maintained.

6. A non-human animal, tissue or cells according to claim 1, wherein said human DNA sequence comprises a sequence that is at least 70% identical to the sequence of SEQ ID NO:2 or the sequence of SEQ ID NO:3.

7. A non-human animal, tissue or cells according to any preceding claim, wherein said human DNA sequence is inserted into the mouse PXR locus, such that expression of human PXR is under the control of the mouse PXR promoter.

8. A non-human animal, tissue or cells according to any preceding claim, further incorporating at least one of any one or more of the following human DNA sequences encoding::

   (i) a further transcription factor

   (ii) a phase-1 drug-metabolising enzyme;

   (iii) a phase-2 drug-metabolising enzyme;

   and/or

   (iv) a drug transporter protein.

9. A non-human animal, tissue or cells according to claim 8, wherein said at least one further transcription factor is one or more of CAR, AhR, and/or PPARα.

10. A non-human animal, tissue or cells according to claim 9, wherein said animal, tissue or cells incorporate a human DNA sequence encoding CAR, which human DNA sequence comprises at least part of intron 2, intron 3, intron 4, intron 5, intron 6, intron 7 and/or intron 8 of the human CAR gene; and/or optionally said human DNA sequence retains the intron-exon structure between exons 2 and 9; and/or optionally said human DNA sequence is at least 70% identical to the sequence of SEQ ID NO:4; and/or optionally said human DNA sequence is inserted into the mouse CAR locus, such that expression of human CAR is under the control of the mouse CAR promoter.

11. A non-human animal, tissue or cells according to any one of claims 9 or 10, wherein said animal, tissue or cells incorporate a human DNA sequence encoding AhR, which human DNA sequence comprises at least exons 3-11 of the human AhR gene and/or which human DNA sequence is inserted into the mouse AhR locus, such that expression of human AhR is under the control of the mouse AhR promoter.

12. A non-human animal, tissue or cells according to any one of claims 9-11 wherein said animal, tissue or cells incorporate a human DNA sequence encoding PPARα, which human DNA sequence includes at least part of intron 5 and/or intron 6 of the human PPARα gene, and, optionally, in which human DNA sequence the intron-exon structure between exons 3 and 8 of the human PPARα gene is maintained and/or which human DNA sequence is inserted into the mouse PPARα locus, such that expression of human PPARα is under the control of the mouse PPARα promoter.

13. A non-human animal, tissue or cells according to any one of claims 8-12, wherein said at least one phase-1 drug-metabolising enzyme is a cytochrome P450 isoform, optionally selected from the group comprising CYP1A, CYP3A, CYP2B, CYP2C, CYP2D, and CYP2E isoforms, optionally selected from one or more of CYP1A1, CYP1A2, CYP3A4, CYP3A5, CYP3A9, CYP2C8, CYP2C9, CYP2B6, CYP2B10, CYP2C19, CYP2D6, CYP2E1, and/or said human DNA sequence encoding a phase-1 drug-metabolising enzyme comprises a cytochrome P450 gene cluster, optionally selected from the CYP3A cluster, the CYP2D cluster, the CYP2C cluster, and/or the CYP1A cluster.

14. A non-human animal, tissue or cells according to any one of claims 8-13, wherein said at least one phase-2 drug-metabolising enzyme is a glucuronyl transferase, the UGT1A gene, the UGT1A gene cluster, a glutathione transferase, a glutathione S-transferase

(GST), a sulphonyl transferase, and/or an acetyl transferase.

15. A non-human animal, tissue or cells according to any one of claims 8-14, wherein said at least one drug transporter protein is a multi-drug resistance protein (MDR), MDR1, MDR1a, MDR1b, a multi-drug resistance-associated protein (MRP), MRP1, MRP2, an organic anion transporting polypeptide (OATP), and/or BCRP (the transmembrane ABC-type half-transporter ABCG2).

16. A non-human animal, tissue or cells derived therefrom incorporating a human DNA sequence encoding PXR and in which the endogenous gene for CAR has been annulled.

17. A non-human animal, tissue or cells derived therefrom incorporating a human DNA sequence encoding CAR and in which the endogenous gene for PXR has been annulled, wherein said DNA sequence encoding human CAR is optionally defined as in claim 10.

18. A non-human animal, tissue or cells derived therefrom in which the endogenous genes for both PXR and CAR have been annulled.

19. A non-human animal, tissue or cells derived therefrom incorporating

(i) a human DNA sequence encoding the transcription factor PXR;

(ii) a human DNA sequence encoding the transcription factor CAR;

(iii) a human DNA sequence encoding the transcription factor AhR; and

(iv) a human DNA sequence encoding the transcription factor PPARα.

20. A non-human animal, tissue or cells according to claim 19, wherein said human DNA sequence encoding the transcription factor PXR is defined as in any one of claims 1-7, and/or said DNA sequence encoding human CAR is defined as in claim 10 and/or said DNA sequence encoding human AhR is defined as in claim 11, and/or said human DNA sequence encoding the transcription factor PPARα is defined as in claim 12.

21. A non-human animal, tissue or cells according to any one of claims 19 or 20, further incorporating one or more human DNA sequences encoding

(i) one or more phase-1 drug-metabolising enzymes or one or more cytochrome P450 gene clusters, optionally as defined in claim 13; and/or

(ii) one or more phase-2 drug-metabolising enzymes, optionally as defined in claim 14, and/or

(iii) one or more drug transporter proteins, optionally as defined in claim 15.

22. A non-human animal, tissue or cells derived therefrom incorporating

(i) a human DNA sequence encoding the transcription factor PXR; and

(ii) a human DNA sequence encoding CYP3A4;

23. A non-human animal, tissue or cells according to claim 22, in which an endogenous gene equivalent to CYP3A4 has been annulled.

24. A non-human animal, tissue or cells according to claim 23 wherein the animal is a mouse and said endogenous gene equivalent to CYP3A4 is Cyp3a11.

25. A non-human animal, tissue or cells according to any one of claims 22-24, wherein the human DNA sequence encoding CYP3A4 is under the control of the endogenous promoter of an equivalent gene in the non-human animal.

26. A non-human animal, tissue or cells according to any one of claims 22-25, wherein the animal is a mouse and the human DNA sequence encoding CYP3A4 is inserted into the mouse Cyp3a11 locus such that the expression of the human DNA sequence encoding CYP3A4 is under the control of the mouse Cyp3a11 promoter.

27. A non-human animal, tissue or cells according to any one of claims 22-25, wherein the animal is a mouse and the human DNA sequence encoding CYP3A4 is inserted into the mouse Cyp3a25 locus such that the expression of the human DNA sequence encoding CYP3A4 is under the control of the human CYP3A4 promoter or optionally under the control of the mouse Cyp3a25 promoter.

28. A non-human animal, tissue or cells according to any one of claims 22-24, wherein the human DNA sequence encoding CYP3A4 is under the control of the human CYP3A4 promoter.

29. A non-human animal, tissue or cells according to any one of claims 22-24, wherein the human DNA sequence encoding CYP3A4 is inserted into the mouse Rosa 26 locus, such that expression of human CYP3A4 is under the control of the human CYP3A4 promoter.

30. A non-human animal, tissue or cells according to any one of claims 22-24, wherein the human DNA sequence encoding CYP3A4 is inserted into the mouse Cyp3a cluster, such that expression of human CYP3A4 is under the control of a human CYP3A4 promoter.

31. A non-human animal, tissue or cells derived therefrom according to any one of claims 22-30, wherein the human DNA sequence encoding human CYP3A4 comprises at least part of intron 1 of the human CYP3A4 gene.

32. The non-human animal, tissue or cells of any one of claims 22-31, further incorporating one or more human DNA sequences encoding

(i) one or more further transcription factors, optionally selected from the group consisting of CAR, optionally as defined in claim 10, AhR, optionally as defined in claim 11, and PPARα, optionally as defined in claim 12; and/or

(ii) one or more further phase-1 drug-metabolising enzymes or one or more cytochrome P450 gene clusters, optionally as defined in claim13; and/or

(iii) one or more phase-2 drug-metabolising enzymes, optionally as defined in claim 14, and/or

(iv) one or more drug transporter proteins, optionally as defined in claim 15.

33. A non-human animal, tissue or cells derived therefrom incorporating

(i) a human DNA sequence encoding CYP2C9; and

(ii) a human DNA sequence encoding the transcription factor PXR;

and/or

(iii) a human DNA sequence encoding the transcription factor CAR.

34. A non-human animal, tissue or cells according to claim 33, wherein the DNA sequence encoding CYP2C9 is under the control of the 12kb human CYP2C9 promoter, and/or wherein said DNA sequence encoding CYP2C9 comprises at least part of intron 4 of the human CYP2C9 gene; and/or wherein said DNA sequence encoding CYP2C9 is inserted either into the mouse Rosa26 locus or into the mouse Cyp2c cluster.

35. A non-human animal, tissue or cells according to any one of claims 33 or 34, wherein the animal is a mouse and the human DNA sequence encoding CYP2C9 is inserted into the mouse Cyp2c55 locus such that the expression of the human DNA sequence encoding CYP2C9 is under the control of the human CYP2C9 or optionally under the control of the mouse Cyp2c55 promoter.

36. A non-human animal, tissue or cells derived therefrom incorporating

(i) a human DNA sequence encoding CYP2D6; and

(ii) a human DNA sequence encoding the transcription factor PXR;

and/or

(iii) a human DNA sequence encoding the transcription factor CAR.

37. A non-human animal, tissue or cells according to claim 36, wherein the DNA sequence encoding CYP2D6 is under the control of the human CYP2D6 promoter, and/or wherein said DNA sequence encoding CYP2D6 is inserted into the mouse Cyp2d cluster.

38. A non-human animal, tissue or cells derived therefrom incorporating

(i) a human DNA sequence encoding CYP1A1, and

(ii) a human DNA sequence encoding CYP1A2.

39. A non-human animal, tissue or cells according to claim 38, wherein said human DNA sequences are inserted into the mouse Cyp1a cluster, such that expression of human CYP1A1 is under the control of the human CYP1A1 promoter and human CYP1A2 is under the control of the human CYP1A2 promoter.

40. A non-human animal, tissue or cells derived therefrom incorporating

(i) a human DNA sequence encoding the transcription factor PXR;

(ii) a human DNA sequence encoding the transcription factor CAR; and

(iii) a human DNA sequence encoding the CYP3A gene cluster.

41. A non-human animal, tissue or cells according to claim 40, wherein the human DNA sequence encoding the human CYP3A cluster is inserted into the mouse Cyp3a cluster, such that expression of the human CYP3A cluster is under the control of human CYP3A promoters.

42. A non-human animal, tissue or cells derived therefrom incorporating

(i) a human DNA sequence encoding the transcription factor PXR;

(ii) a human DNA sequence encoding the transcription factor CAR; and

(iii) a human DNA sequence encoding the CYP2C gene cluster.

43. A non-human animal, tissue or cells derived therefrom which has been humanised for the CYP2D cluster.

44. A non-human animal, tissue or cells derived therefrom which has been humanised for the CYP2C cluster.

45. A non-human animal, tissue or cells derived therefrom which has been humanised for the CYP1A cluster.

46. A non-human animal, tissue or cells according to any one of claims 43-45, further

incorporating one or more human DNA sequences encoding

(i) one or more transcription factors, optionally selected from the group consisting of PXR, optionally as defined in any one of claims 1 to 7, CAR, optionally as defined in claim 10, AhR, optionally as defined in claim 11, and PPARα, optionally as defined in claim 12; and/or

(ii) one or more phase-1 drug-metabolising enzymes and/or one or more further cytochrome P450 gene clusters, optionally as defined in claim 13; and/or

(iii) one or more phase-2 drug-metabolising enzymes, optionally as defined in claim 14, and/or

(iv) one or more drug transporter proteins, optionally as defined in claim 15.

47. A non-human animal, tissue or cells according to claim 45 further incorporating a human DNA sequence encoding the transcription factor AhR, optionally as defined in claim 11.

48. A mouse, tissue or cells derived therefrom in which the endogenous gene for Cyp3a11 has been annulled.

49. A mouse, tissue or cells derived therefrom in which the endogenous gene for Cyp1a1 and/or the endogenous gene for Cyp1a2 have been annulled.

50. A non-human animal, tissue or cells derived therefrom in which the endogenous CYP3A cluster has been annulled.

51. The non-human animal, tissue or cells of any one of claims 17, 18, 38, 39, or 48-50, further incorporating one or more human DNA sequences encoding

(i) one or more transcription factors, optionally selected from the group consisting of PXR, optionally as defined in any one of claims 1 to 7, CAR, optionally as defined in claim 10, AhR, optionally as defined in claim 11, and PPARα, optionally as defined in claim12; and/or

(ii) one or more phase-1 drug-metabolising enzymes or one or more cytochrome P450 gene clusters, optionally as defined in claim 13; and/or

(iii) one or more phase-2 drug-metabolising enzymes, optionally as defined in claim 14, and/or

(iv) one or more drug transporter proteins, optionally as defined in claim 15.

52. A non-human animal, tissue or cells derived therefrom incorporating a human DNA

sequence encoding the human UGT1 cluster, wherein said human DNA sequence is inserted into the mouse Ugt1 cluster, optionally such that expression of the human UGT1 cluster is under the control of human UGT1 promoters.

53. A non-human animal, tissue or cells according to claim 52, further incorporating one or more human DNA sequences encoding

(i) one or more transcription factors, optionally selected from the group consisting of PXR, optionally as defined in any one of claims 1 to 7, CAR, optionally as defined in claim 10, AhR, optionally as defined in claim 11, and PPARα, optionally as defined in claim12; and/or

(ii) one or more phase-1 drug-metabolising enzymes or one or more cytochrome P450 gene clusters, optionally as defined in claim 13; and/or

(iii) one or more further phase-2 drug-metabolising enzymes, optionally as defined in claim 14, and/or

(iv) one or more drug transporter proteins, optionally as defined in claim 15.

54. A non-human animal, tissue or cells derived therefrom incorporating

(i) a human DNA sequence encoding the transcription factor PXR; and

(ii) a human DNA sequence encoding the drug transporter MRP2.

55. A non-human animal, tissue or cells according to claim 54, wherein expression of said human DNA sequence encoding the drug transporter MRP2 is under the control of the endogenous promoter of the non-human animal.

56. A non-human animal, tissue or cells according to claim 55, wherein said animal is a mouse and said DNA sequence encoding MRP2 is optionally inserted into the mouse Mrp2 locus, such that expression of human MRP2 is under the control of the mouse Mrp2 promoter.

57. A non-human animal, tissue or cells according to any one of claims 52 or 55, further incorporating

(i) a human DNA sequence encoding the transcription factor CAR; and/or

(ii) a human DNA sequence encoding the drug transporter MDR1a and/or

(iii) a human DNA sequence encoding the drug transporter MDR1b.

58. A non-human animal, tissue or cells derived therefrom incorporating a human DNA

sequence encoding the drug transporter protein MDR1, and optionally further incorporating one or more further human DNA sequences encoding human PXR, human CAR, wherein said DNA sequence encoding MDR1 is optionally inserted into the mouse Mdr1a locus, such that expression of human MDR1 is under the control of the mouse Mdr1a promoter.

59. A non-human animal, tissue or cells derived therefrom incorporating a human DNA sequence encoding the drug transporter protein MDR1, and optionally further incorporating one or more further human DNA sequences encoding human PXR, human CAR, wherein said DNA sequence encoding MDR1 is optionally inserted into the mouse Mdr1b locus, such that expression of human MDR1 is under the control of the mouse Mdr1b promoter.

60. A non-human animal, tissue or cells derived therefrom incorporating a human DNA sequence encoding the drug transporter protein MDR1a, and optionally further incorporating one or more further human DNA sequences encoding human PXR, human CAR and/or human MDR1b.

61. A non-human animal, tissue or cells derived therefrom incorporating a human DNA sequence encoding the drug transporter protein MDR1b, and optionally further incorporating one or more further human DNA sequences encoding human PXR, human CAR, and/or human MDR1a.

62. A non-human animal, tissue or cells derived therefrom incorporating human DNA sequences encoding human PXR and/or human CAR, in which the endogenous genes for MDR1a and/or MDR1b have been annulled.

63. A non-human animal, tissue or cells according to any one of claims 16, 52 or 58-62 further incorporating one or more human DNA sequences encoding

(i) one or more further transcription factors, optionally selected from the group consisting of CAR, optionally as defined in claim 10, AhR, optionally as defined in claim 11, and/or PPARα, optionally as defined in claim12; and/or

(ii) one or more phase-1 drug-metabolising enzymes optionally as defined in claim 13 and/or one or more cytochrome P450 gene clusters, optionally as defined in claim 13; and/or

(iii) one or more phase-2 drug-metabolising enzymes, optionally as defined in

claim 14, and/or

(iv) one or more further drug transporter proteins, optionally as defined in claim 15.

64. A transgenic mouse, tissue or cells derived therefrom incorporating a human DNA sequence encoding PXR under the control of an endogenous promoter, and optionally having its equivalent endogenous murine PXR genes annulled, which mouse, tissue or cell:

(i) is more sensitive to rifampicin-mediated induction of the expression and/or activity of a cytochrome P450 than the corresponding wild-type mouse, tissue or cells derived therefrom; and/or

(ii) is less sensitive to dexamethasone-mediated or pregnenolone-16α carbonitrile-mediated or clotrimazole-mediated induction of the expression and/or activity of a cytochrome P450 than the corresponding wild-type mouse, tissue or cells derived therefrom.

65. A transgenic mouse, tissue or cells derived therefrom according to claim 64, wherein the cytochrome P450 is a Cyp3a enzyme.

66. A transgenic mouse, tissue or cells derived therefrom incorporating a human DNA sequence encoding CAR under the control of an endogenous promoter, and optionally having its equivalent endogenous murine CAR genes annulled, which mouse, tissue or cell:

(i) is more sensitive to CITCO-mediated induction of the expression and/or activity of a cytochrome P450 than the corresponding wild-type mouse, tissue or cells derived therefrom; and/or

(ii) is less sensitive to TCPOBOP-mediated induction of the expression and/or activity of a cytochrome P450 than the corresponding wild-type mouse, tissue or cells derived therefrom.

67. A transgenic mouse, tissue or cells derived therefrom according to claim 66, wherein the cytochrome P450 is a Cyp2b enzyme.

68. A transgenic mouse, tissue or cells derived therefrom incorporating human DNA sequences encoding PXR and CAR under the control of endogenous promoters, and optionally having its equivalent endogenous PXR and CAR genes annulled, which mouse,

tissue or cell:

(i) is more sensitive to rifampicin-mediated induction of the expression and/or activity of a cytochrome P450 than the corresponding wild-type mouse, tissue or cells derived therefrom; or

(ii) is less sensitive to dexamethasone-mediated or pregnenolone-16α carbonitrile-mediated or clotrimazole-mediated induction of the expression and/or activity of a cytochrome P450 than the corresponding wild-type mouse, tissue or cells derived therefrom; and

(iii) is more sensitive to CITCO-mediated induction of the expression and/or activity of a cytochrome P450 than the corresponding wild-type mouse, tissue or cells derived therefrom; or

(iv) is less sensitive to TCPOBOP-mediated induction of the expression and/or activity of a cytochrome P450 than the corresponding wild-type mouse, tissue or cells derived therefrom.

69. A transgenic mouse, tissue or cells derived therefrom according to claim 68, wherein the cytochrome P450 referred to in parts (i) and (ii) is a Cyp3a enzyme, and the cytochrome P450 referred to in parts (iii) and (iv) is a Cyp2b enzyme.

70. A transgenic mouse, tissue or cells derived therefrom incorporating a human DNA sequence encoding PXR under the control of an endogenous promoter, and optionally having its equivalent endogenous PXR gene annulled, which displays increased dexamethasone-mediated hepatotoxicity relative to the corresponding wild-type mouse.

71. A non-human animal, tissue or cells derived therefrom incorporating a reporter gene sequence under the control of a human CYP3A4 promoter sequence, and wherein said non-human animal, tissue or cells further incorporate

(i) a human DNA sequence encoding the transcription factor PXR; and/or

(ii) a human DNA sequence encoding the transcription factor CAR.

72. A non-human animal, tissue or cells according to claim 64, wherein said animal is a mouse and said reporter gene and human CYP3A4 promoter sequence are randomly integrated into the mouse genome.

73. A transgenic mouse, tissue or cells derived therefrom incorporating a reporter gene sequence under the control of a mouse Cyp3a11 promoter sequence, wherein the reporter

gene is inserted into the mouse Cyp3a11 locus, and wherein said mouse, tissue or cells optionally further incorporate

(i) a human DNA sequence encoding the transcription factor PXR; and/or

(ii) a human DNA sequence encoding the transcription factor CAR.

74. A non-human animal, tissue or cells derived therefrom incorporating a reporter gene sequence under the control of a human CYP2B6 promoter sequence, wherein the reporter gene and a human CYP2B6 promoter sequence are optionally inserted into the locus of an endogenous equivalent gene of the non-human animal, tissue or cells, and wherein said non-human animal, tissue or cells optionally further incorporate

(i) a human DNA sequence encoding the transcription factor PXR; and/or

(ii) a human DNA sequence encoding the transcription factor CAR.

75. A non-human animal, tissue or cells according to claim 74, wherein said animal is a mouse and said reporter gene and human CYP2B6 promoter sequence are inserted at the mouse Cyp2b6 locus.

76. A non-human animal, tissue or cells derived therefrom incorporating a reporter gene sequence under the control of a human CYP2D6 promoter sequence, wherein the reporter gene and a human CYP2D6 promoter sequence are optionally inserted into the locus of an endogenous equivalent gene of the non-human animal, tissue or cells, and wherein said non-human animal, tissue or cells optionally further incorporate

(i) a human DNA sequence encoding the transcription factor PXR; and/or

(ii) a human DNA sequence encoding the transcription factor CAR.

77. A non-human animal, tissue or cells according to claim 76, wherein said animal is a mouse and said reporter gene and human CYP2D6 promoter sequence are inserted into the mouse Cyp2d6 locus,

78. A non-human animal, tissue or cells derived therefrom incorporating a reporter gene sequence under the control of a human MDR1 promoter sequence, wherein the reporter gene and a human MDR1 promoter sequence are optionally inserted into the mouse Rosa26 locus, and wherein said non-human animal, tissue or cells optionally further incorporate

(i) a human DNA sequence encoding the transcription factor PXR; and/or

(ii) a human DNA sequence encoding the transcription factor CAR.

79. A non-human animal, tissue or cells derived therefrom incorporating a reporter gene sequence under the control of a human MDR1 promoter sequence, and wherein said non-human animal, tissue or cells optionally further incorporate

(i) a human DNA sequence encoding the transcription factor PXR; and/or

(ii) a human DNA sequence encoding the transcription factor CAR.

80. A non-human animal, tissue or cells according to claim 79, wherein said animal is a mouse and said reporter gene and human MDR1 promoter sequence are inserted into the mouse Rosa26 locus.

81. A non-human animal, tissue or cells according to any one of claims 79-80, wherein said human human MDR1 promoter sequence comprises both upstream and downstream promoter sequences.

82. A non-human animal, tissue or cells according to any one of claims 79-81, further incorporating a reporter gene sequence under the control of a human CYP3A4 promoter sequence, optionally according to claim 72.

83. A non-human animal, tissue or cells according to claim 82, further incorporating

(i) a reporter gene sequence under the control of a human CYP2B6 promoter sequence optionally according to any one of claims 74 or 75;

(ii) a reporter gene sequence under the control of a human CYP2D6 promoter sequence optionally according to any one of claims 76 or 77;

84. A non-human animal, tissue or cells according to any one of claims 33-35, 41, 42, or 64-83, further incorporating one or more human DNA sequences encoding

(i) one or more further transcription factors, optionally selected from the group consisting of AhR, optionally as defined in claim 11, and PPARα, optionally as defined in claim 12; and/or

(ii) one or more further phase-1 drug-metabolising enzymes or one or more further cytochrome P450 gene clusters, optionally as defined in claim 13; and/or

(iii) one or more phase-2 drug-metabolising enzymes, optionally as defined in claim 14, and/or

(iv) one or more drug transporter proteins, optionally as defined in claim 15.

85. A non-human animal, tissue or cells according to any one of claims 16, 19, 22-32, 33-42, 52-63, or 64-84 wherein said human DNA sequence encoding the transcription factor PXR is defined as in any one of claims 1-7.

86. A non-human animal, tissue or cells according to any one of claims 17, 19, 33-42, 57-62, or 64-84, wherein said human DNA sequence encoding the transcription factor CAR is defined as in claim 10.

87. A non-human animal, tissue or cells according to any one of the preceding claims, wherein any one of the endogenous equivalent genes has been annulled.

88. A non-human animal, tissue or cells according to any one of the preceding claims, wherein any one of the endogenous equivalent genes has not been annulled.

89. A non-human animal, tissue or cells according to any one of the preceding claims, wherein one or more of the CYP3A cluster, the CYP2C cluster, CYP2D cluster, or the CYP1A cluster have been replaced by a DNA sequence encoding at least one human cytochrome P450 enzyme.

90. A non-human animal, tissue or cells according to any one of the preceding claims, wherein one or more of said human DNA sequences is a complete human genomic gene sequence.

91. A non-human animal, tissue or cells according to any one of the preceding claims, wherein one or more of said human DNA sequences is a partial human genomic gene sequence.

92. A non-human animal, tissue or cells according to any one of the preceding claims, wherein one or more of said human DNA sequences is cDNA.

93. A non-human animal, tissue or cells according to any one of the preceding claims, wherein the non-human animal is a rodent, optionally a rat or a mouse, or is a non-human primate.

94. A non-human animal, tissue or cells according to any one of the preceding claims, wherein expression of one or more of said human DNA sequences is under the control of the corresponding human promoter.

95. A non-human animal, tissue or cells according to any one of the preceding claims, wherein expression of one or more of said human DNA sequences is under the control of the corresponding endogenous promoter of the non-human animal or mouse.

96. A method for investigating xenobiotic metabolism or toxicity, comprising the use of:

(i) a non-human animal, tissue or cells derived therefrom incorporating a

human DNA sequence encoding a protein involved in drug metabolism under the control of an endogenous regulatory sequence and optionally having its endogenous equivalent gene annulled;

(ii) a corresponding non-human animal, tissue or cells derived therefrom whose endogenous equivalent gene has been annulled but which does not incorporate a human DNA sequence encoding a corresponding protein involved in drug metabolism; and

(iii) optionally a corresponding wild-type non-human animal, tissue or cells derived therefrom.

97. A method according to claim 96, which comprises the use of:

(i) two or more, three or more, four or more, or five or more non-human animals, tissues or cells derived therefrom incorporating respectively two or more, three or more, four or more, or five or more human DNA sequences encoding different proteins involved in drug metabolism under the control of endogenous regulatory sequences, each non-human animal, tissue or cells optionally having its endogenous equivalent gene annulled;

(ii) two or more, three or more, four or more, or five or more corresponding non-human animals, tissues or cells derived therefrom whose endogenous equivalent genes have been annulled but which do not incorporate human DNA sequences encoding the relevant drug metabolism proteins; and

(iii) optionally a corresponding wild-type non-human animal, tissue or cells derived therefrom.

98. A method for investigating xenobiotic metabolism or toxicity, comprising the use of:

(i) a first non-human animal, tissue or cells derived therefrom incorporating a human DNA sequence encoding a protein involved in drug metabolism under the control of an endogenous human regulatory sequence, the non-human animal, tissue or cells optionally having its endogenous equivalent gene annulled;

(ii) a second non-human animal, tissue or cells derived incorporating a human DNA sequence encoding a protein involved in drug metabolism under the control of an endogenous non-human animal regulatory sequence, the non-human animal, tissue or cells optionally having its endogenous equivalent

gene annulled;

(iii) optionally a corresponding non-human animal, tissue or cells derived therefrom whose endogenous equivalent gene has been annulled but which does not incorporate a human DNA sequence encoding a corresponding protein involved in drug metabolism; and

(iv) optionally a corresponding wild-type non-human animal, tissue or cells derived therefrom.

99. A method for investigating xenobiotic metabolism or toxicity, comprising the use of at least three types of non-human animal, tissue or cells derived therefrom, wherein each type of non-human animal, tissue or cells comprises a different genetic modification affecting the amino acid sequence or expression of a protein involved in drug metabolism.

100. A transgenic non-human animal, tissue or cells derived therefrom, that comprises a DNA sequence that is at least 70% identical over its entire length to a nucleic acid molecule as recited in SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3 or SEQ ID NO:4, or a DNA sequence that is complementary to such a nucleic acid molecule.

101. A transgenic non-human animal, tissue or cells according to claim 100, wherein the DNA sequence is stably integrated at the locus of the endogenous equivalent gene.

102. A nucleic acid targeting vector that comprises a DNA sequence that is at least 70% identical over its entire length to a nucleic acid molecule as recited in SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3 or SEQ ID NO:4, or a DNA sequence that is complementary to such a nucleic acid molecule, and which further comprises 5' and 3' flanking nucleic acid sequences that are homologous to 5' and 3' regions in the locus of the endogenous equivalent gene, and which optionally further comprises nucleic acid sequence elements that permit conditional deletion of the human DNA sequence after its integration in the locus of the endogenous equivalent gene.

103. A non-human animal, tissue or cells derived therefrom containing a nucleic acid targeting vector according to claim 102.

104. A method for investigating xenobiotic metabolism or toxicity, comprising the use of:

(i) a first non-human animal, tissue or cells derived therefrom comprising a first endogenous regulatory sequence (e.g. a mouse or human promoter) operatively linked to a DNA sequence whose expression can conveniently

be measured by assay of transcription and/or translation products; and

(ii) a second non-human animal, tissue or cells derived therefrom comprising a second endogenous regulatory sequence (e.g. a mouse or human promoter) different to said first endogenous regulatory sequence operatively linked to a DNA sequence whose expression can conveniently be measured by assay of transcription and/or translation products,

(iii) wherein the first and second endogenous regulatory sequences are regulatory sequences normally associated with genes encoding proteins involved in drug metabolism.

105. A method for investigating xenobiotic metabolism or toxicity, comprising the use of at least three types of non-human animal, tissue or cells derived therefrom, wherein each type of non-human animal, tissue or cells comprises a different endogenous regulatory sequence operatively linked to a DNA sequence whose expression can conveniently be measured by assay of transcription and/or translation products, and wherein each endogenous regulatory sequence is a regulatory sequence normally associated with a gene encoding a protein involved in drug metabolism.

106. A method according to any one of claims 104 or 105, wherein the DNA sequence whose expression can conveniently be measured by assay of transcription and/or translation products is the same in the different non-human animals, tissues or cells.

107. A method according to any of claims 104-106, which comprises administering the same drug at the same dose to the different types of non-human animal and comparing the metabolism or toxicity of that drug between the different animals.

108. A method for investigating xenobiotic metabolism or toxicity, comprising administering a drug compound to two or more non-human animals, tissues or cells derived therefrom, wherein each non-human animal, tissue or cells comprises a human DNA sequence encoding a protein involved in drug metabolism under the control of an endogenous regulatory sequence, and optionally has its endogenous equivalent gene annulled.

109. A method according to claim 108, which further includes a step of comparing the experimental results obtained for different non-human animals, tissues or cells.

110. A transgenic non-human animal, tissue or cells derived therefrom, optionally a mouse, tissue or cells derived therefrom, having a genotype selected from the group

consisting of:

huPXR/huCAR; huPXR/huCAR/huAhR; huPXR/huCAR/huPPARα; huPXR/huCAR/huAhR/huPPARα;

koPXR/huCAR; huPXR/koCAR; koPXR/koCAR; koPXR/koCAR/koAhR; koPXR/koCAR/koPPARα; koPXR/koCAR/koAhR/koPPARα;

huCYP2C9/huPXR; huCYP2D6; huCYP2D6/huPXR; huCYP3A4/huPXR; huCYP3A cluster; huCYP3A cluster/huPXR; huCYP3A cluster/huPXR/huCAR; huCYP3A4/huPXR/huCAR; huCYP2C cluster; huPXR/huCYP2C cluster; huCYP2C cluster/huPXR/huCAR; huCYP1A1/huCYP1A2; huCYP2D6; koCyp1A cluster; huCYP1A1/huCYP1A2/huAhR; huCYP1A1/CYP1A2/huPXR/huCAR; huCYP2D6/huPXR/huCAR; koCyp2d Cluster; huCYP3A cluster/huCYP2C cluster/huCYP2D6/huPXR/huCAR

koCyp3a11/huPXR; huCYP3A4/koCyp3a11/huPXR; koCyp3a cluster/huPXR; koCyp2c cluster/huPXR; huCYP3A4/koCyp3a11/huPXR/huCAR; huCYP3A4/koCyp3a cluster/huPXR/huCAR; huCYP2C9/huPXR/huCAR; huCYP2C9/huPXR/huCAR/koCyp2c cluster; koCyp3a cluster/koCyp2c cluster/koCyp2d cluster; huPXR/huCAR/koCyp3a cluster/koCyp2c cluster/koCyp2d cluster

huUGT/huPXR/huCAR; huUGT1/huPXR/huCAR

huMDR1/mdr1a$^{-/-}$/huPXR; huMDR1/mdr1b$^{-/-}$/huPXR; huMDR1/mdr1a$^{-/-}$/mdr1b$^{-/-}$/huPXR; huMRP2/huPXR; huMRP2/huPXR/huCAR; huMRP2/ huMDR1/mdr1a$^{-/-}$/mdr1b$^{-/-}$/huPXR/huCAR; huCYP3A cluster/huMDR1/mdr1a$^{-/-}$/mdr1b$^{-/-}$/huPXR/huCAR; huCYP3A cluster/huMDR1/mdr1a$^{-/-}$/mdr1b$^{-/-}$/huUGT/huMRP2/huPXR/huCAR; huCYP3A cluster/huCYP2C cluster/huMDR1/mdr1a$^{-/-}$/mdr1b$^{-/-}$/huPXR/huCAR; huUGT/huMRP2/huPXR/huCAR; huMDR1/mdr1a$^{-/-}$/mdr1b$^{-/-}$/huPXR/huCAR; huMRP2/huPXR/huCAR; huPXR/huCAR/huMDR1a/b; huMDR1a; huMDR1b; huMDR1a/huMDR1b (*i.e.*, huMDR1a/b);

huPXR/huCAR/koMDR1a/b;

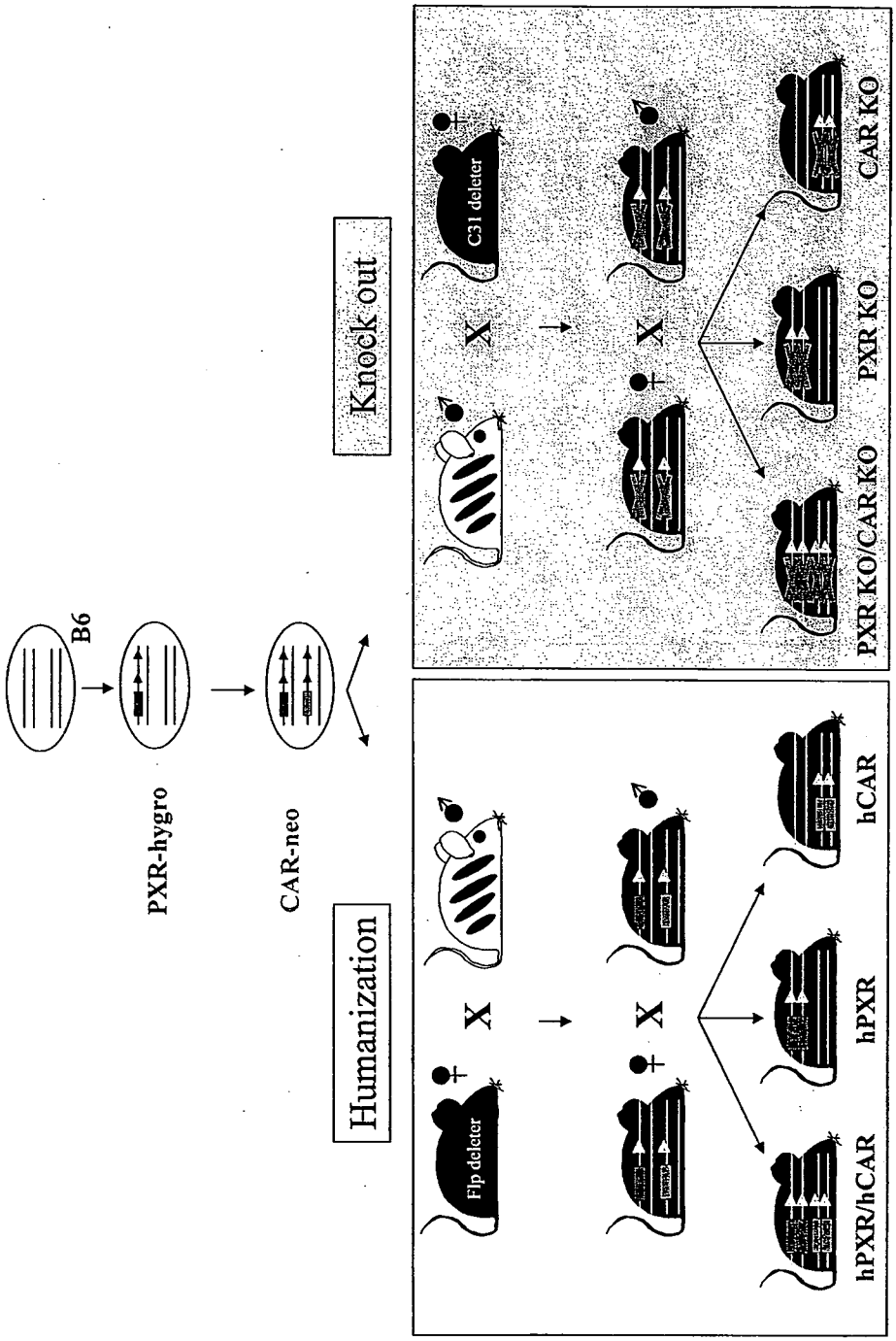
rCYP2B6/huCAR; rCyp3a11/huPXR; rCyp3a11/huPXR; rCYP3A4/huPXR; rCYP2C9/huPXR; rCYP2D6/huPXR; rCYP2C19/huPXR; rCYP3A4/huPXR/huCAR;

rCYP2C9/huPXR/huCAR; rCYP2D6/huPXR/huCAR; rCYP2C19/huPXR/huCAR; rCYP2B6/huPXR/huCAR; rCYP3A4/huCYP2B6/huPXR/huCAR; rCYP3A4/huCYP2C9/huPXR/huCAR; rCYP2D6/huCYP2C19/huPXR/huCAR; rCYP3A4/huCYP2C9/huCYP2D6/huCYP2C19/huPXR/huCAR; rCYP3A4/huCYP2D6/huPXR/huCAR; rCYP2B6/huMDR1/mdr1a$^{-/-}$/ mdr1b$^{-/-}$ /huPXR/huCAR; rCYP3A4/huCYP2C9/huCYP2B6/huMDR1/mdr1a$^{-/-}$/mdr1b$^{-/-}$ /huPXR/huCAR; rCYP3A4/huCYP2B6/MDR1/huPXR/huCAR; rCYP3A4/huCYP2D6/huCYP2B6/huMDR1/mdr1a$^{-/-}$/mdr1b$^{-/-}$/huPXR/huCAR; huPXR/rCYP3A4; rCyp3a11; huPXR/huCAR/rCYP3A4; hCAR/rCYP2B6; huPXR/huCAR/rCYP2B6; huPXR/huCAR/rCYP2D6; hPXR/rCYP2D6;

rMDR1/huPXR; rMDR1/huPXR/huCAR; rMDR1; huPXR/huCAR/rMDR1; huPXR/huCAR/rCYP2B6/r2D6/r3A4/rMDR1.

FIG. 1

Humanization and Knock out of PXR and CAR



PXR-humanization: targeting strategy

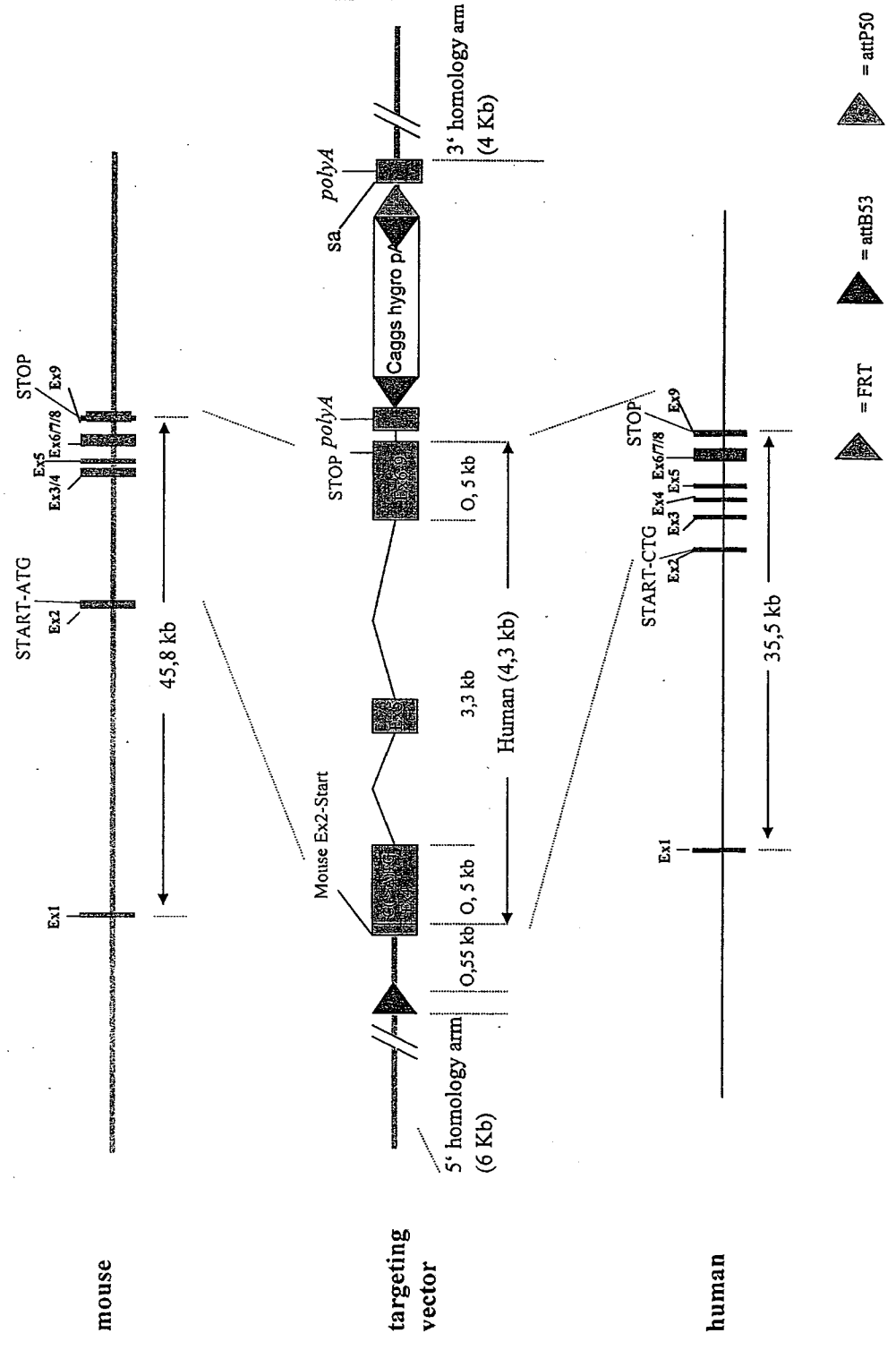


FIG. 2

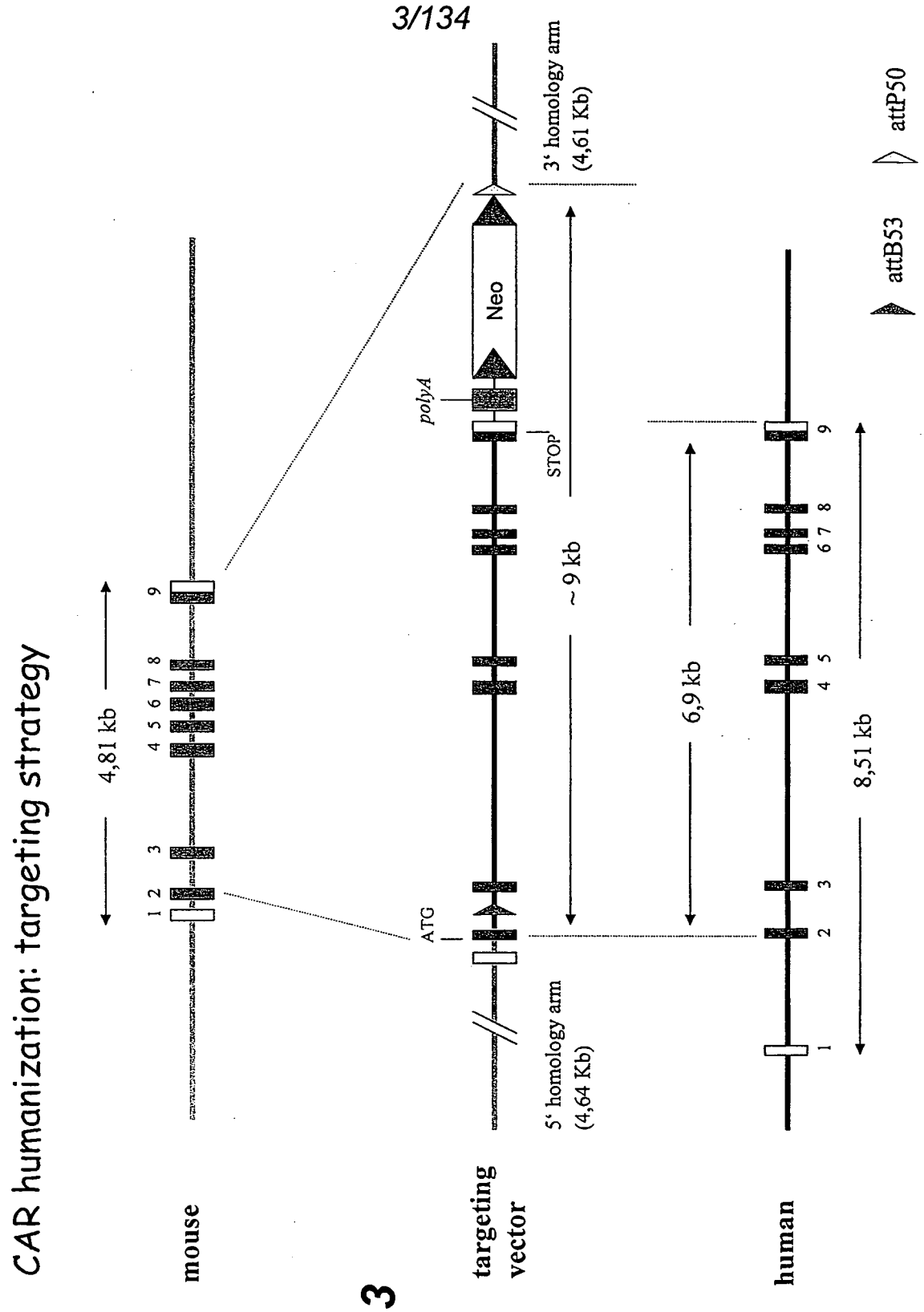

CAR humanization: targeting strategy
mouse
4,81 kb
1 2 3 4 5 6 7 8 9
targeting vector
ATG
polyA
Neo
STOP
5' homology arm (4,64 Kb)
3' homology arm (4,61 Kb)
~ 9 kb
6,9 kb
8,51 kb
human
1 2 3 4 5 6 7 8 9
attB53
attP50

FIG. 3

FIG. 4 (Mdr1a)

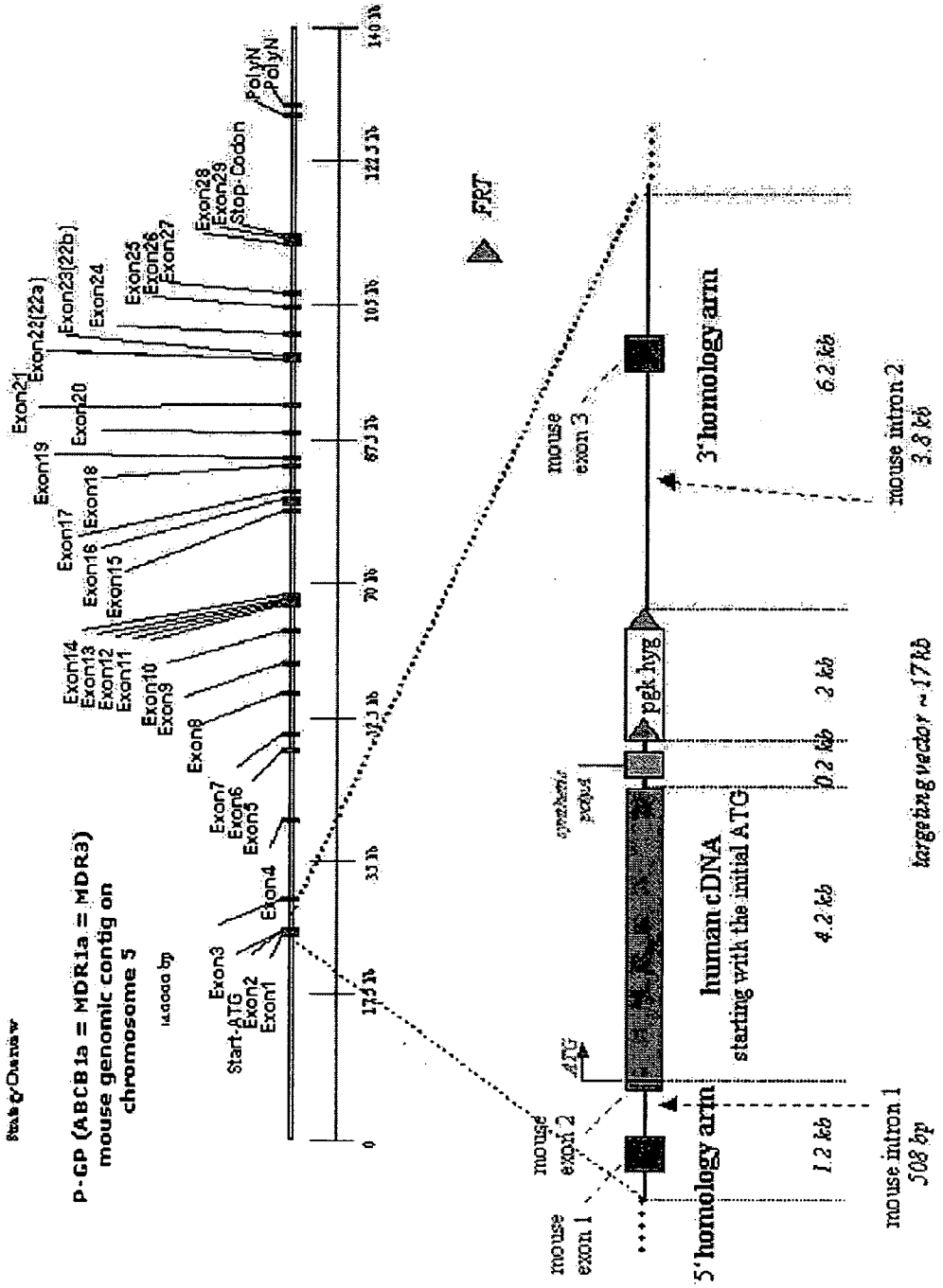

P-GP (ABCB1a = MDR1a = MDR3)
mouse genomic contig on
chromosome 5
Exon1
Start-ATG
Exon2
Exon3
Exon4
Exon5
Exon6
Exon7
Exon8
Exon9
Exon10
Exon11
Exon12
Exon13
Exon14
Exon15
Exon16
Exon17
Exon18
Exon19
Exon20
Exon21
Exon22(22a)
Exon23(22b)
Exon24
Exon25
Exon26
Exon27
Exon28
Exon29
Stop-Codon
PolyN
PolyN
FRT
mouse exon 1
mouse exon 2
ATG
human cDNA
starting with the initial ATG
mouse exon 3
pgk-hyg
5' homology arm
1.2 kb
4.2 kb
0.2 kb
2 kb
3' homology arm
6.2 kb
mouse intron 1
508 bp
mouse intron 2
3.8 kb
targeting vector ~ 17 kb

FIG. 5 (Mdr1b)

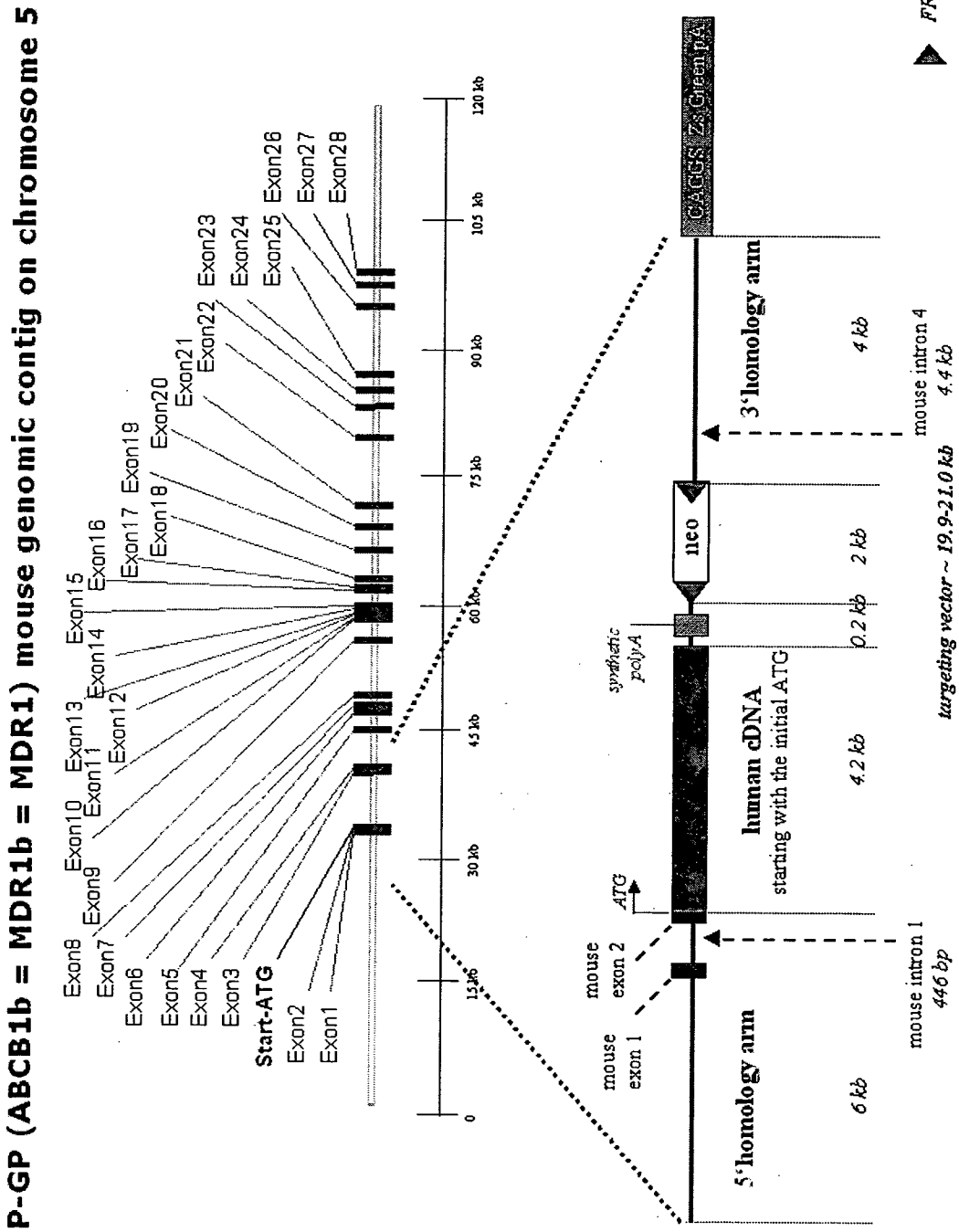

P-GP (ABCB1b = MDR1b = MDR1) mouse genomic contig on chromosome 5
Exon1
Exon2
Start-ATG
Exon3
Exon4
Exon5
Exon6
Exon7
Exon8
Exon9
Exon10
Exon11
Exon12
Exon13
Exon14
Exon15
Exon16
Exon17
Exon18
Exon19
Exon20
Exon21
Exon22
Exon23
Exon24
Exon25
Exon26
Exon27
Exon28
0
15 kb
30 kb
45 kb
60 kb
75 kb
90 kb
105 kb
120 kb
5'homology arm
6 kb
mouse exon 1
mouse exon 2
ATG
mouse intron 1
446 bp
human cDNA
starting with the initial ATG
4.2 kb
synthetic polyA
0.2 kb
neo
2 kb
CAGGS Zs Green pA
3'homology arm
4 kb
mouse intron 4
4.4 kb
FRT
targeting vector ~ 19.9-21.0 kb

FIG. 6 (MRP2)

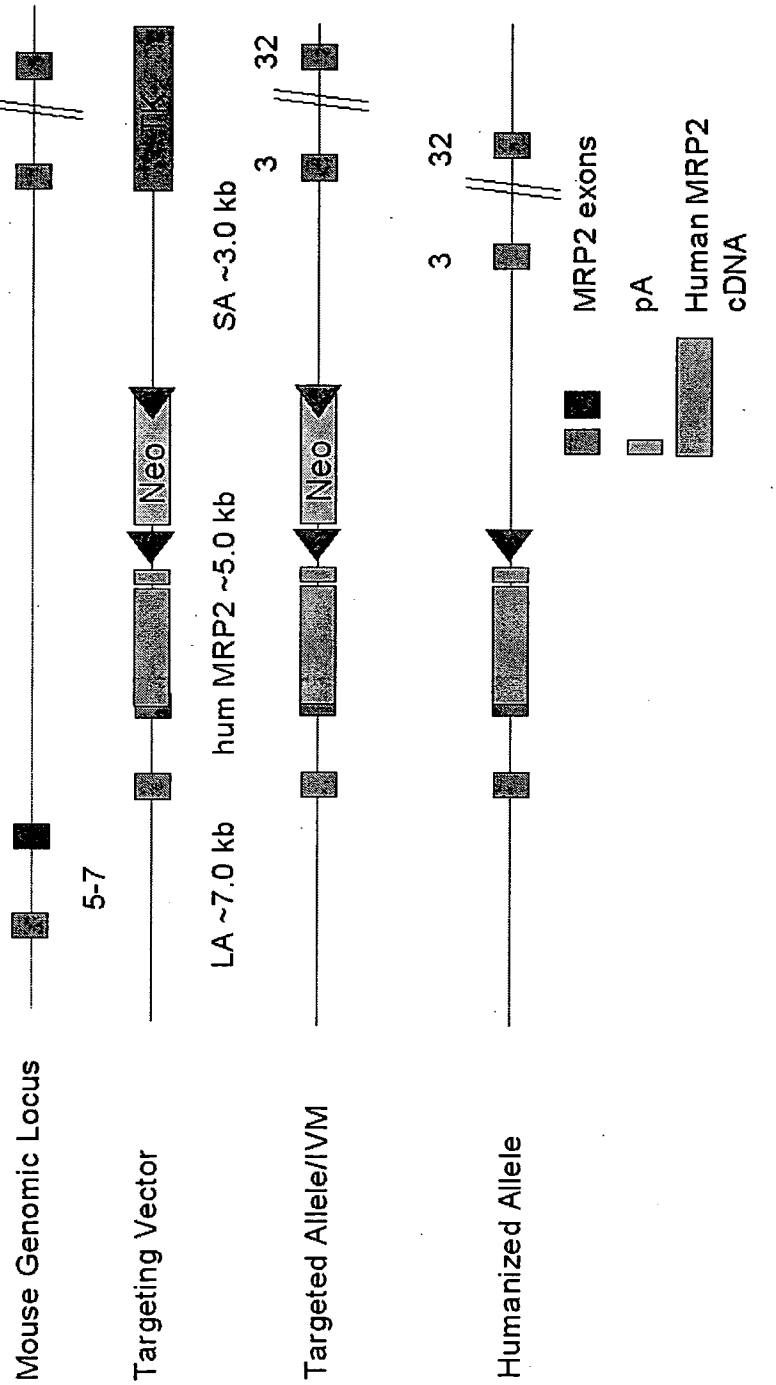

MRP2 Conditional KI Project
Mouse Genomic Locus
1
2
5-7
3
32
Targeting Vector
TK
Neo
LA ~7.0 kb
hum MRP2 ~5.0 kb
SA ~3.0 kb
Targeted Allele/IVM
Neo
Humanized Allele
MRP2 exons
pA
Human MRP2 cDNA

FIG. 7 (PXR)

PXR-humanization: targeting strategy

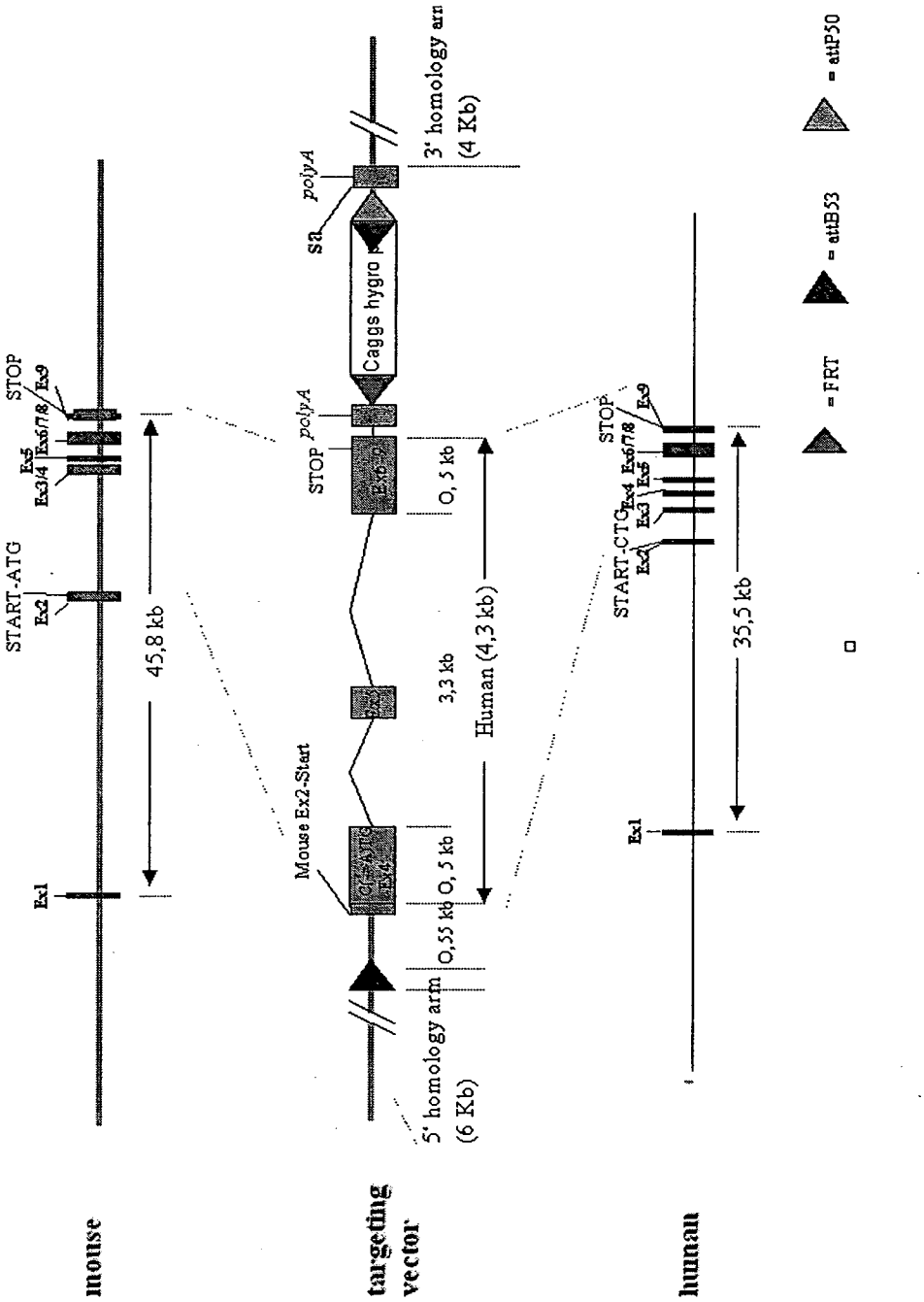


FIG. 8 (CAR)

CAR humanization: targeting strategy

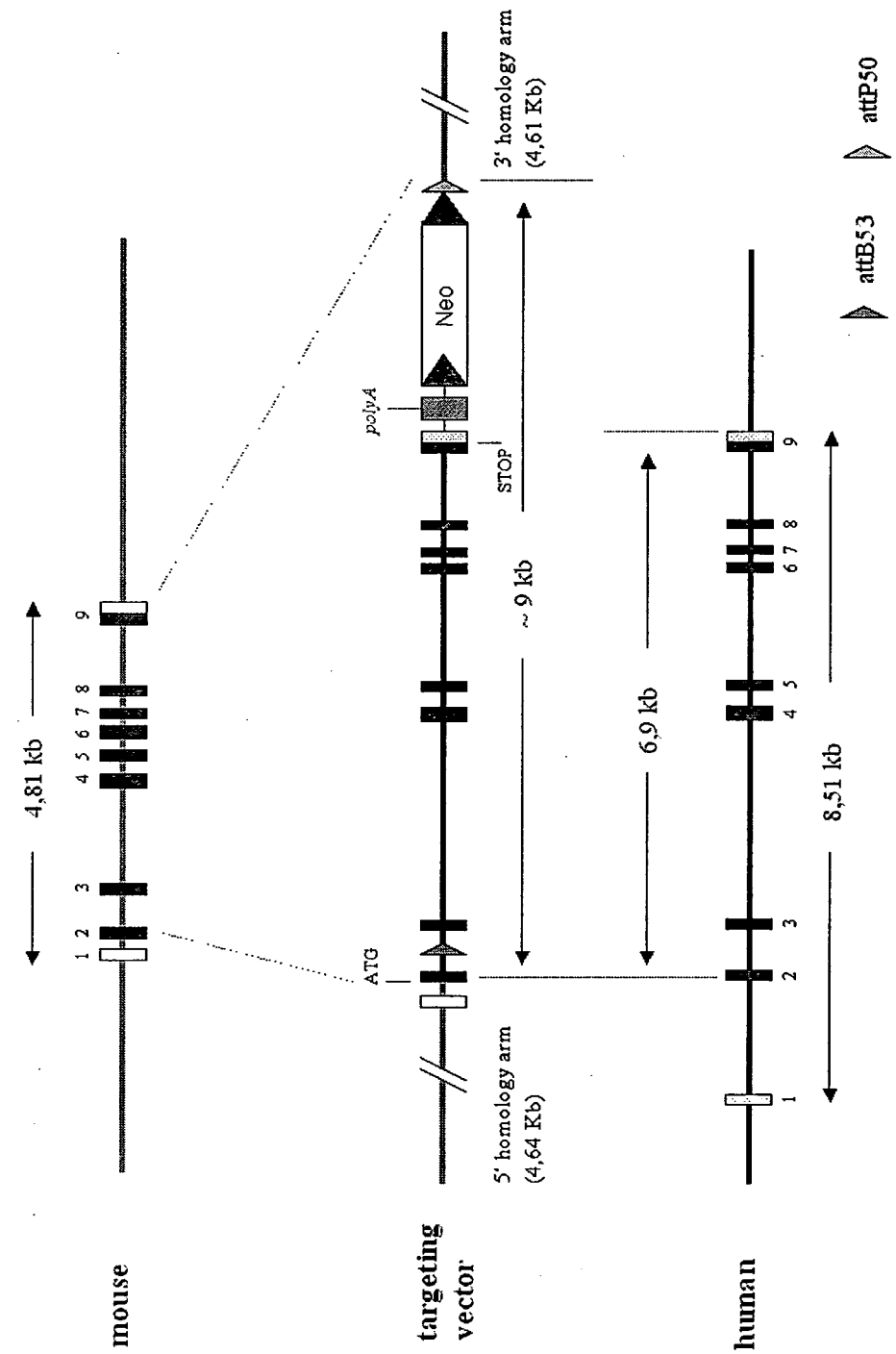


FIG. 9 (CYP3A4)

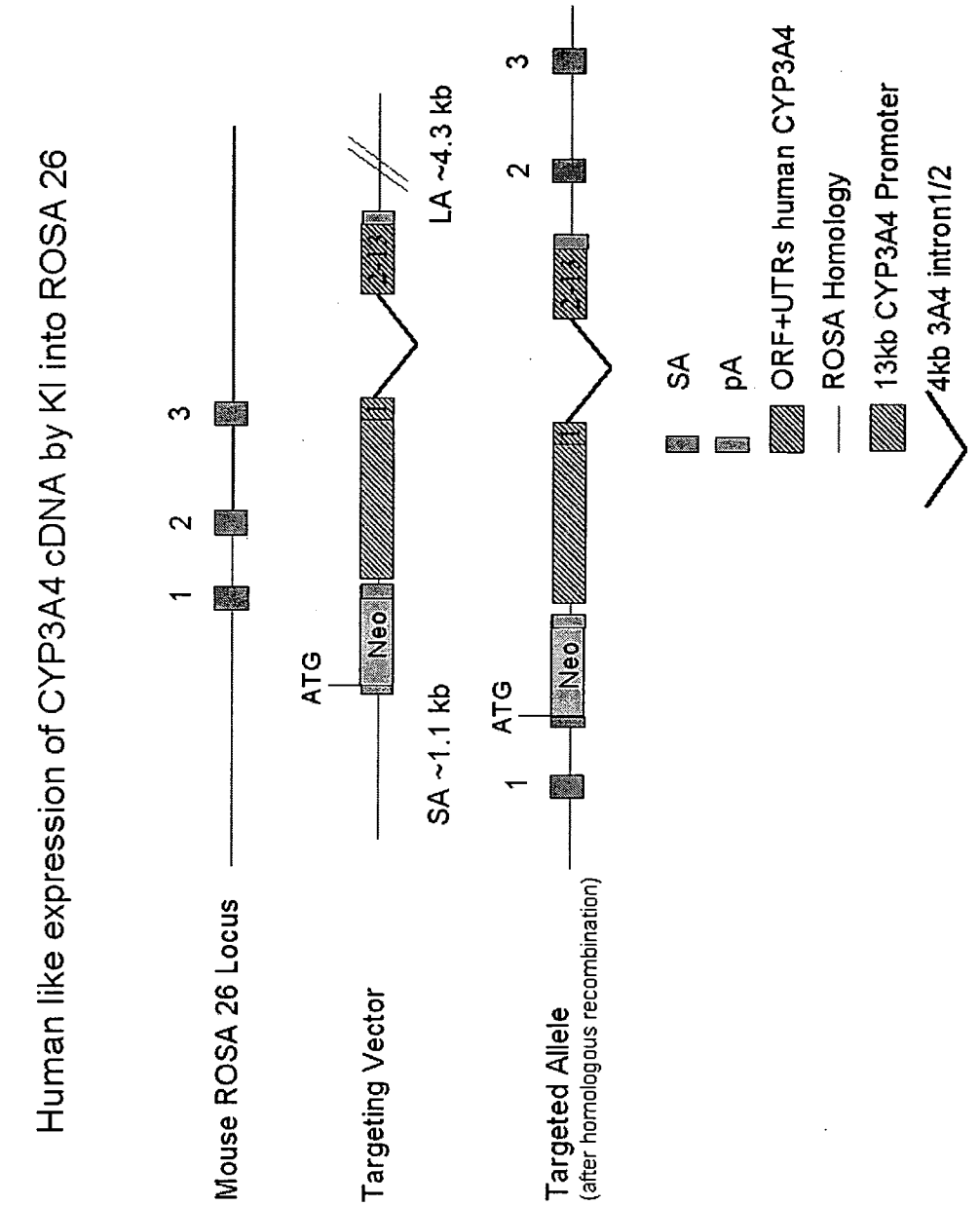

Human like expression of CYP3A4 cDNA by KI into ROSA 26
1 2 3
Mouse ROSA 26 Locus
ATG
Neo
Targeting Vector
SA ~1.1 kb
LA ~4.3 kb
1 ATG
2 3
Neo
Targeted Allele
(after homologous recombination)
SA
pA
ORF+UTRs human CYP3A4
ROSA Homology
13kb CYP3A4 Promoter
4kb 3A4 intron1/2

FIG. 10 (CYP2C9)

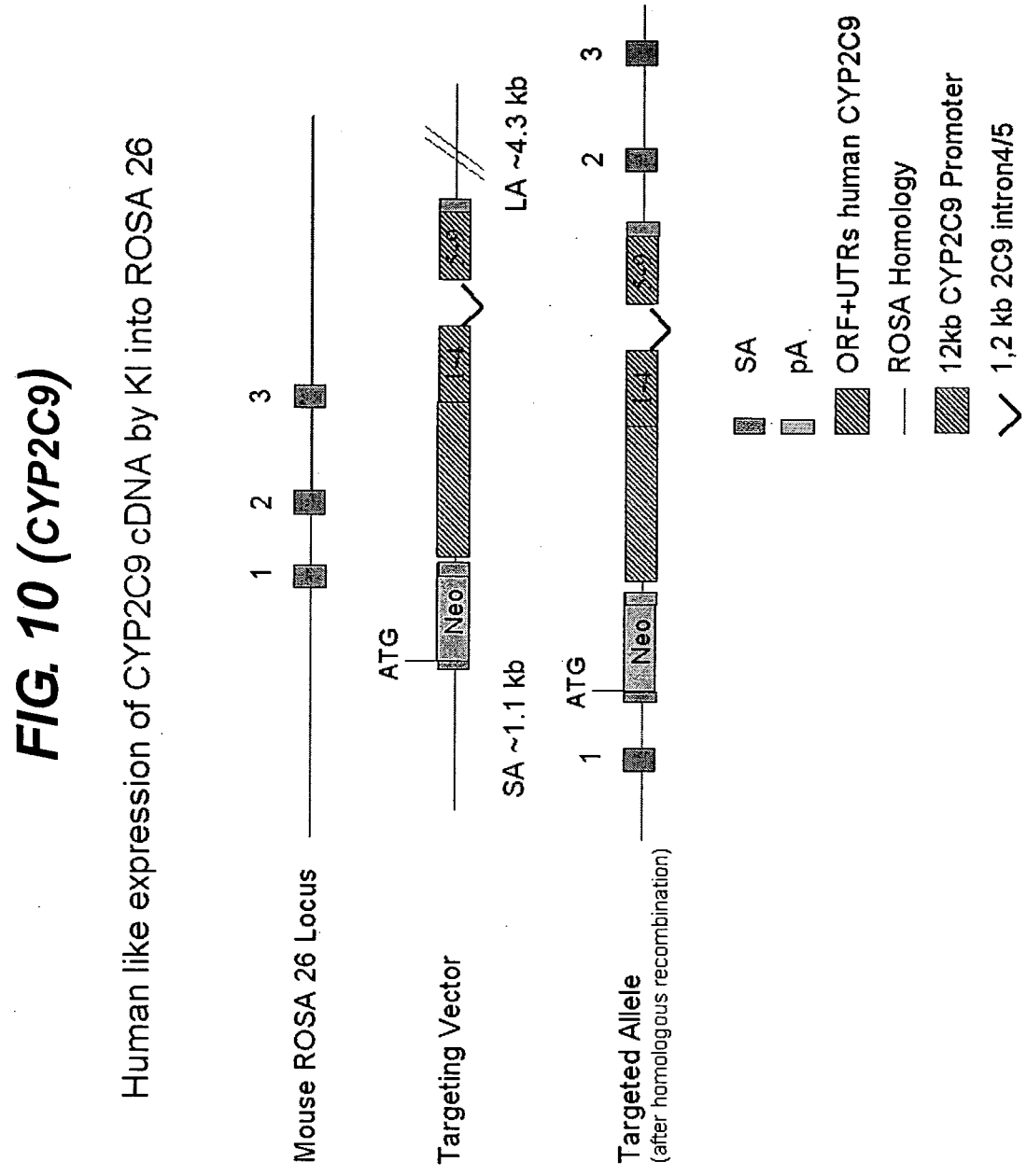


FIG. 11 (Overall strategy for cluster exchange)

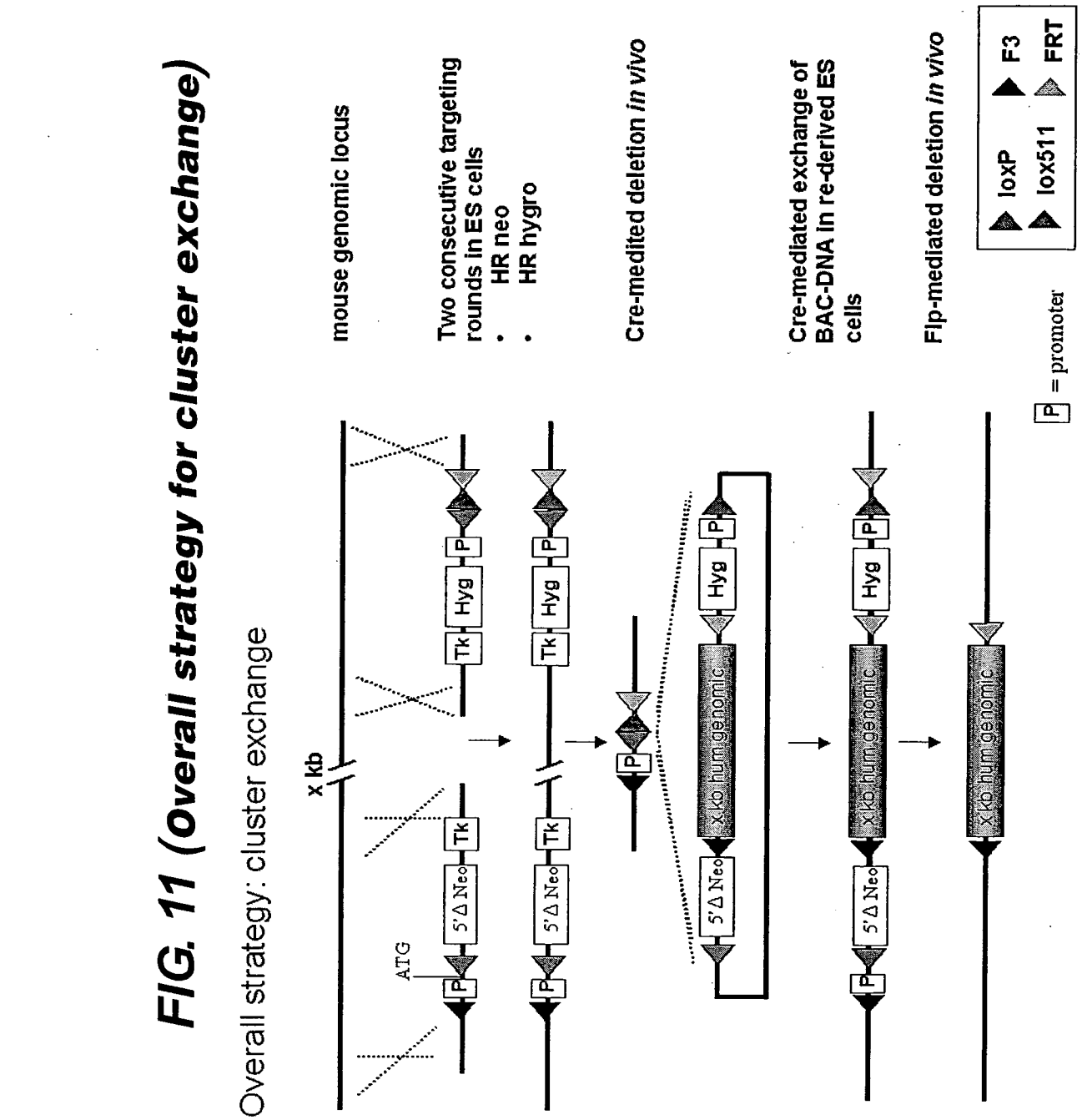


FIG. 12 (Reporter project strategy)

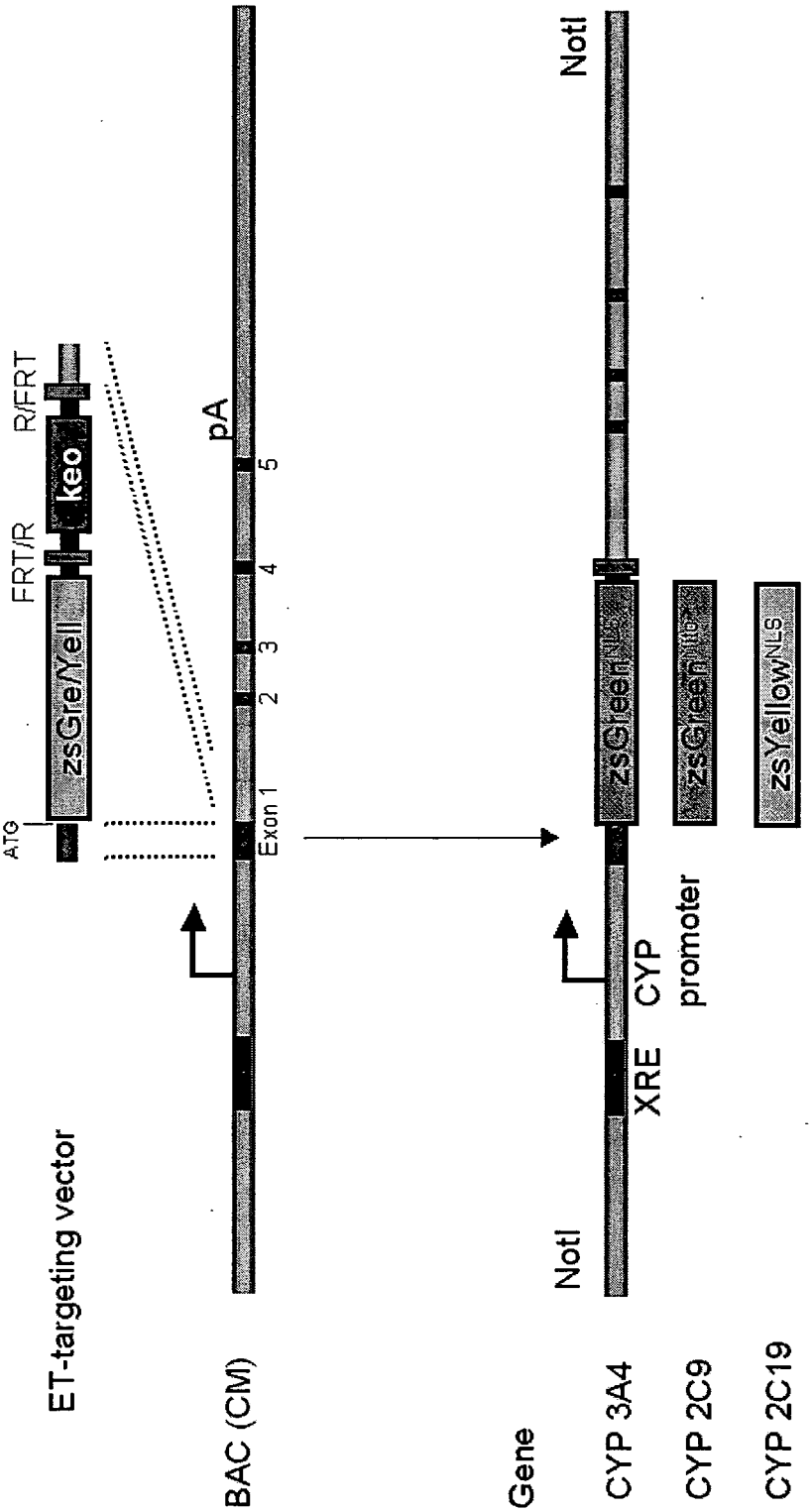


FIG. 13 (Reporter project strategy for CYP2D6 and CYP2B6)

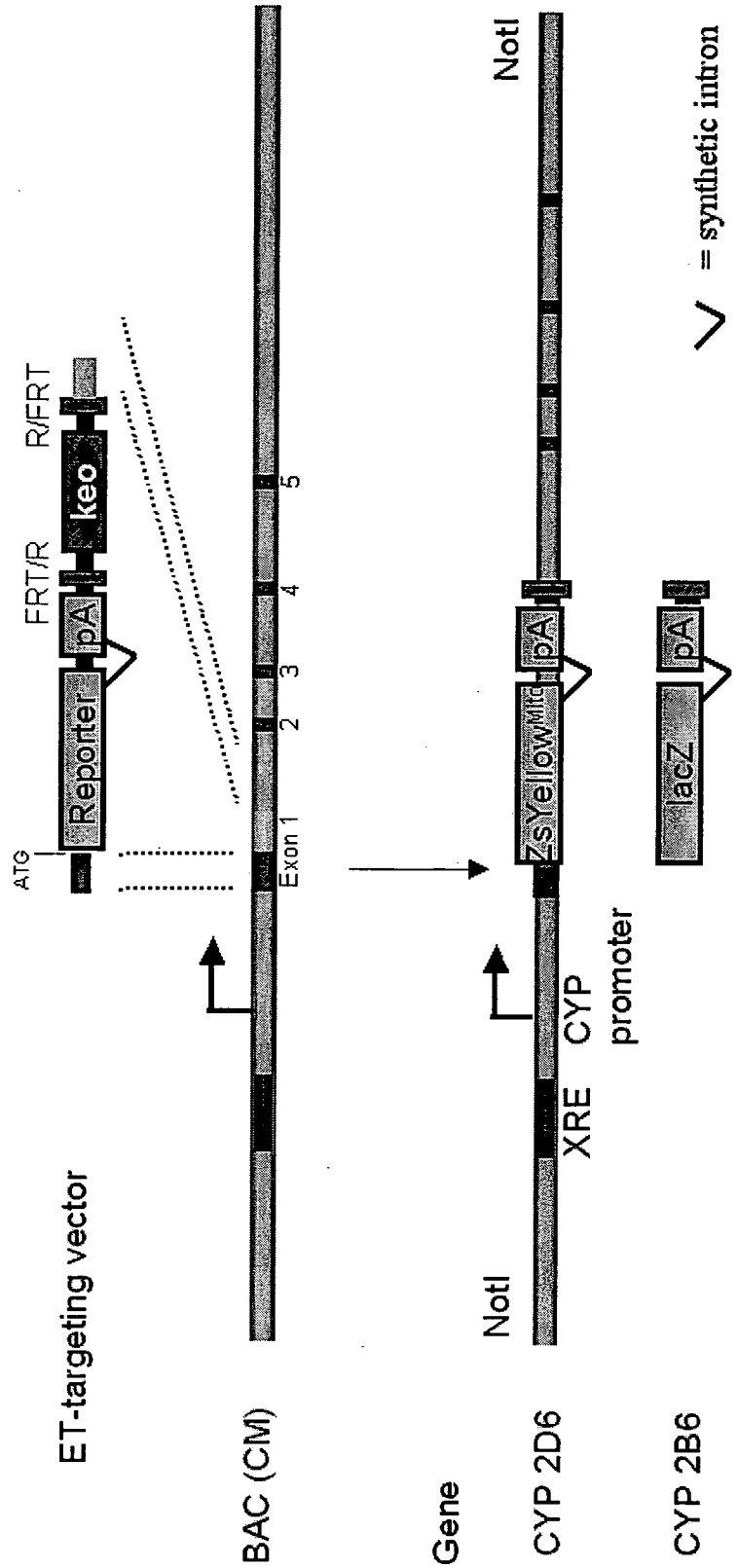


FIG. 14 (reporter project strategy for MDR1)

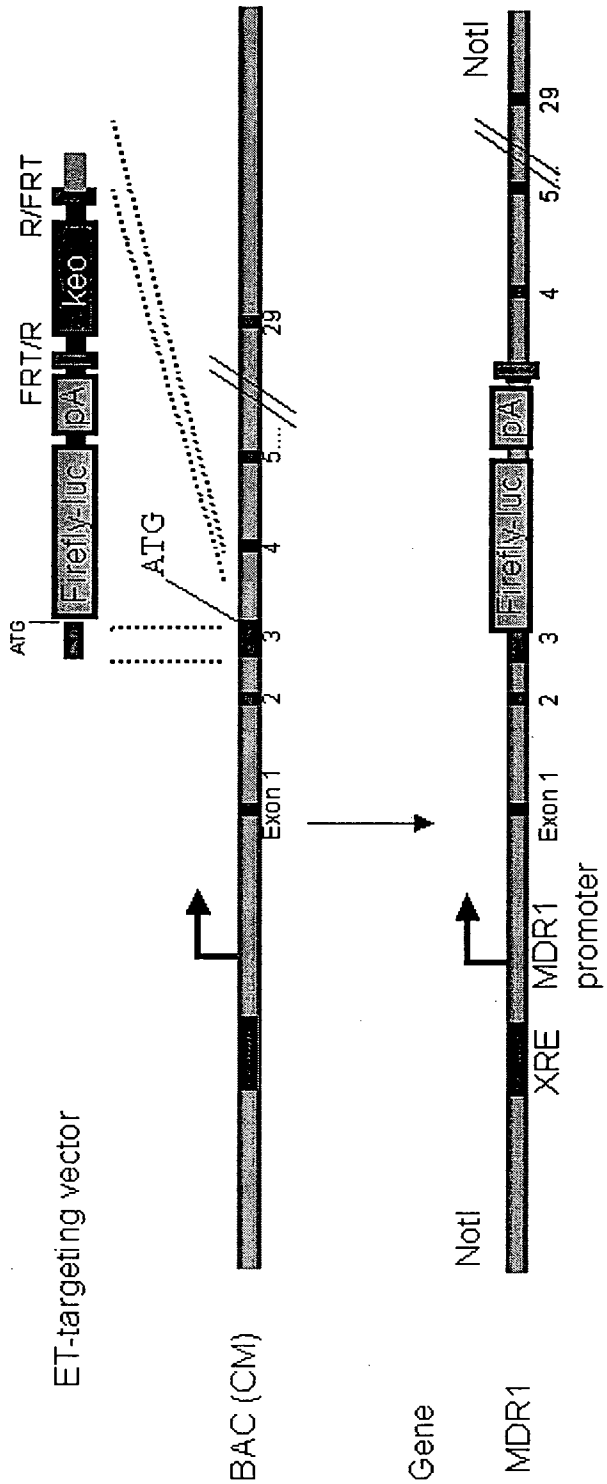
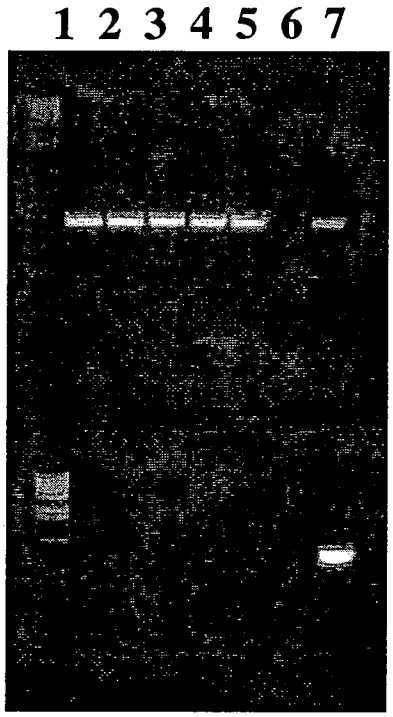


FIG. 15 (Example of a PXR typing PCR)



ID	
1	32599
2	32600
3	32601
4	32606
5	32607
6	H2O
7	+ control

Primer combination	Detected Genotype	Size of expected fragment
PxTf1/PxTHr1	Humanized PXR	386 bp
PxTf1/PxTMr1	Mouse PXR	700 bp

Gene	TaqMan® Genomic Assay catalogue #	TaqMan® Genomic Assay batch #
Human PXR	hPXR_Hs00243666_ml	295782
Mouse PXR	mPXR _Mm00803092_ml	295782
Mouse β-actin	β-actin_Mm607939_sl	312686

TaqMan® Genomic Assays used.

FIG. 16A

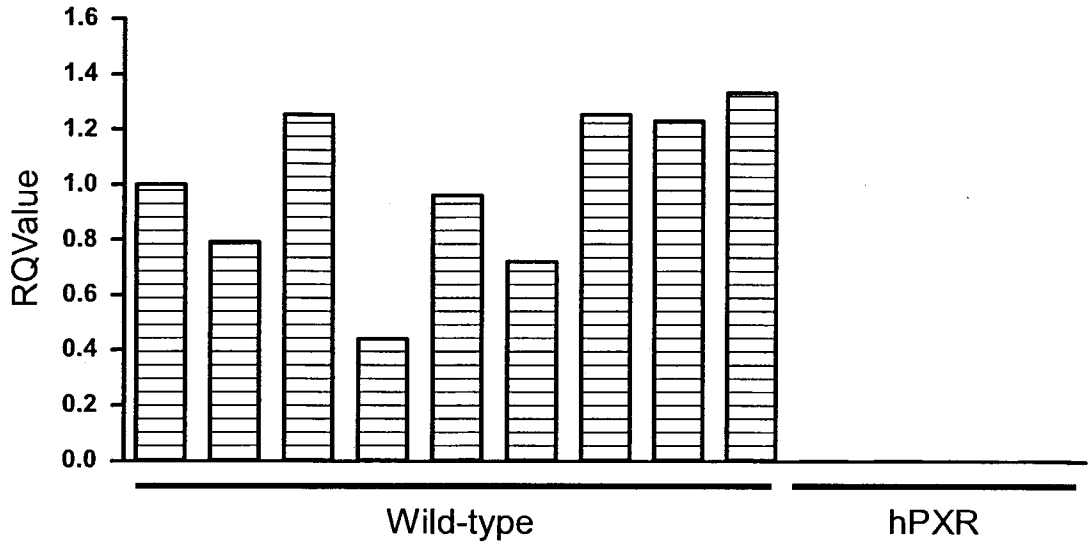


FIG. 16B

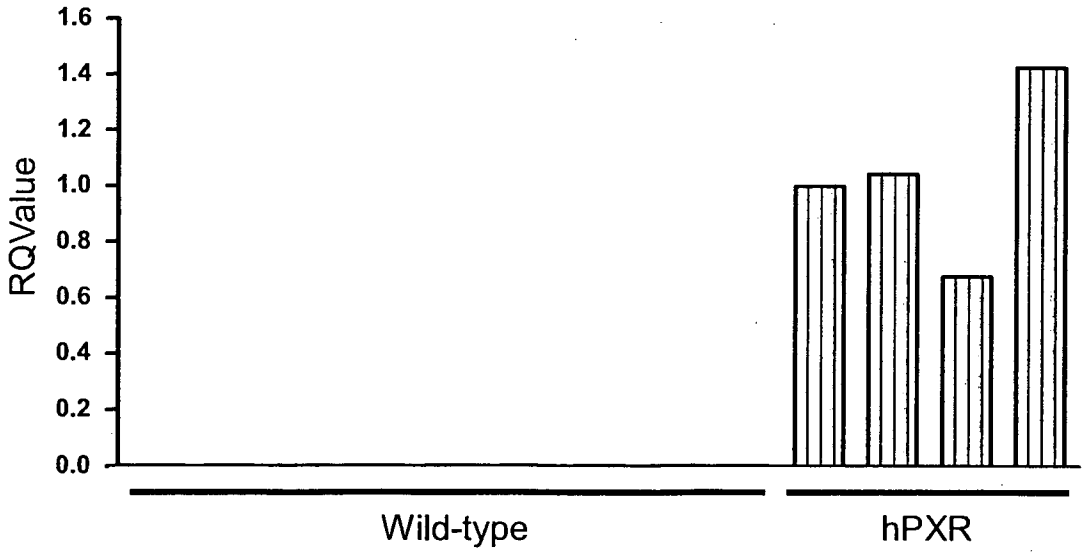


FIG. 16C

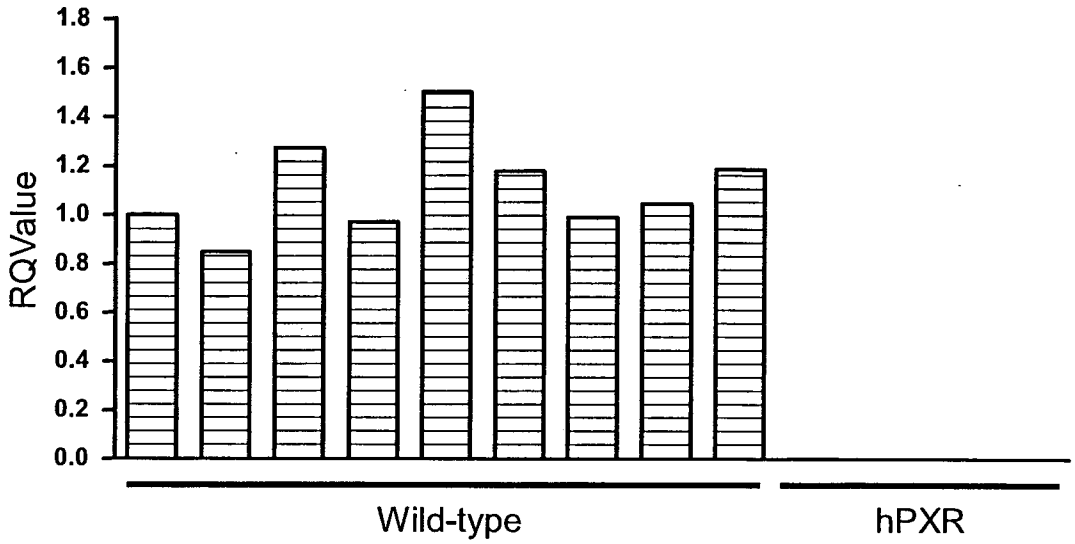


FIG. 16D

FIG. 17

MW- Molecular weight marker 1kb ladder (Advantage).

1. RT-PCR product. Two major bands of 1.3 and 1.1 kb were observed, additional minor bands were observed of ~1.2 and 1.0kb.

FIG. 18

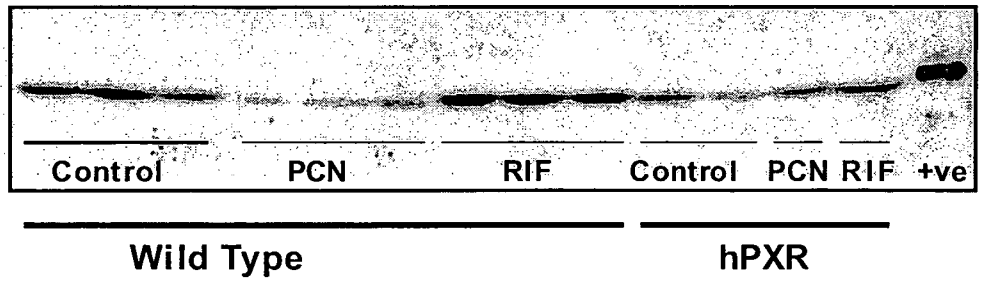
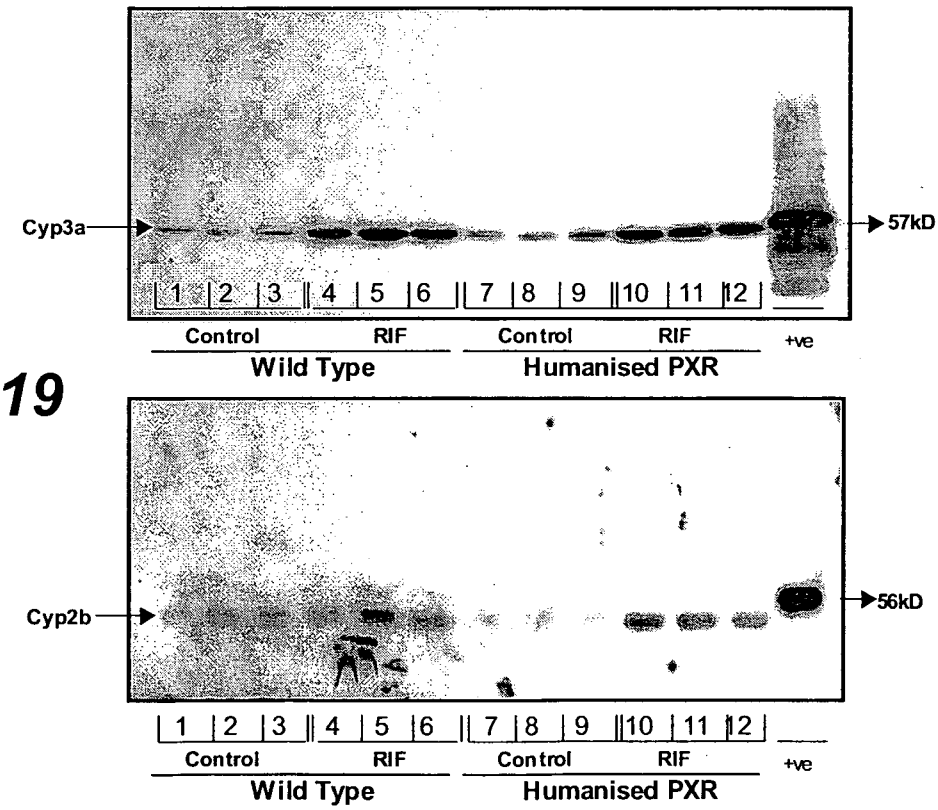


FIG. 19



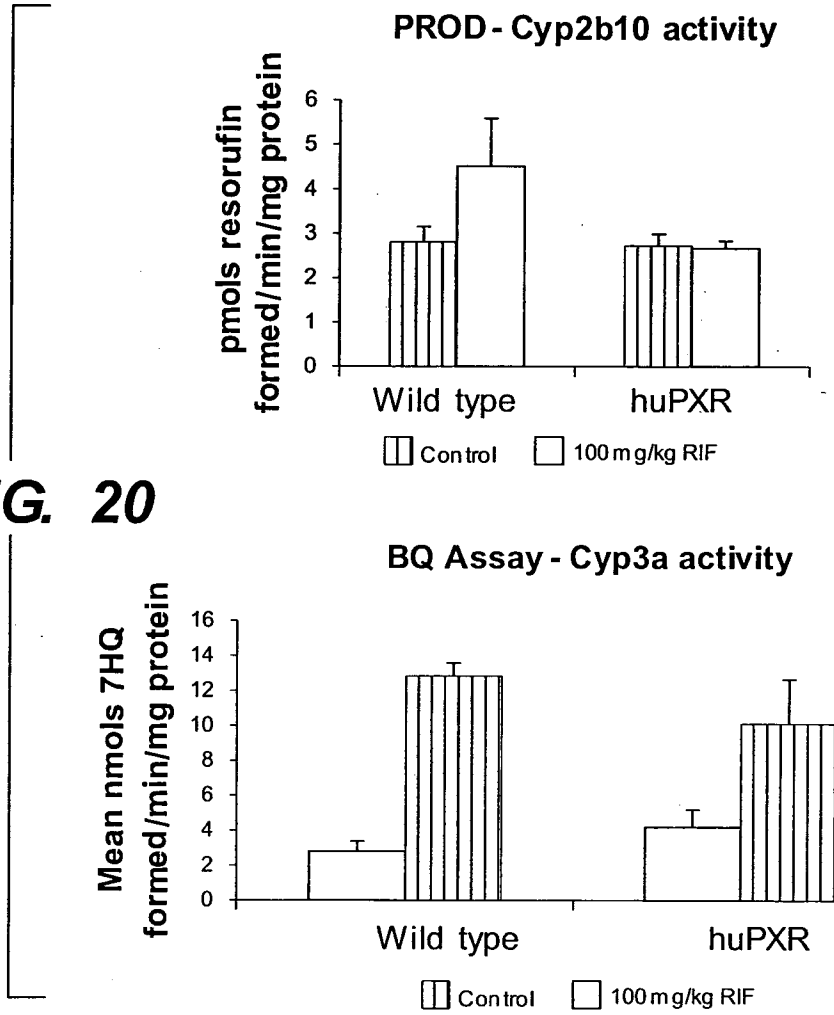

PROD - Cyp2b10 activity
pmols resorufin formed/min/mg protein
0
1
2
3
4
5
6
Wild type
huPXR
Control
100 mg/kg RIF
BQ Assay - Cyp3a activity
Mean nmols 7HQ formed/min/mg protein
0
2
4
6
8
10
12
14
16
Wild type
huPXR
Control
100 mg/kg RIF

FIG. 20

FIG. 21

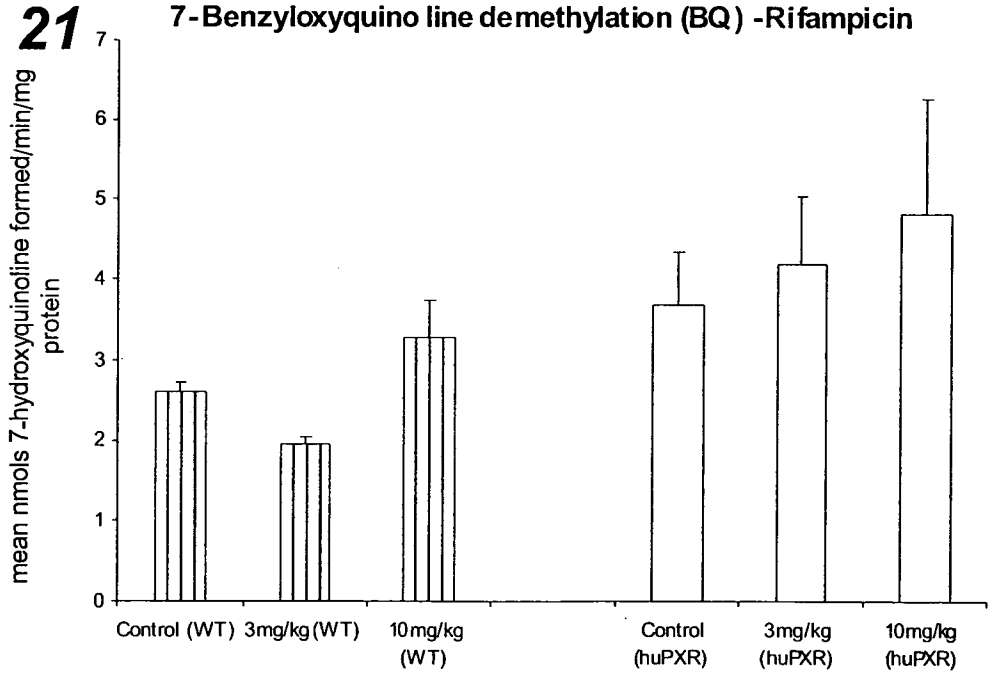

7-Benzyloxyquino line demethylation (BQ) -Rifampicin
mean nmols 7-hydroxyquinoline formed/min/mg protein
0
1
2
3
4
5
6
7
Control (WT)
3mg/kg (WT)
10mg/kg (WT)
Control (huPXR)
3mg/kg (huPXR)
10mg/kg (huPXR)

FIG. 22

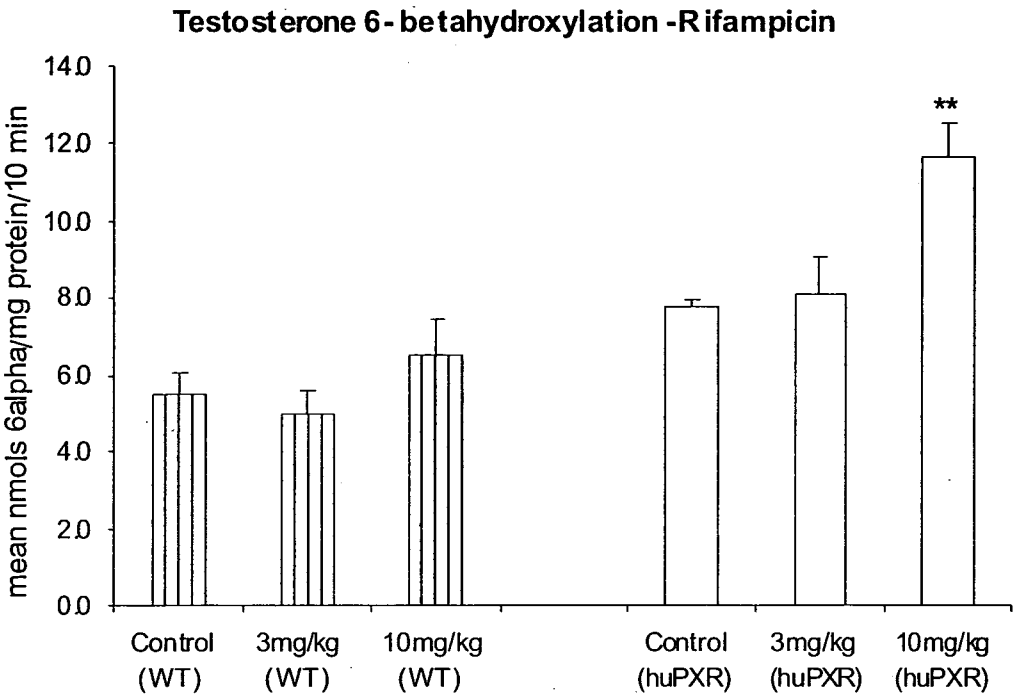

Testosterone 6-betahydroxylation -Rifampicin
mean nmols 6alpha/mg protein/10 min
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
**
Control (WT)
3mg/kg (WT)
10mg/kg (WT)
Control (huPXR)
3mg/kg (huPXR)
10mg/kg (huPXR)

FIG. 23

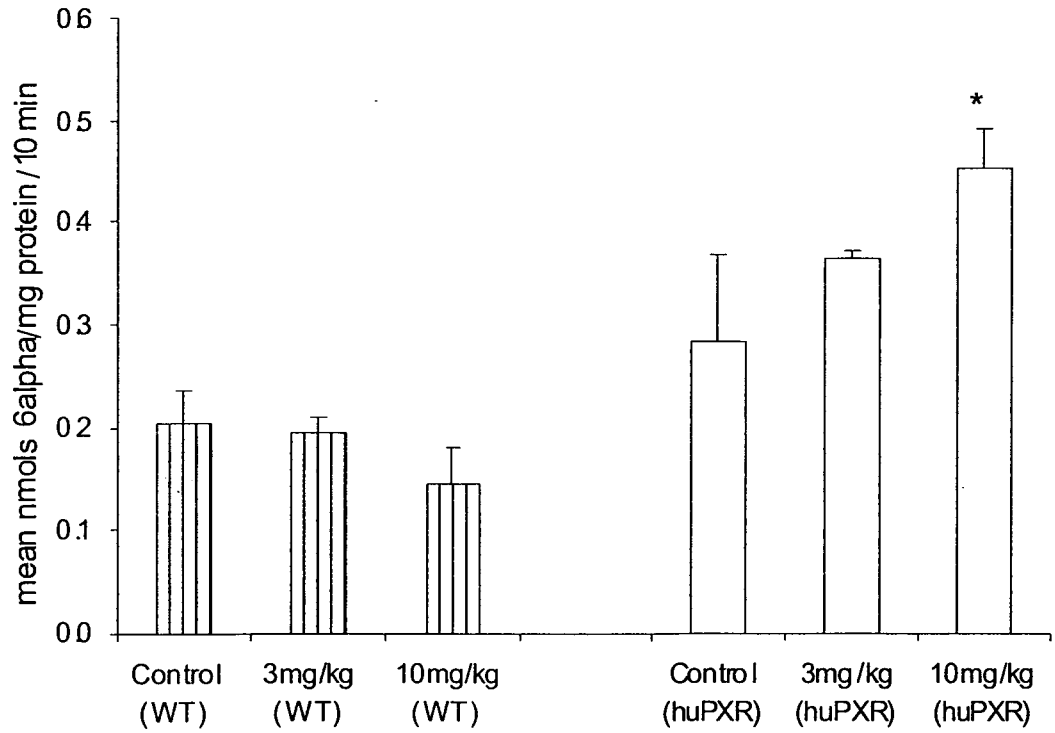

Testosterone 16-beta hydroxylation - Rifampicin
mean nmols 6alpha/mg protein / 10 min
0.6
0.5
0.4
0.3
0.2
0.1
0.0
*
Control (WT)
3mg/kg (WT)
10mg/kg (WT)
Control (huPXR)
3mg/kg (huPXR)
10mg/kg (huPXR)

FIG. 24

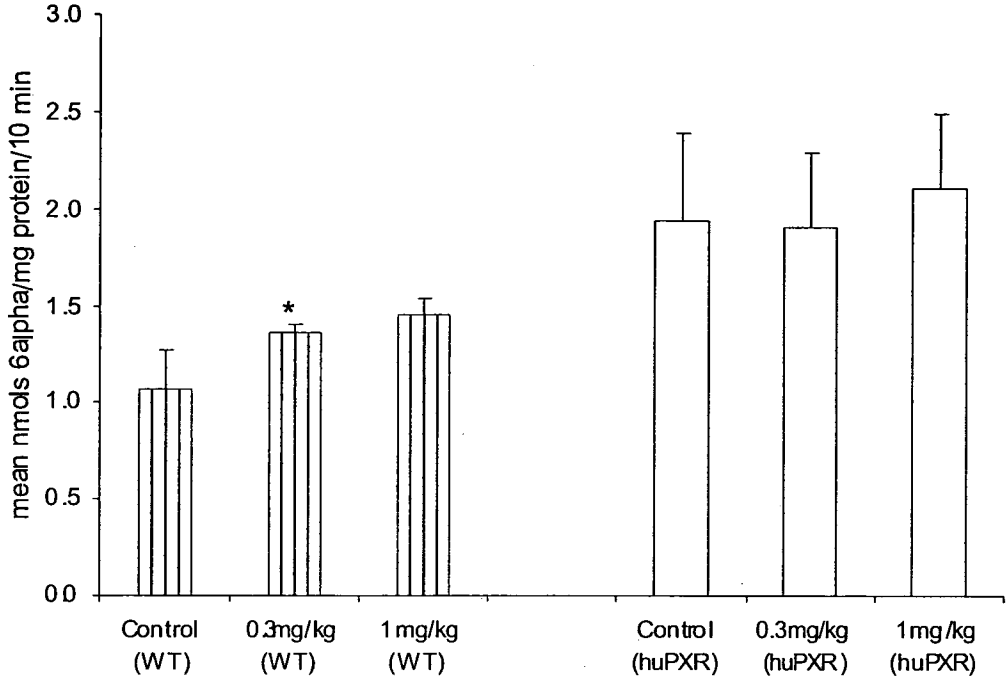

Testosterone 7-alpha hydroxylation -TCPOBOP
mean nmols 6alpha/mg protein/10 min
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
Control (WT)
0.3mg/kg (WT)
1mg/kg (WT)
Control (huPXR)
0.3mg/kg (huPXR)
1mg/kg (huPXR)

FIG. 25

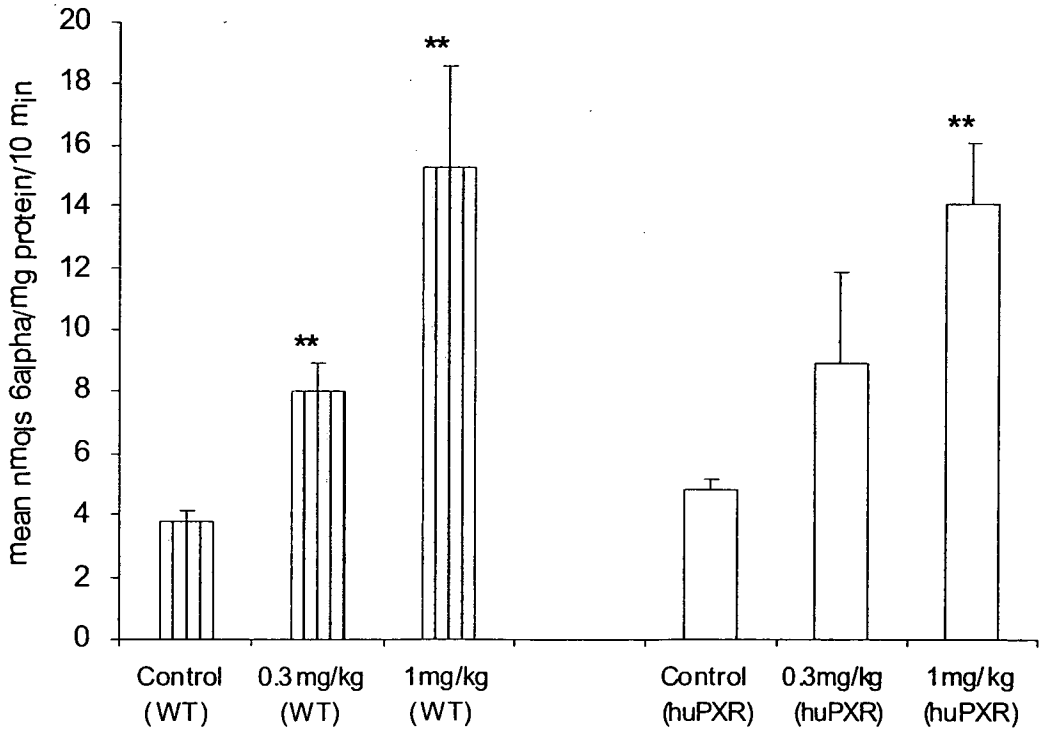

Testosterone 6-beta hydroxylation -TCPOBOP
mean nmols 6alpha/mg protein/10 min
20
18
16
14
12
10
8
6
4
2
0
**
**
**
Control (WT)
0.3mg/kg (WT)
1mg/kg (WT)
Control (huPXR)
0.3mg/kg (huPXR)
1mg/kg (huPXR)

FIG. 26

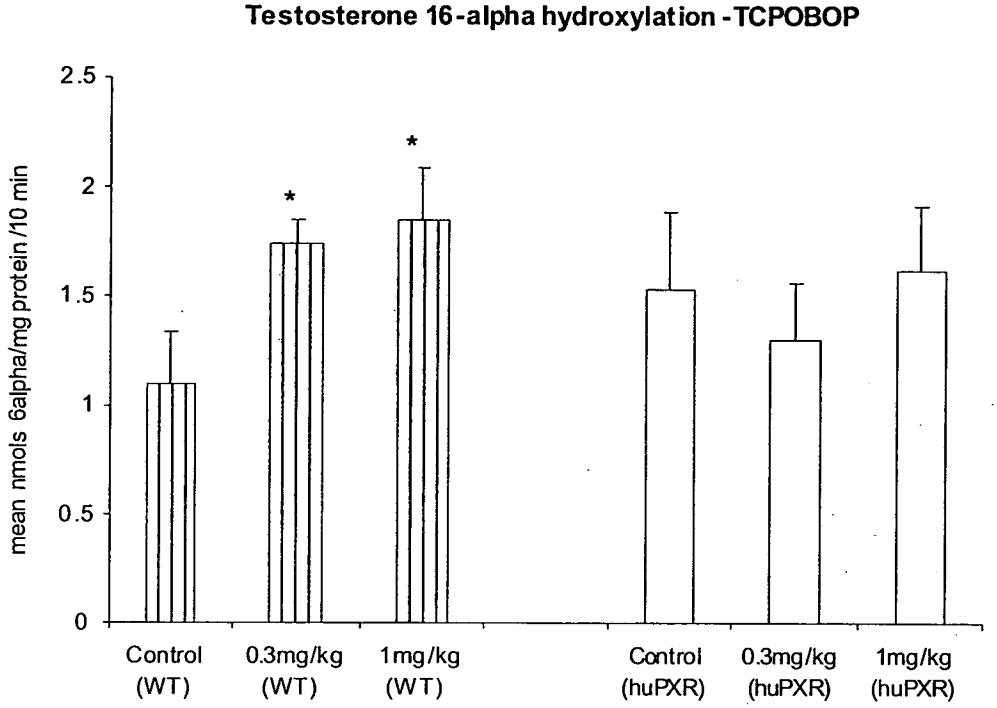

Testosterone 16-alpha hydroxylation -TCPOBOP
mean nmols 6alpha/mg protein /10 min
2.5
2
1.5
1
0.5
0
*
*
Control (WT)
0.3mg/kg (WT)
1mg/kg (WT)
Control (huPXR)
0.3mg/kg (huPXR)
1mg/kg (huPXR)

FIG. 27

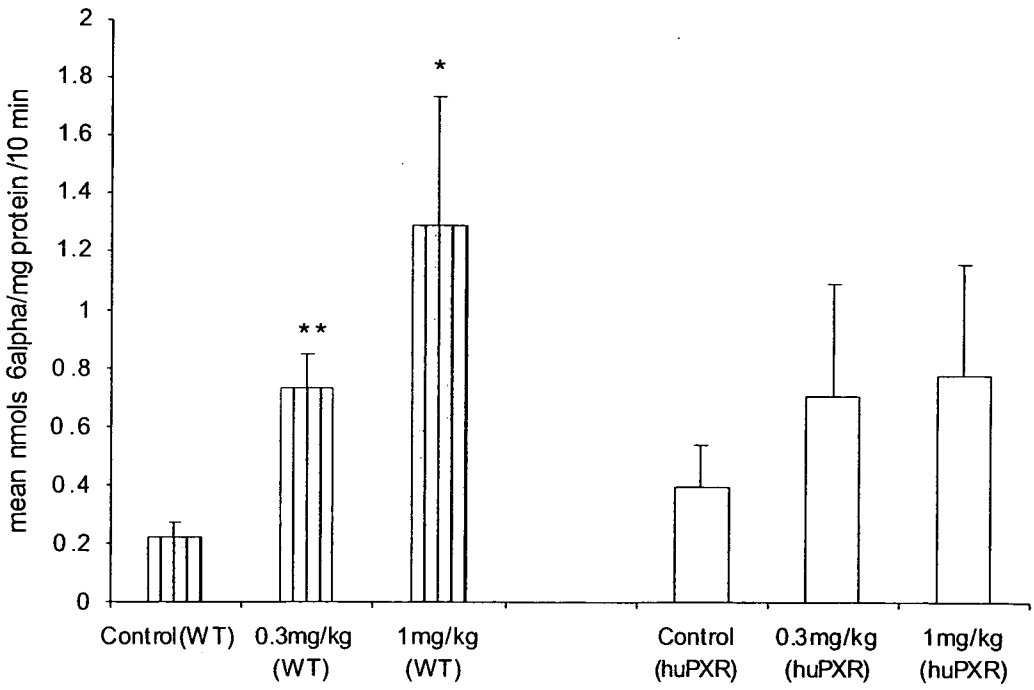
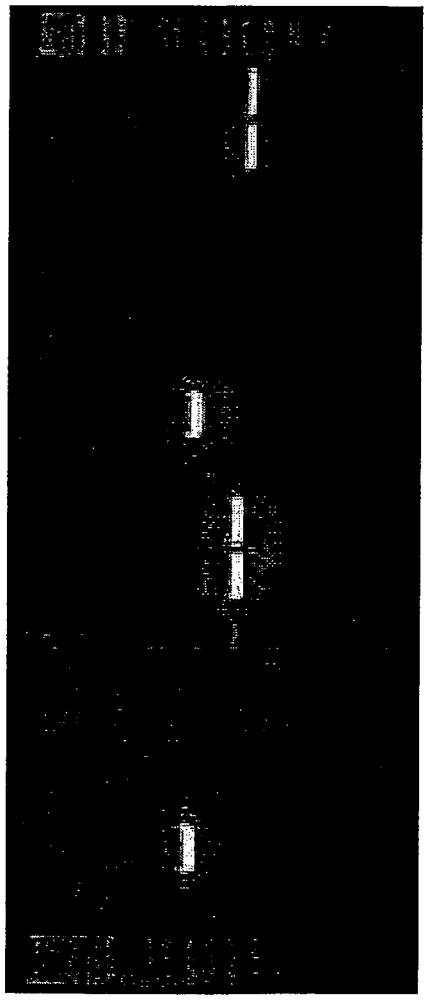

Testosterone 16-beta hydroxylation -TCPOBOP
mean nmols 6alpha/mg protein /10 min
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
**
*
Control(WT)
0.3mg/kg (WT)
1mg/kg (WT)
Control (huPXR)
0.3mg/kg (huPXR)
1mg/kg (huPXR)

FIG. 28

No.	DNA	PCR name	Detected allele	PCR SOP
1	Control(H_2O)	m PXR	Mouse PXR	1057
2	W t			
3	D42749			
4	D42752			
5	Control(H_2O)	PXR hum targ	Humanised PXR	1058
6	W t			
7	D42749			
8	D42752			
9	Control(H_2O)	mCAR	Mouse CAR	1062
10	W t			
11	D42749			
12	D42752			
13	Control(H_2O)	CAR hum	Humanised CAR	1063
14	W t			
15	D42749			
16	D42752			

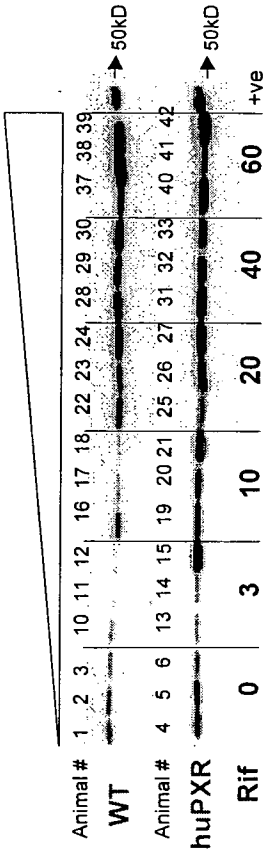
PCR Results

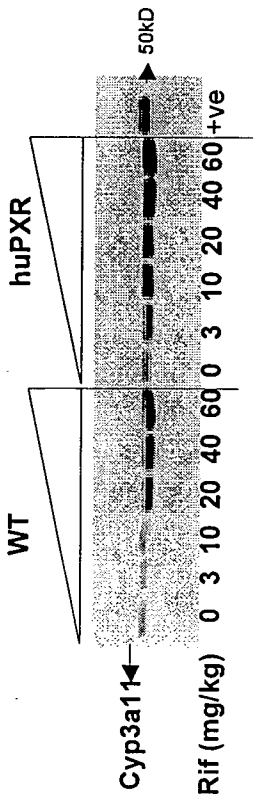
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 M

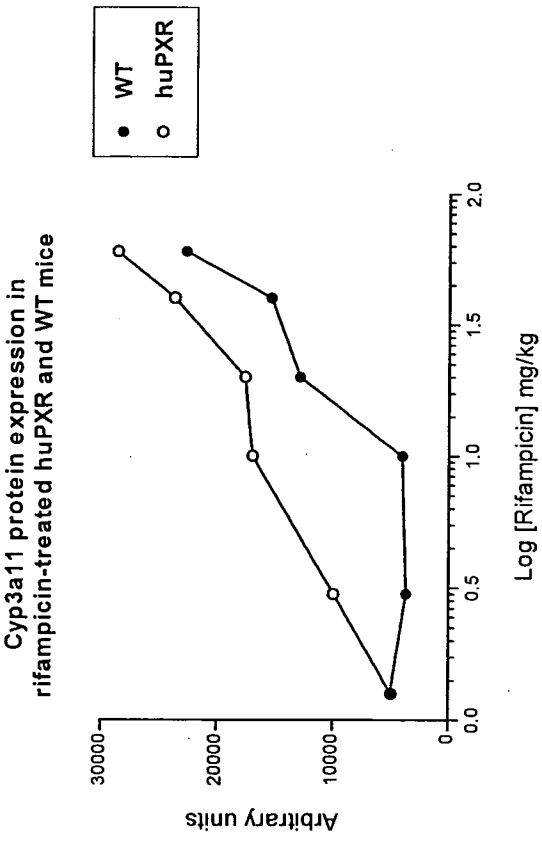


M = Marker

Fig. 29


Animal #
WT
Animal #
huPXR
Rif
50kD
50kD
+ve


WT
huPXR
Cyp3a11
50kD
Rif (mg/kg)
+ve


Cyp3a11 protein expression in rifampicin-treated huPXR and WT mice
WT
huPXR
Arbitrary units
Log [Rifampicin] mg/kg

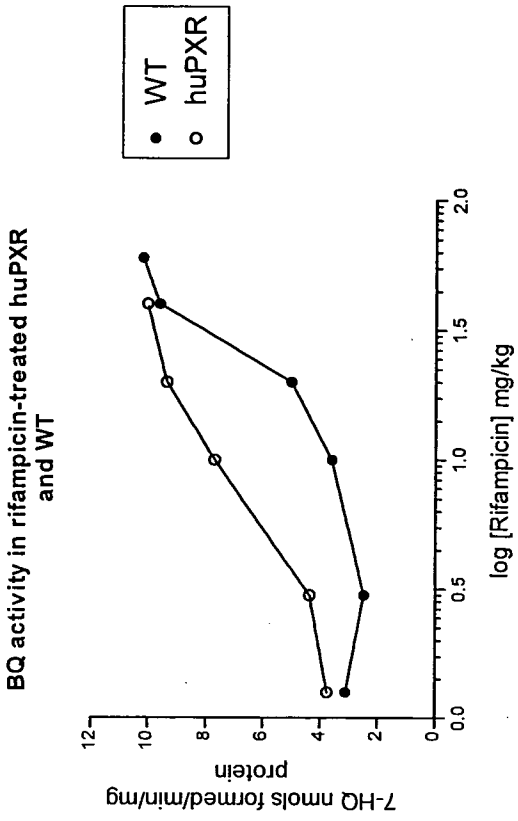

BQ activity in rifampicin-treated huPXR and WT
WT
huPXR
7-HQ nmols formed/min/mg protein
log [Rifampicin] mg/kg

Fig. 30

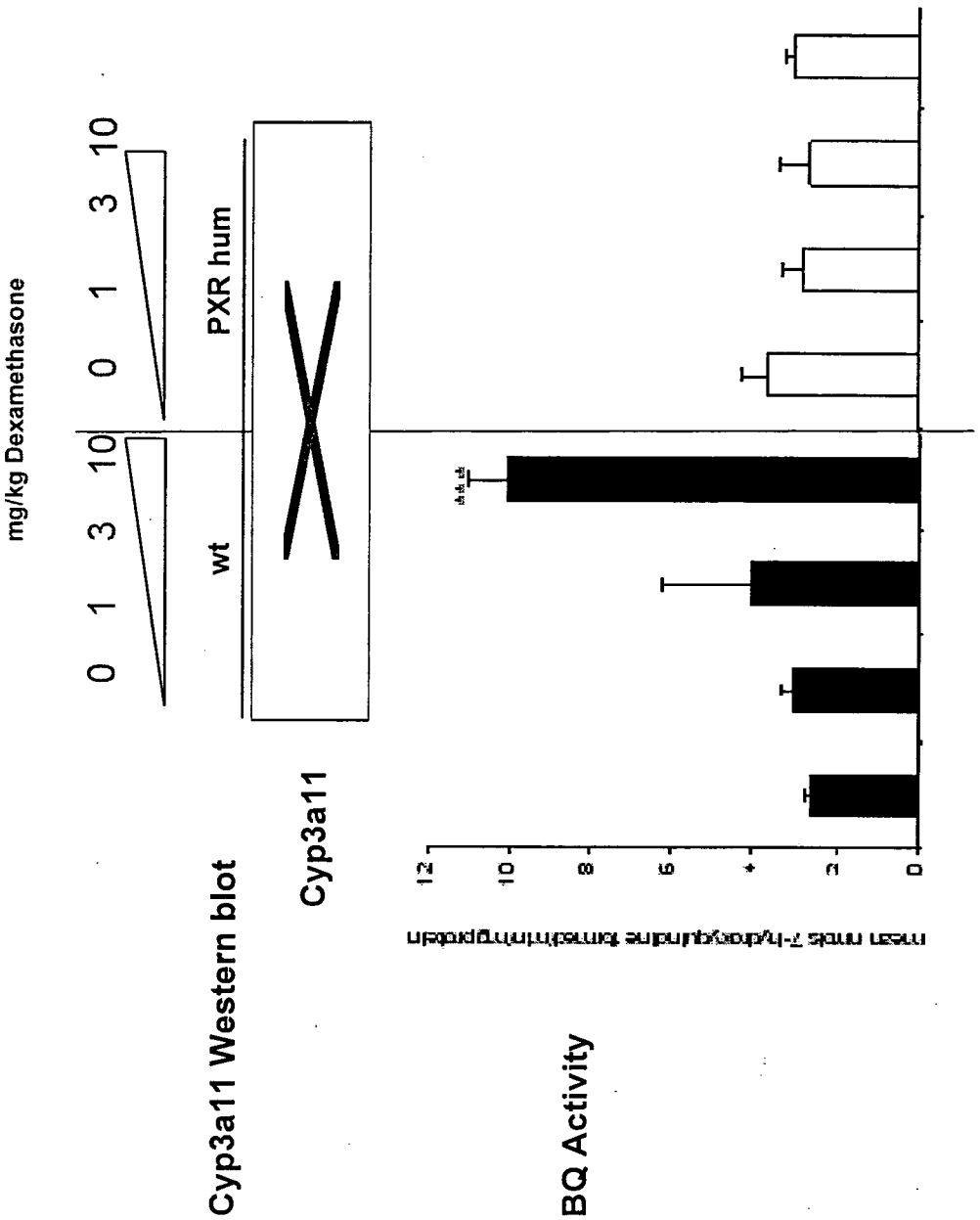

mg/kg Dexamethasone
0 1 3 10 0 1 3 10
wt
PXR hum
Cyp3a11 Western blot
Cyp3a11
BQ Activity
mean mmols 7-hydroxyquinidine formed/min/mg protein
0
2
4
6
8
10
12

Fig. 31

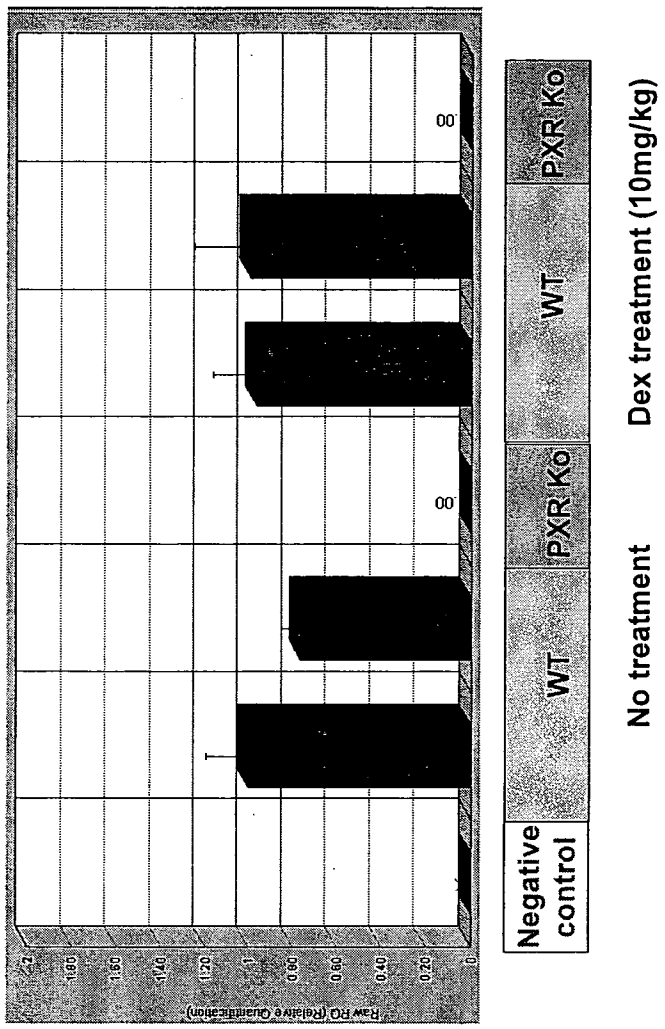

Mouse-specific TaqMan probe
Raw RQ (Relative Quantification)
2
1.80
1.60
1.40
1.20
1
0.80
0.60
0.40
0.20
0
Negative control
WT
PXR Ko
WT
PXR Ko
No treatment
Dex treatment (10mg/kg)
Liver

Fig. 32

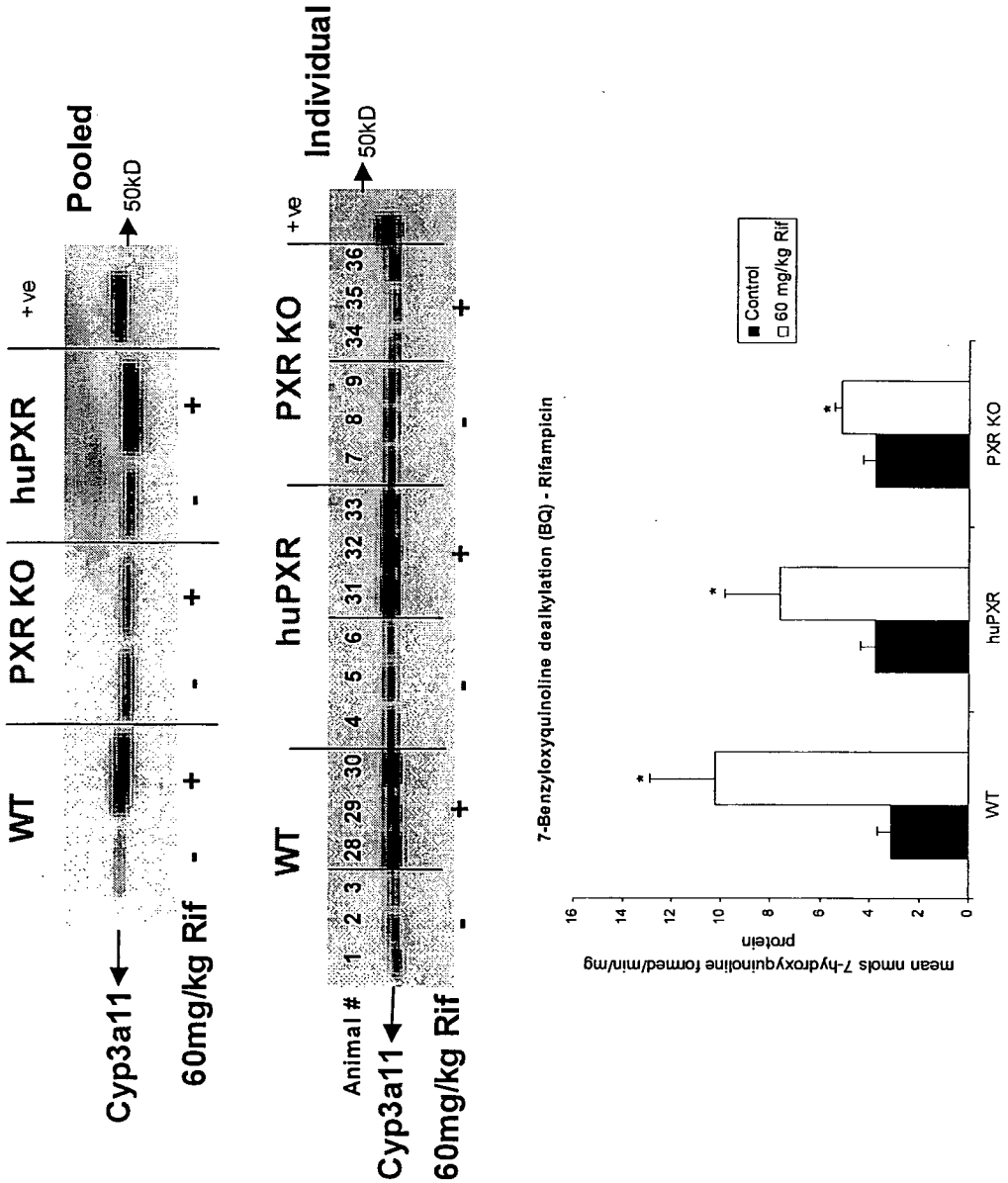

WT
PXR KO
huPXR
+ve
Pooled
50kD
Cyp3a11
60mg/kg Rif
Individual
Animal #
1 2 3 28 29 30 4 5 6 31 32 33 7 8 9 34 35 36
7-Benzyloxyquinoline dealkylation (BQ) - Rifampicin
mean nmols 7-hydroxyquinoline formed/min/mg protein
Control
60 mg/kg Rif
0 2 4 6 8 10 12 14 16
WT
huPXR
PXR KO

Fig. 33

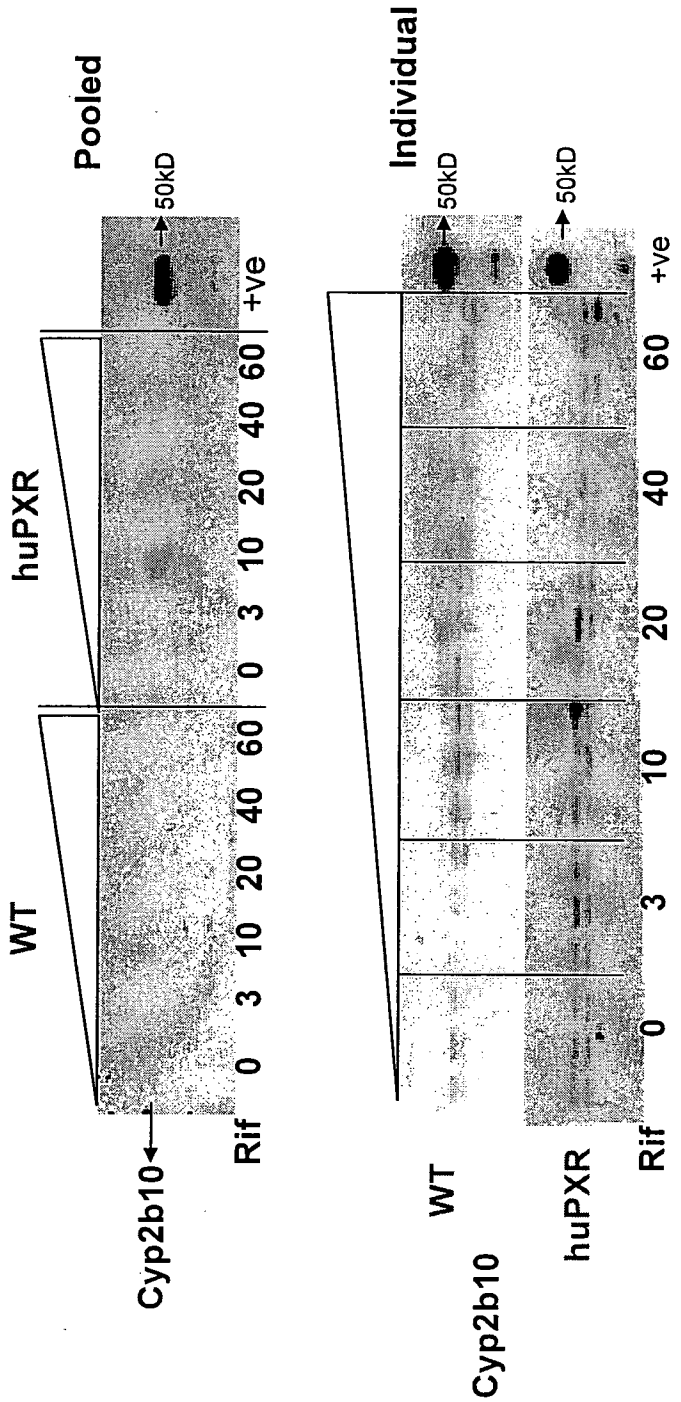

Pooled
50kD
+ve
huPXR
WT
0 3 10 20 40 60 0 3 10 20 40 60
Rif
Cyp2b10
Individual
50kD
50kD
+ve
0 3 10 20 40 60
Rif
WT
huPXR
Cyp2b10

Fig. 34

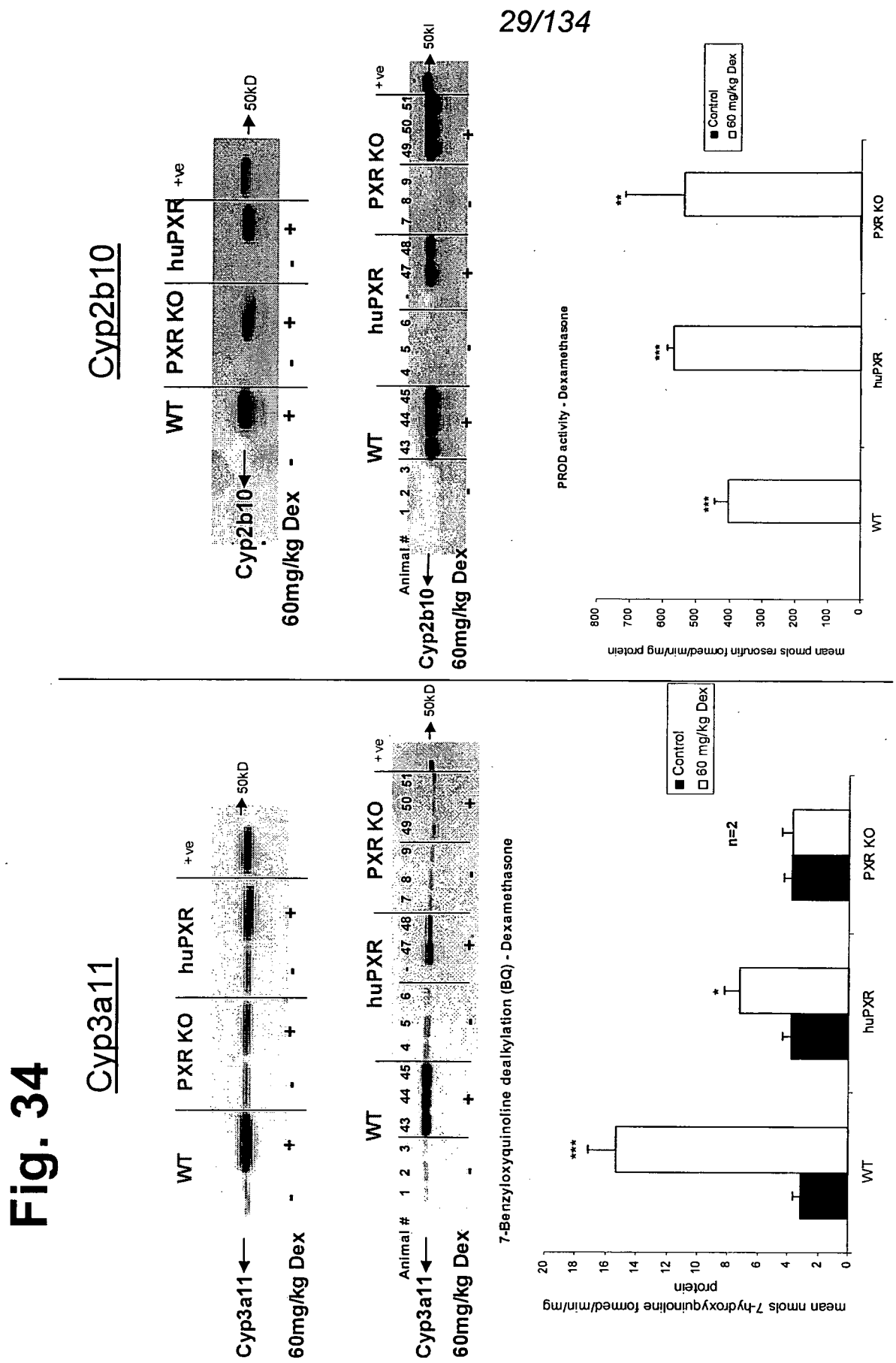
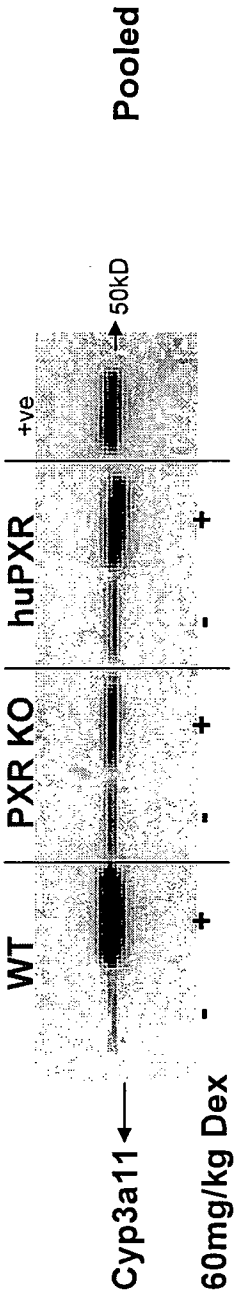

Cyp2b10
WT
PXR KO
huPXR
+ve
Cyp2b10
50kD
60mg/kg Dex
Animal #
PROD activity - Dexamethasone
mean pmols resorufin formed/min/mg protein
Control
60 mg/kg Dex
Cyp3a11
7-Benzyloxyquinoline dealkylation (BQ) - Dexamethasone
mean nmols 7-hydroxyquinoline formed/min/mg protein
n=2

Fig. 35


Pooled
+ve
50kD
WT
PXR KO
huPXR
Cyp3a11
60mg/kg Dex
- + - + - +

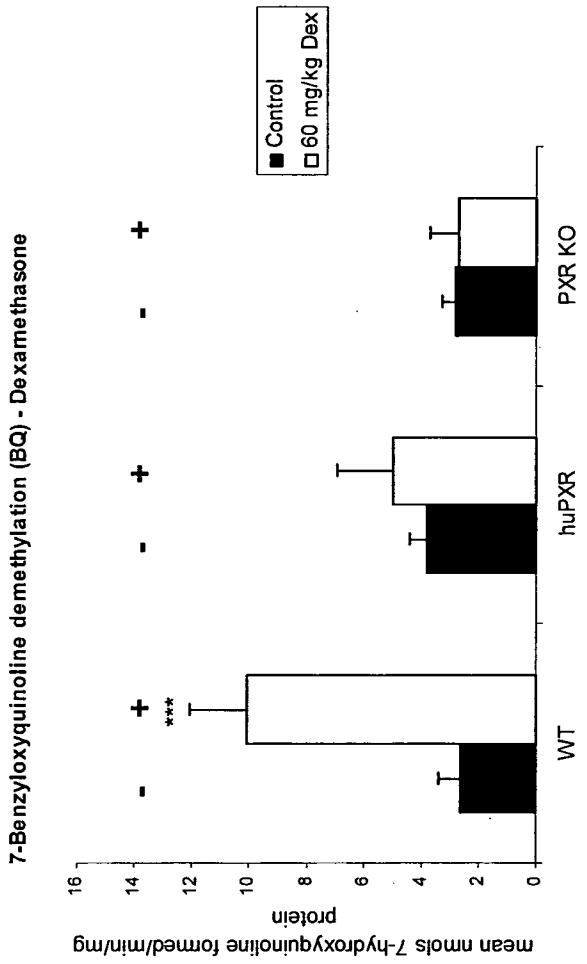

7-Benzyloxyquinoline demethylation (BQ) - Dexamethasone
Control
60 mg/kg Dex
mean nmols 7-hydroxyquinoline formed/min/mg protein
0
2
4
6
8
10
12
14
16
***
- + - + - +
WT
huPXR
PXR KO

Fig. 36

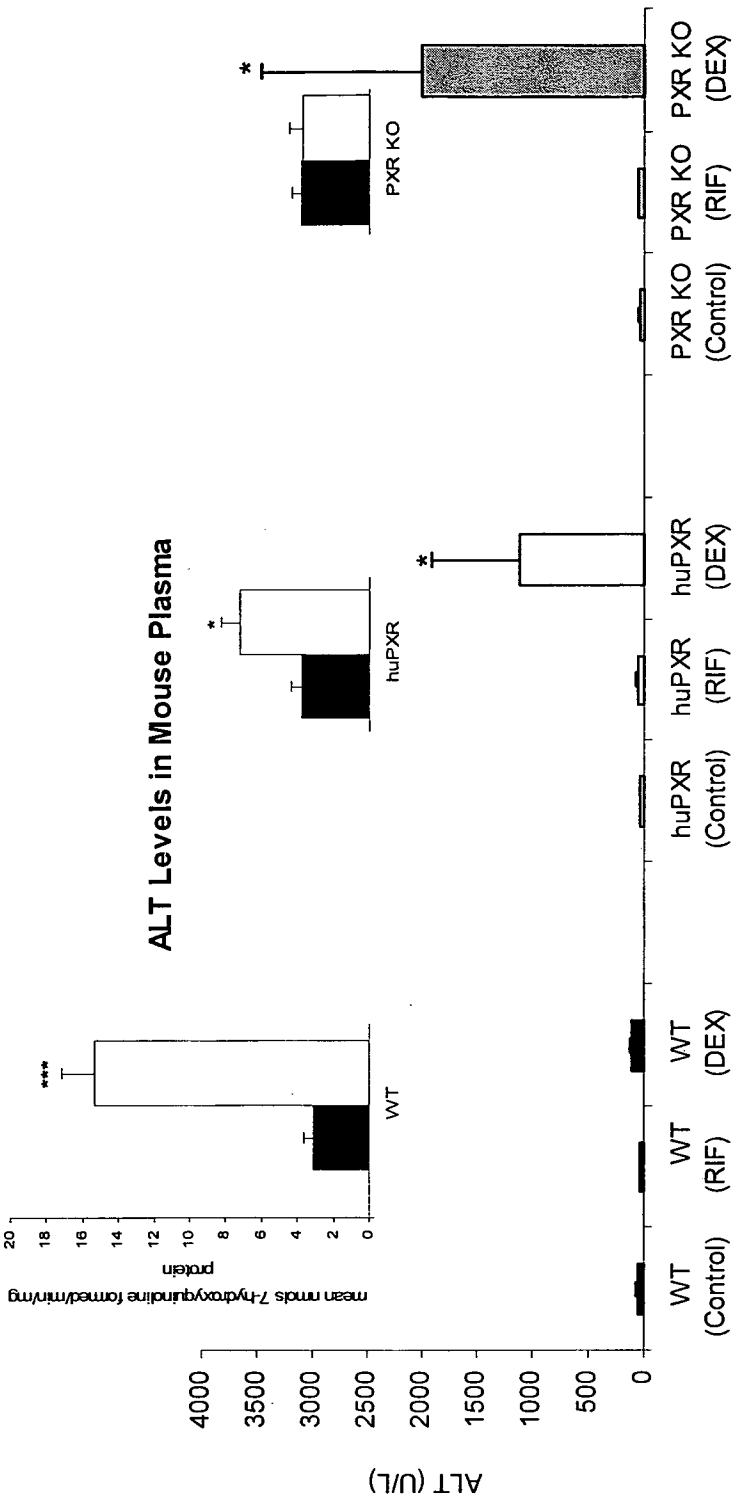

ALT Levels in Mouse Plasma
ALT (U/L)
4000
3500
3000
2500
2000
1500
1000
500
0
WT (Control)
WT (RIF)
WT (DEX)
huPXR (Control)
huPXR (RIF)
huPXR (DEX)
PXR KO (Control)
PXR KO (RIF)
PXR KO (DEX)
mean nmols 7-hydroxyquinidine formed/min/mg protein
20
18
16
14
12
10
8
6
4
2
0
WT
huPXR
PXR KO
***
*

Fig. 37

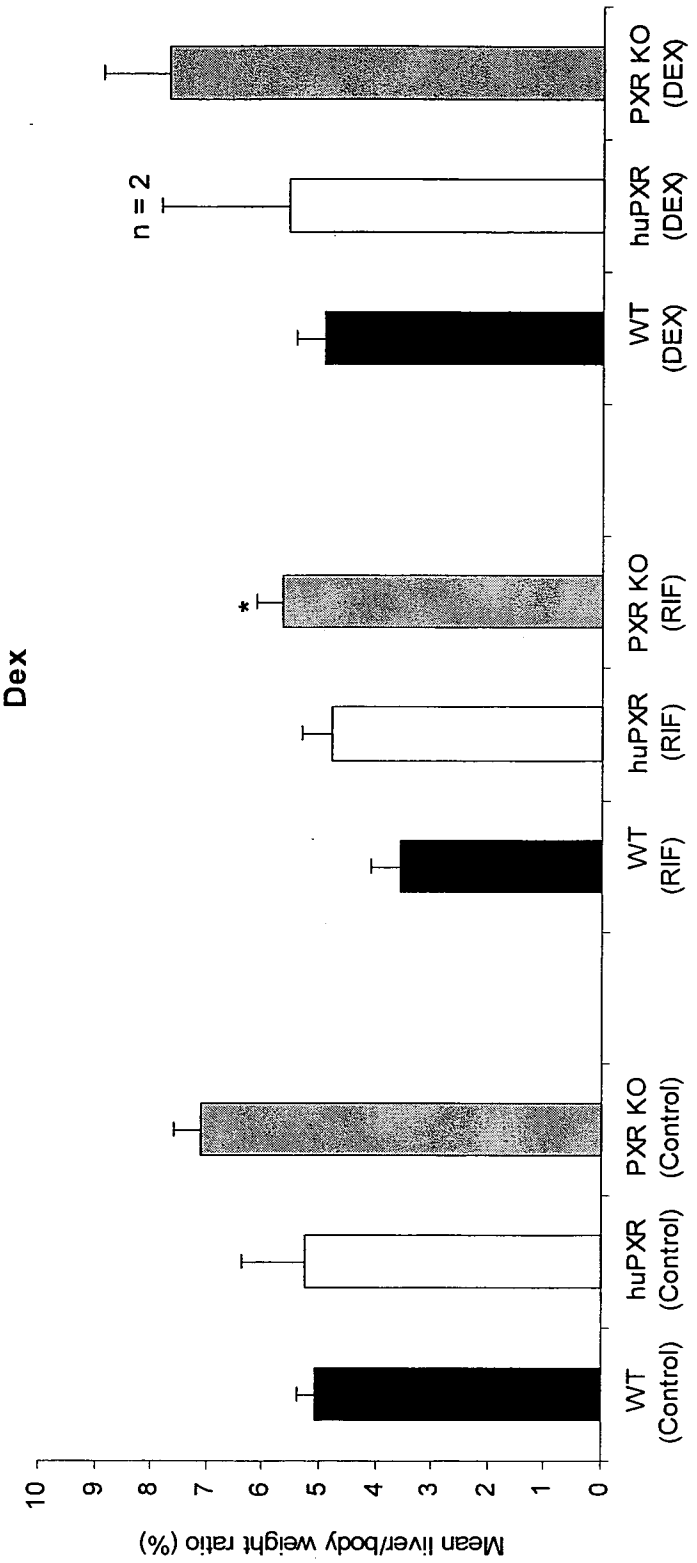
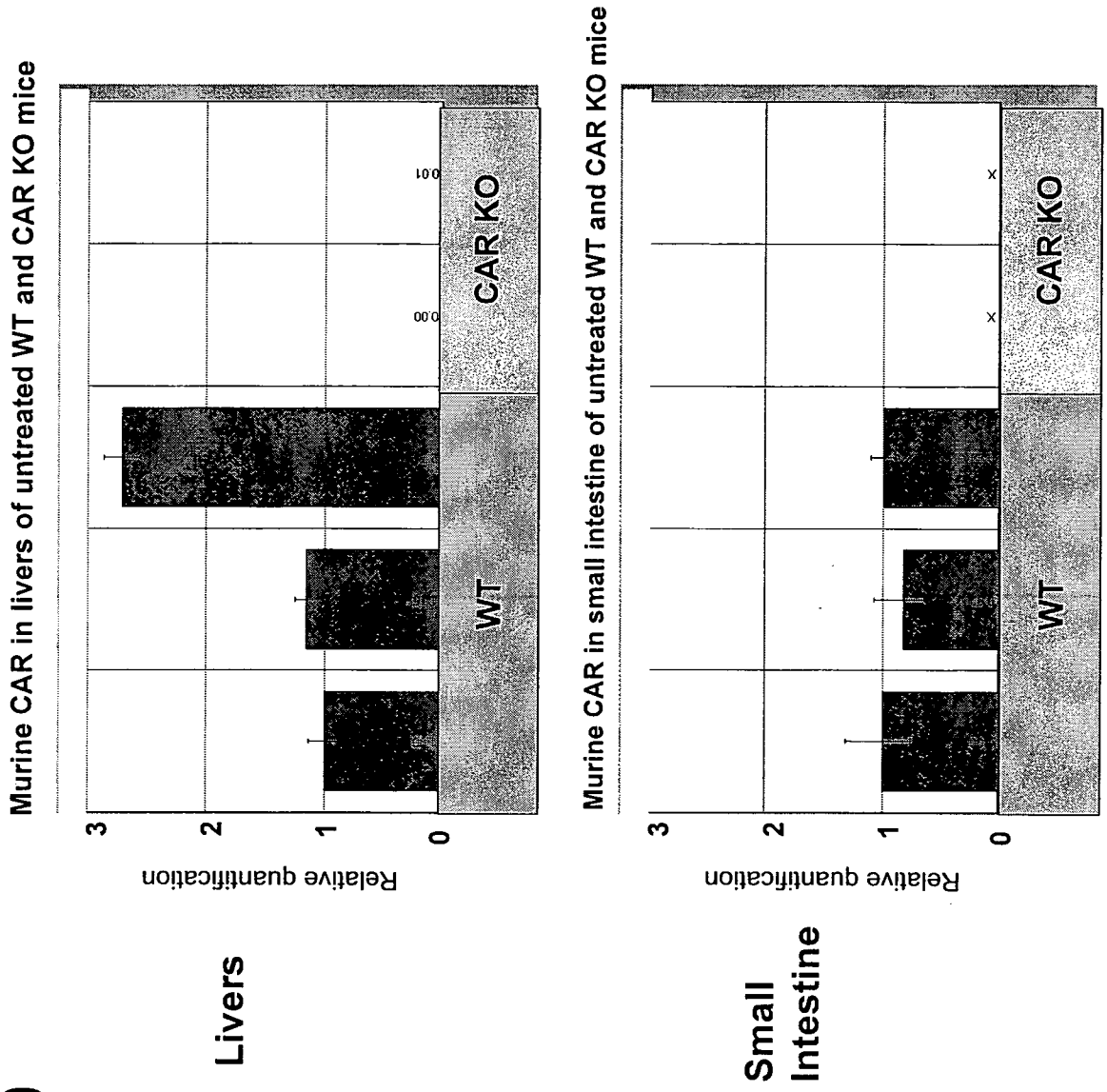

Liver/body weight ratio of WT, huPXR and PXR KO mice treated with 60 mg/kg Rif and Dex
Mean liver/body weight ratio (%)
0
1
2
3
4
5
6
7
8
9
10
n = 2
*
WT (Control)
huPXR (Control)
PXR KO (Control)
WT (RIF)
huPXR (RIF)
PXR KO (RIF)
WT (DEX)
huPXR (DEX)
PXR KO (DEX)

Fig. 38

	3a11		2b10	
	RIF	DEX	RIF	DEX
WT	+++	+++	NC	+++
huPXR	+++	++	NC	+++
PXR KO	NC	NC	NC	+++



Livers

Small Intestine

Fig. 39

Fig. 40

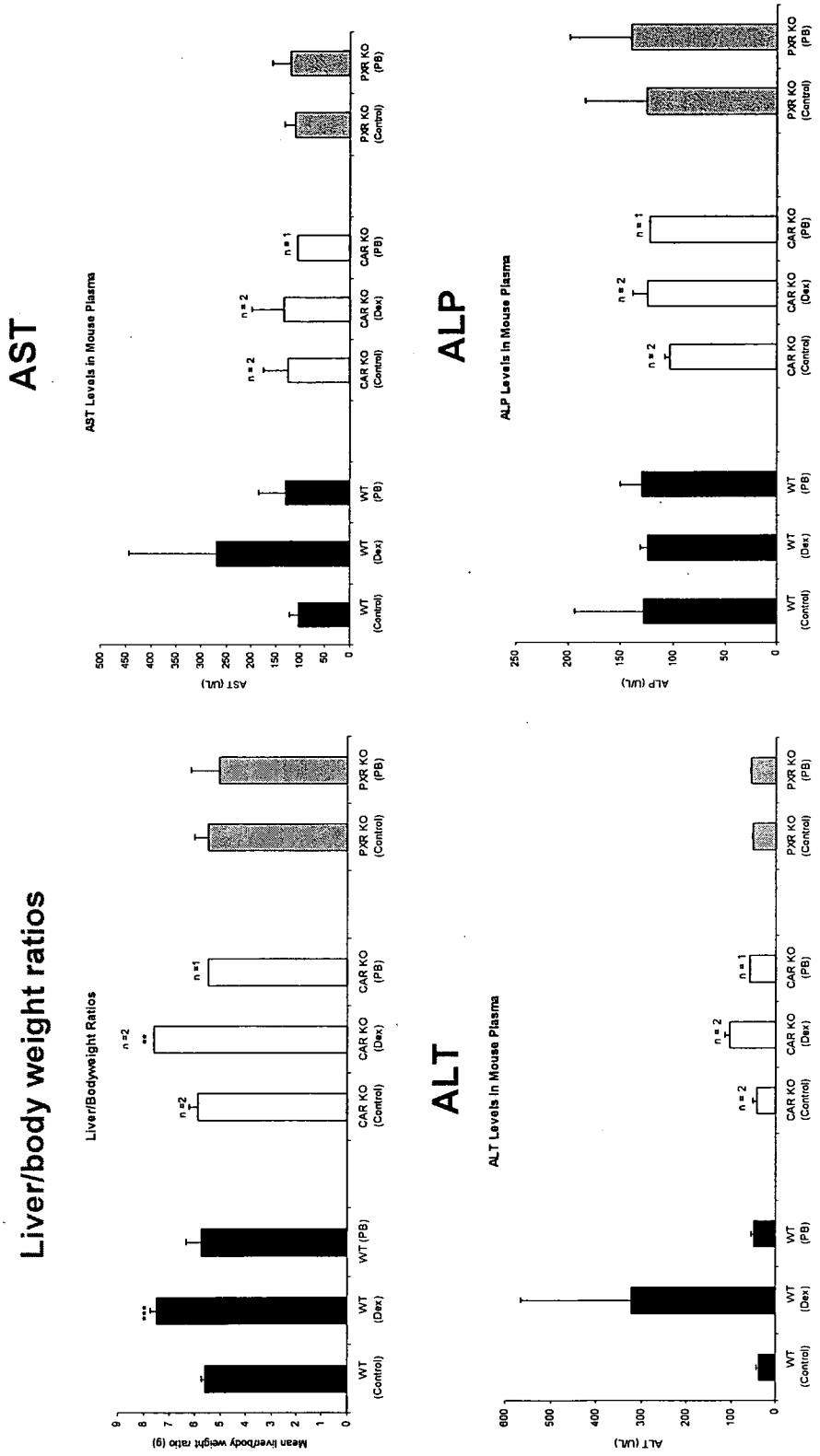
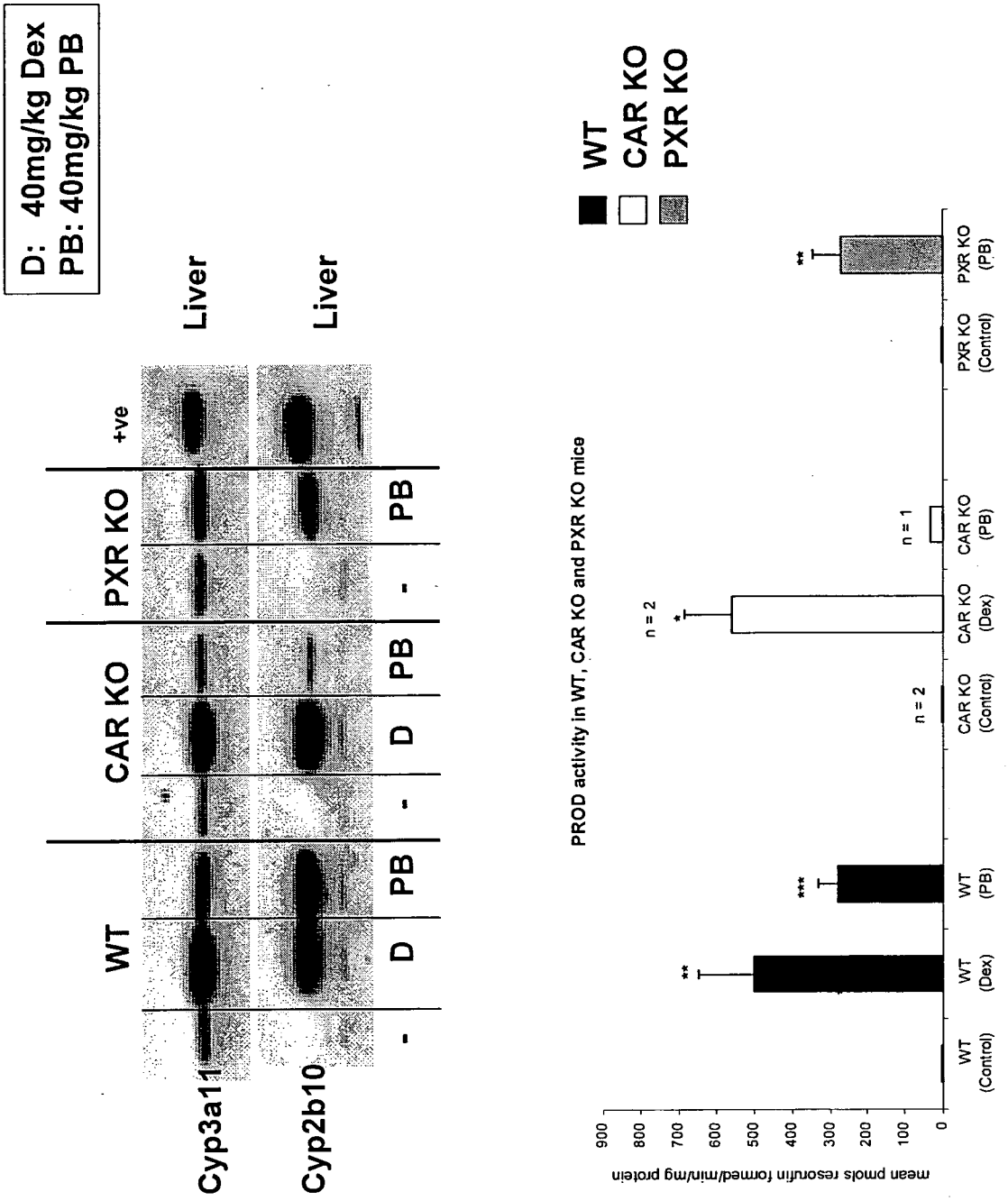

Liver/body weight ratios
Liver/Bodyweight Ratios
Mean liver/body weight ratio (g)
AST
AST Levels in Mouse Plasma
AST (U/L)
ALP
ALP Levels in Mouse Plasma
ALP (U/L)
ALT
ALT Levels in Mouse Plasma
ALT (U/L)
WT (Control)
WT (Dex)
WT (PB)
CAR KO (Control)
CAR KO (Dex)
CAR KO (PB)
PXR KO (Control)
PXR KO (PB)

Fig. 41


D: 40mg/kg Dex
PB: 40mg/kg PB
WT
CAR KO
PXR KO
+ve
Liver
Liver
Cyp3a11
Cyp2b10
-
D
PB
-
D
PB
-
PB
PROD activity in WT, CAR KO and PXR KO mice
WT
CAR KO
PXR KO
mean pmols resorufin formed/min/mg protein
0
100
200
300
400
500
600
700
800
900
n = 2
n = 2
n = 1
**
***
*
**
WT (Control)
WT (Dex)
WT (PB)
CAR KO (Control)
CAR KO (Dex)
CAR KO (PB)
PXR KO (Control)
PXR KO (PB)

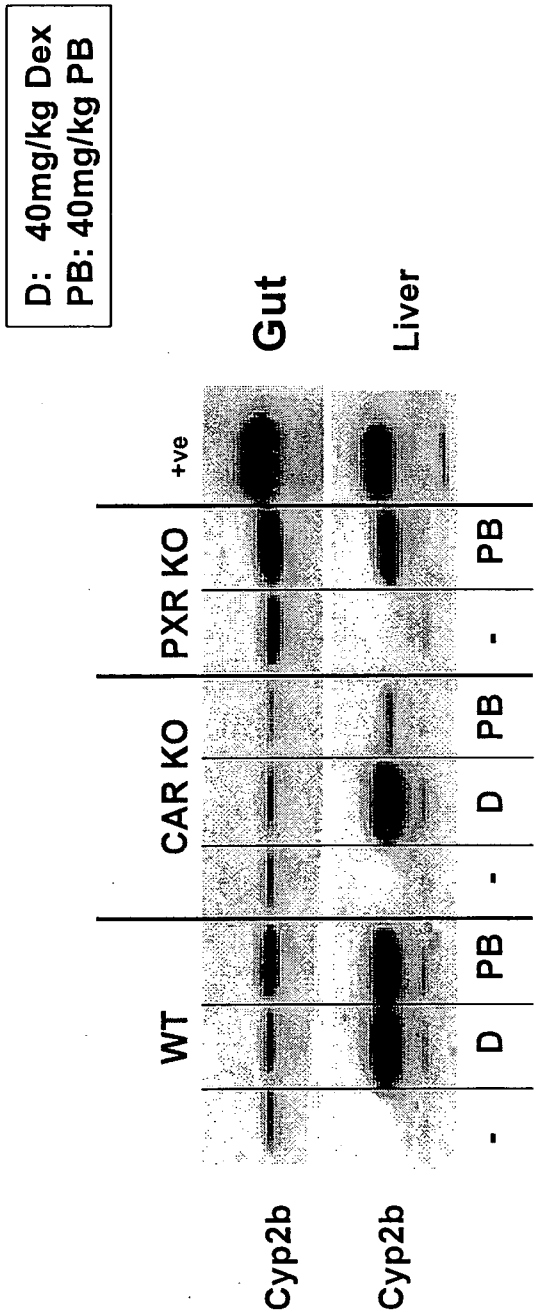

D: 40mg/kg Dex
PB: 40mg/kg PB
Gut
Liver
+ve
PXR KO
CAR KO
WT
PB
-
D
Cyp2b
Cyp2b

Fig. 42

Fig. 43

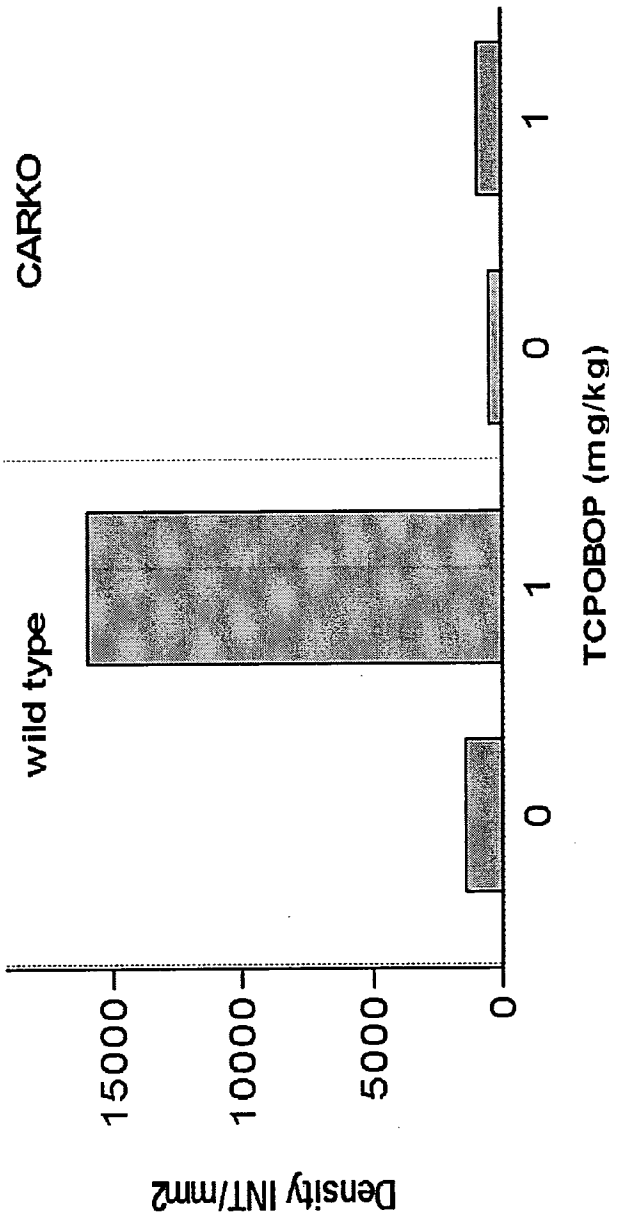
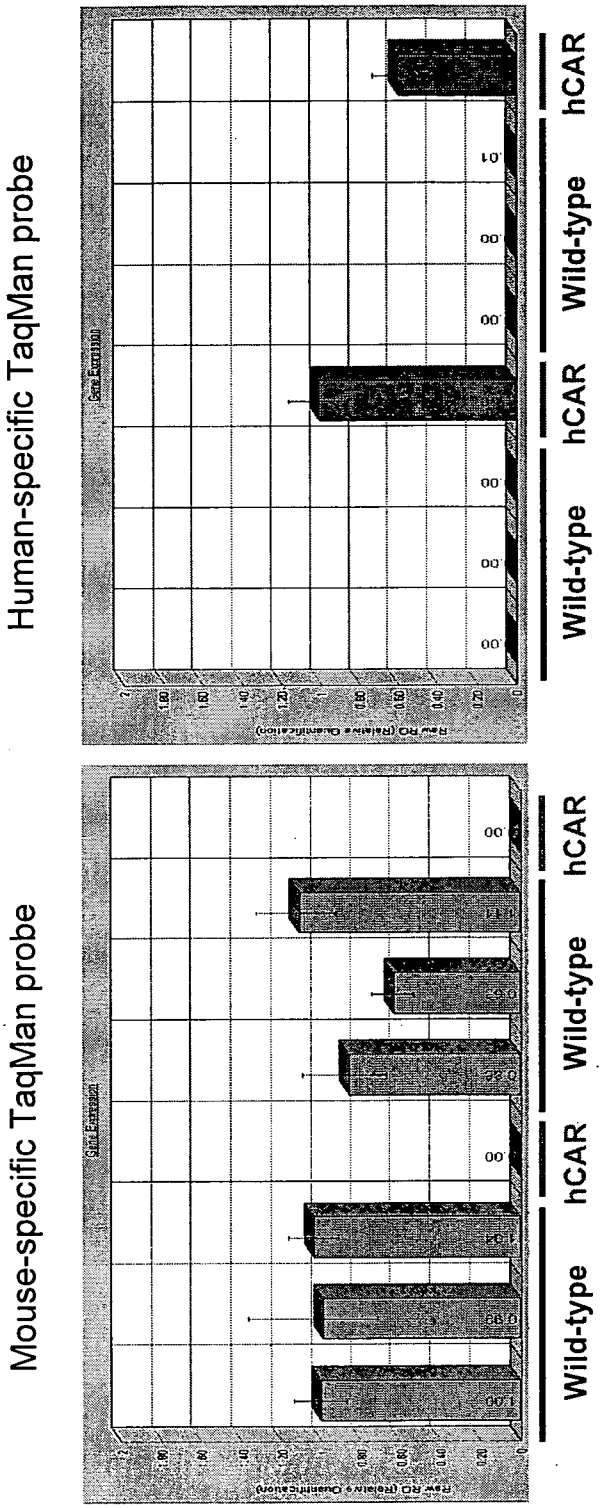

wild type
CARKO
0
1
0
1
TCPOBOP (mg/kg)
0
5000
10000
15000
Density INT/mm2

Fig. 44


Liver
Mouse-specific TaqMan probe
Human-specific TaqMan probe
Wild-type
hCAR
Wild-type
hCAR
Wild-type
hCAR
Wild-type
hCAR

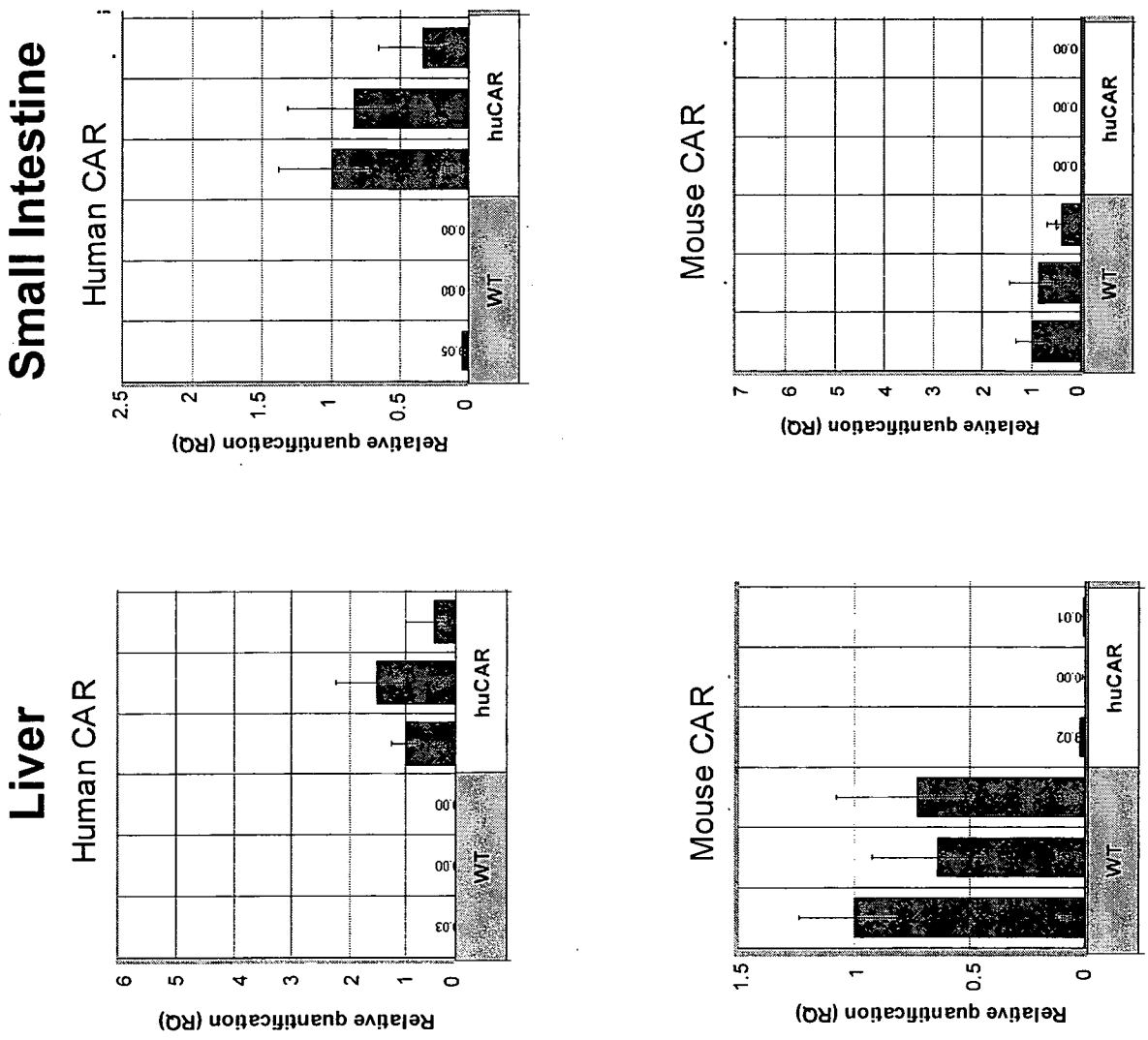

Small Intestine
Human CAR
Relative quantification (RQ)
2.5
2
1.5
1
0.5
0
0.05
0.00
0.00
WT
huCAR
Mouse CAR
Relative quantification (RQ)
7
6
5
4
3
2
1
0
1
0.00
0.00
0.00
WT
huCAR
Liver
Human CAR
Relative quantification (RQ)
6
5
4
3
2
1
0
0.03
0.00
0.00
WT
huCAR
Mouse CAR
Relative quantification (RQ)
1.5
1
0.5
0
0.02
0.00
0.01
WT
huCAR

Fig. 45

Fig. 46

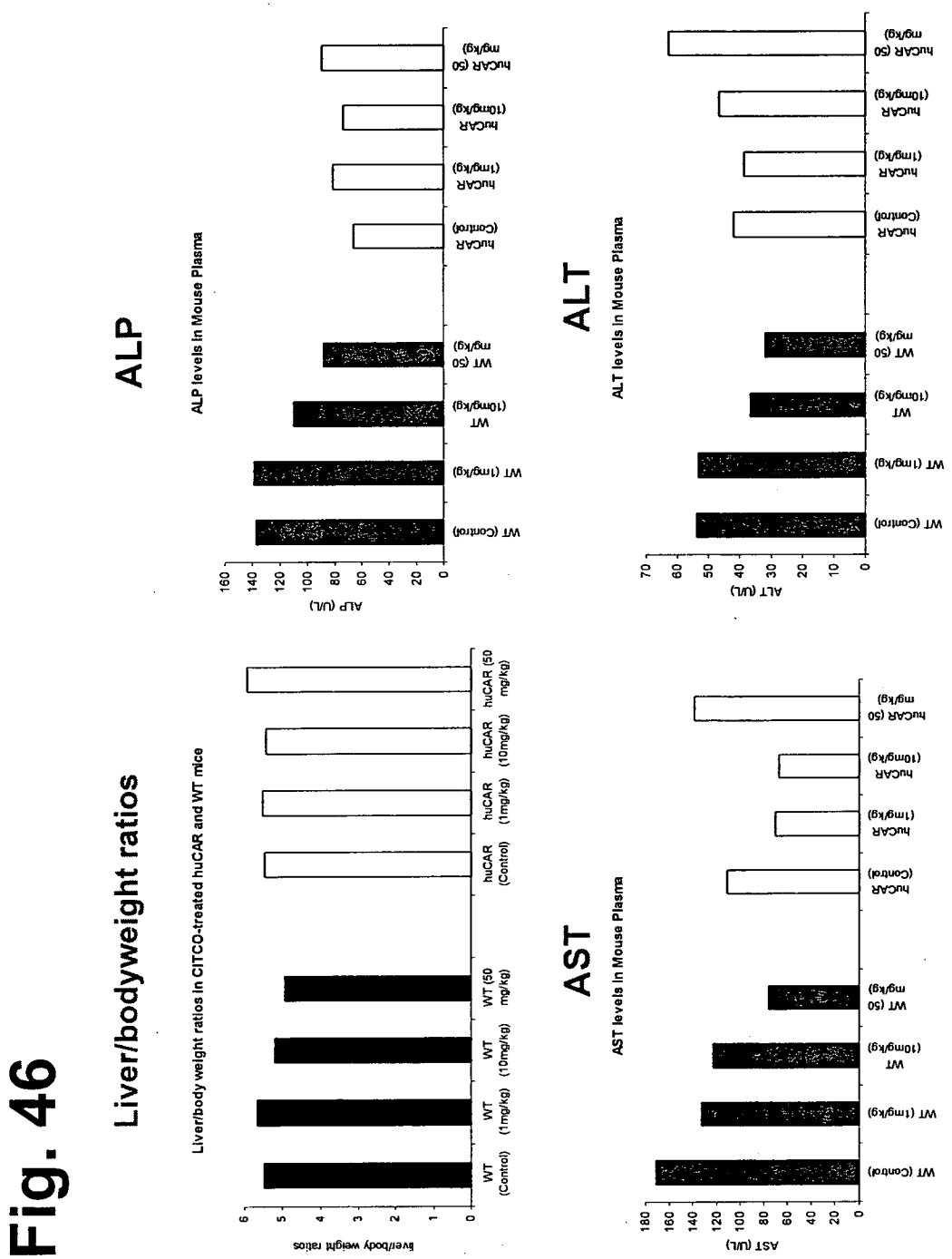

Liver/bodyweight ratios
Liver/body weight ratios in CITCO-treated huCAR and WT mice
liver/body weight ratios
WT (Control)
WT (1mg/kg)
WT (10mg/kg)
WT (50 mg/kg)
huCAR (Control)
huCAR (1mg/kg)
huCAR (10mg/kg)
huCAR (50 mg/kg)
ALP
ALP levels in Mouse Plasma
ALP (U/L)
ALT
ALT levels in Mouse Plasma
ALT (U/L)
AST
AST levels in Mouse Plasma
AST (U/L)

Fig. 47

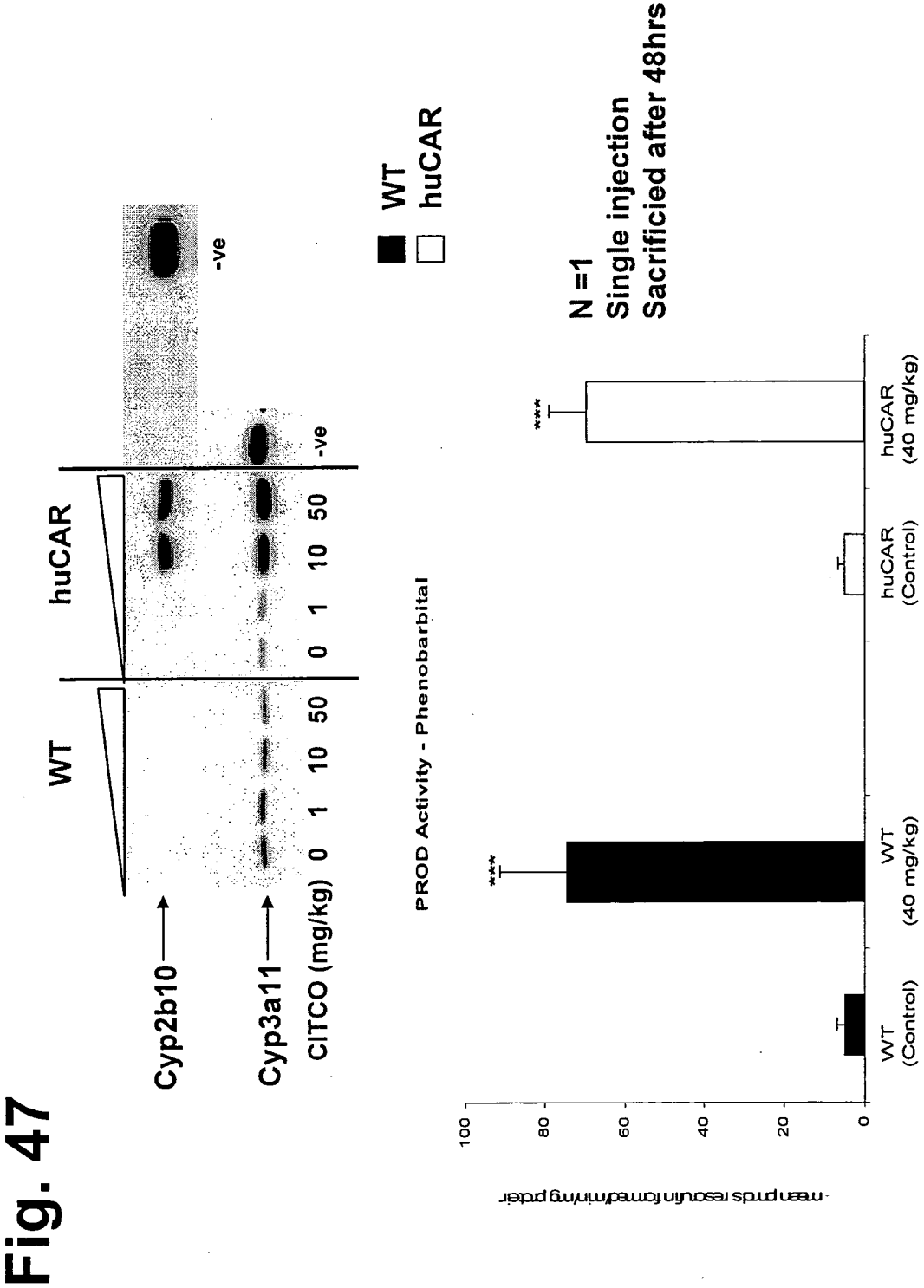

WT
huCAR
-ve
-ve
Cyp2b10
Cyp3a11
CITCO (mg/kg) 0 1 10 50 0 1 10 50
WT
huCAR
N =1
Single injection
Sacrificied after 48hrs
PROD Activity - Phenobarbital
mean pmols resorufin formed/min/mg protein
0
20
40
60
80
100
***
***
WT (Control)
WT (40 mg/kg)
huCAR (Control)
huCAR (40 mg/kg)

Fig. 48

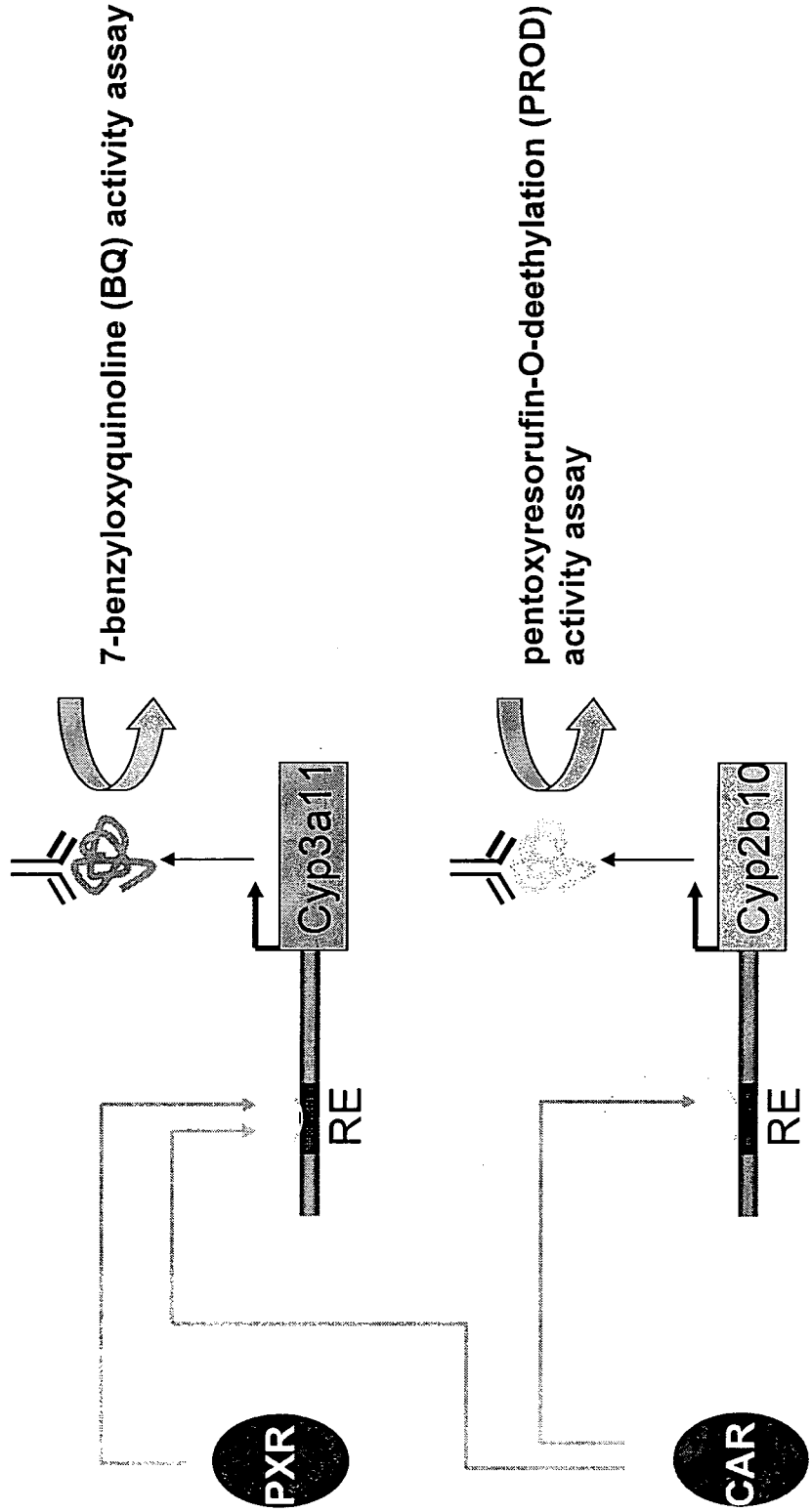

7-benzyloxyquinoline (BQ) activity assay
pentoxyresorufin-O-deethylation (PROD) activity assay
Cyp3a11
Cyp2b10
RE
RE
PXR
CAR

Fig. 49

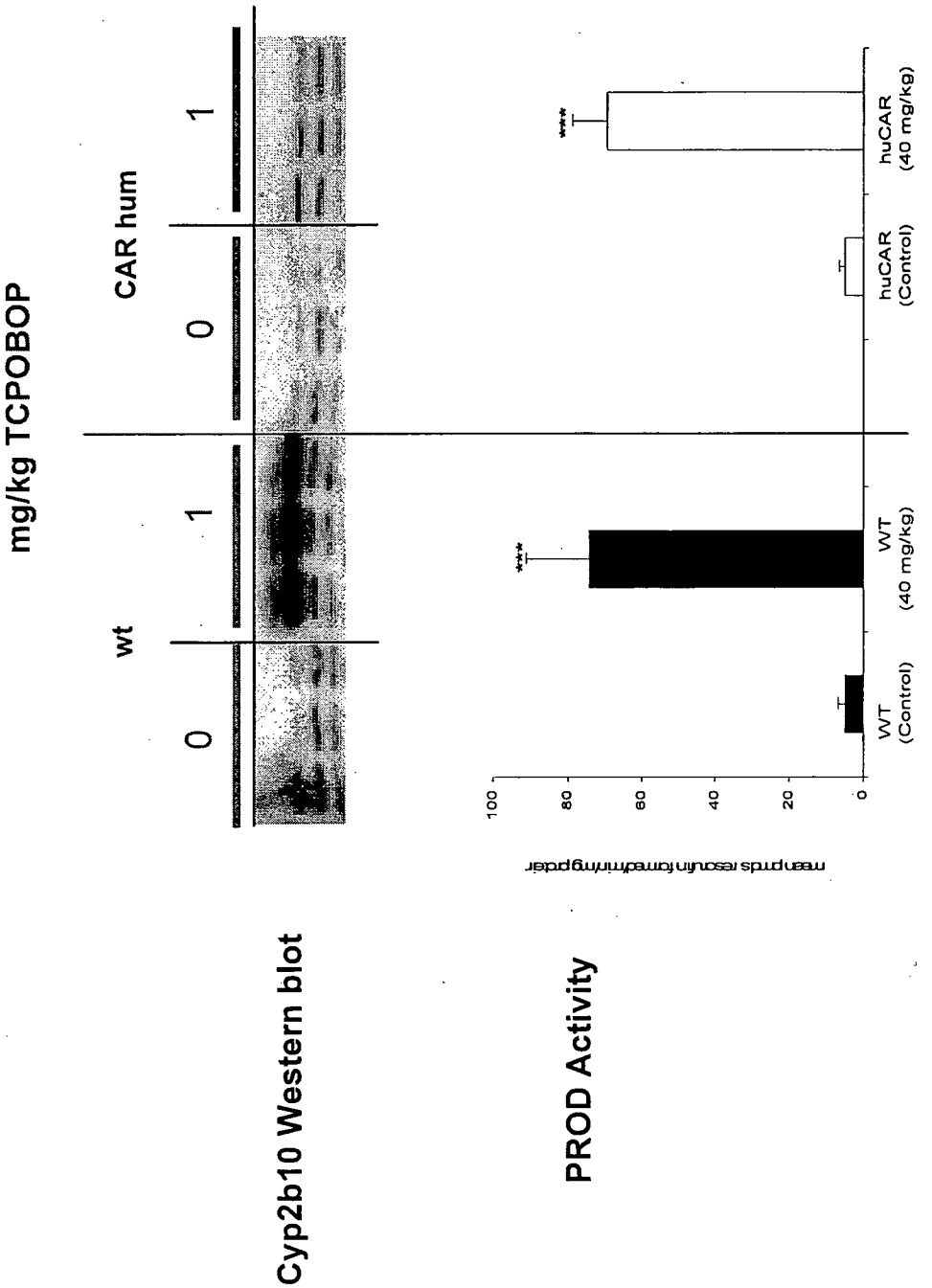

mg/kg TCPOBOP
wt
CAR hum
0
1
0
1
Cyp2b10 Western blot
PROD Activity
mean pmols resorufin formed/min/mg protein
100
80
60
40
20
0
***
***
WT (Control)
WT (40 mg/kg)
huCAR (Control)
huCAR (40 mg/kg)

Fig. 50

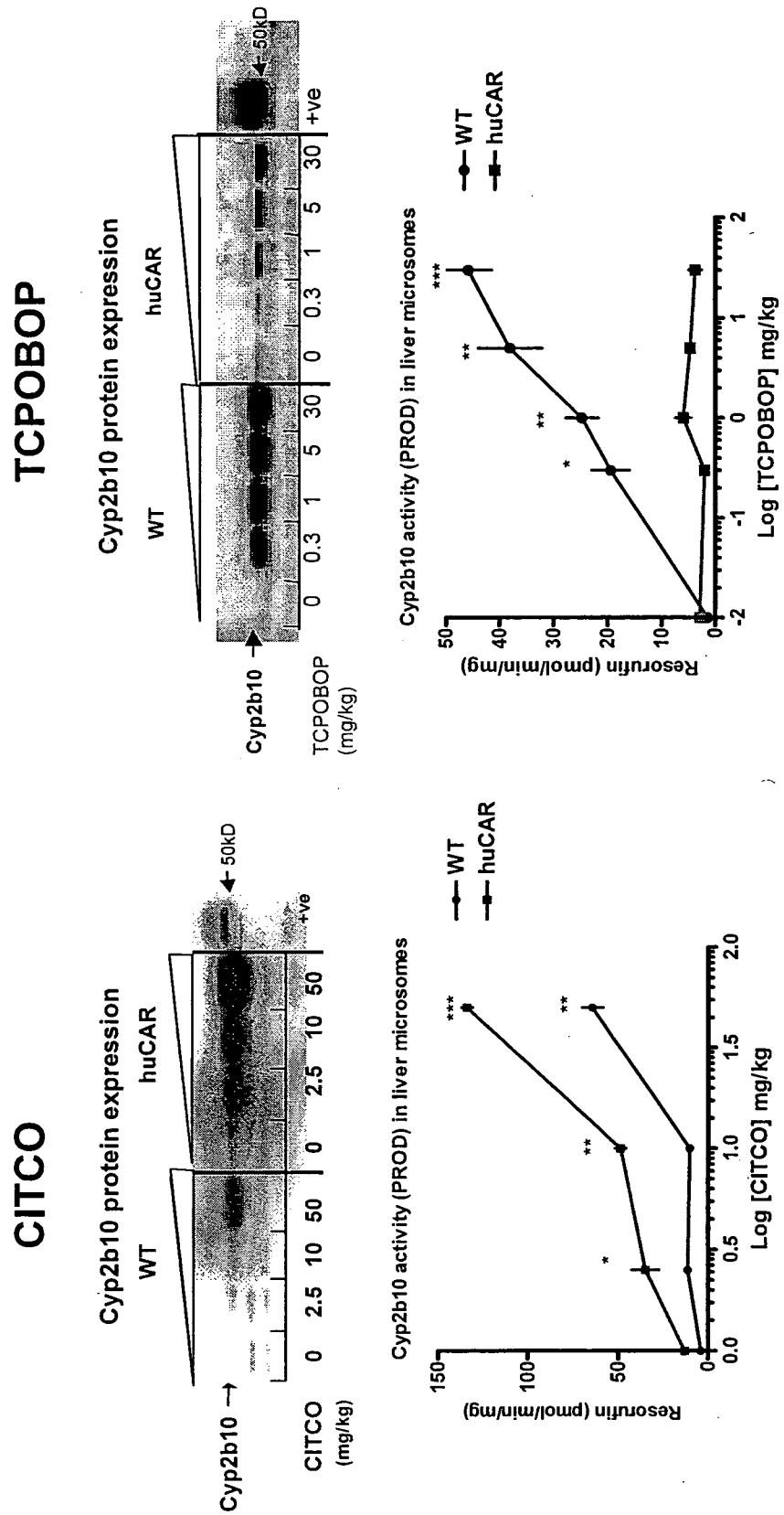

TCPOBOP
Cyp2b10 protein expression
WT
huCAR
50kD
+ve
Cyp2b10
TCPOBOP (mg/kg)
0 0.3 1 5 30 0 0.3 1 5 30
Cyp2b10 activity (PROD) in liver microsomes
Resorufin (pmol/min/mg)
WT
huCAR
Log [TCPOBOP] mg/kg
CITCO
0 2.5 10 50 0 2.5 10 50
Log [CITCO] mg/kg

Fig. 51

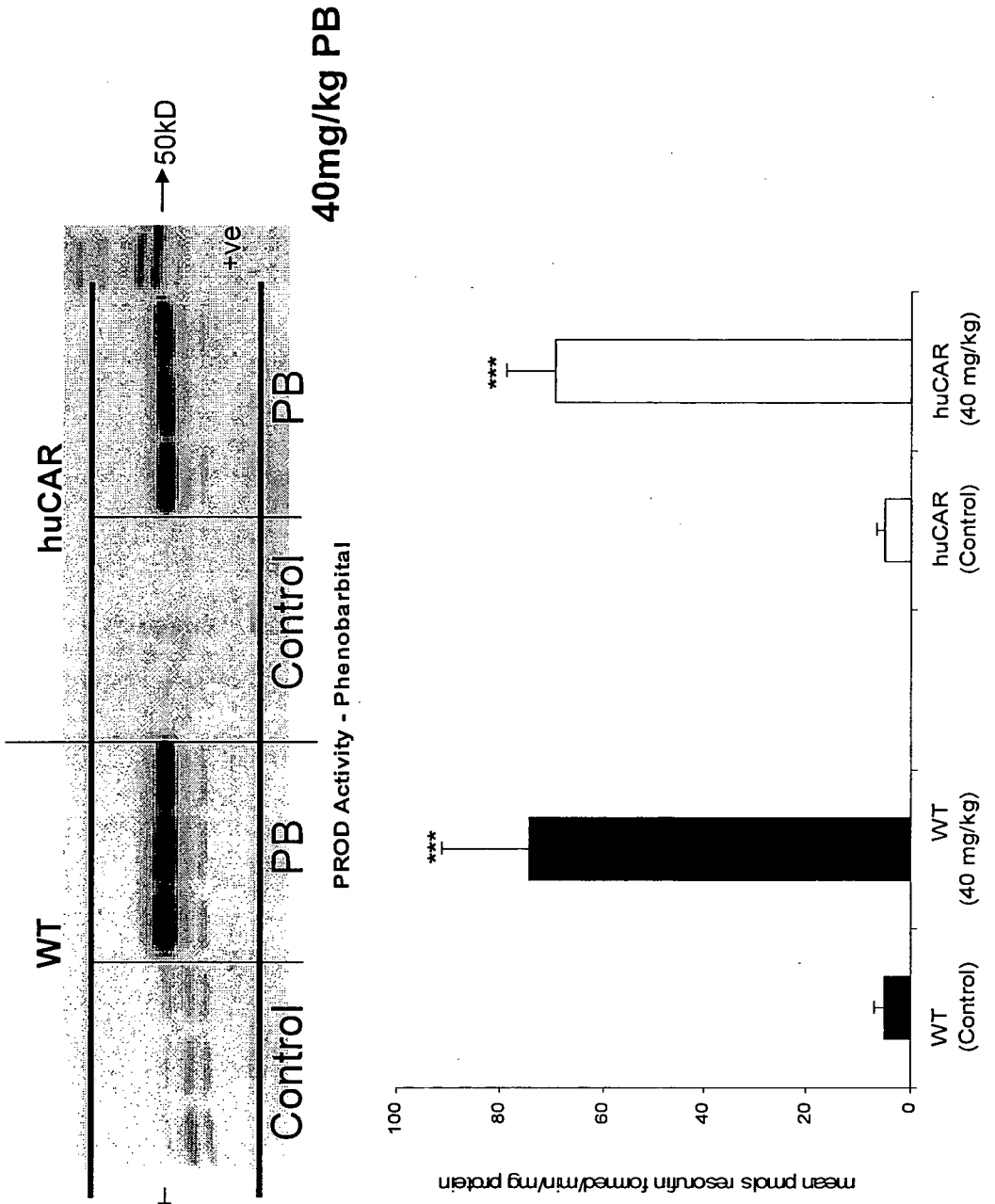
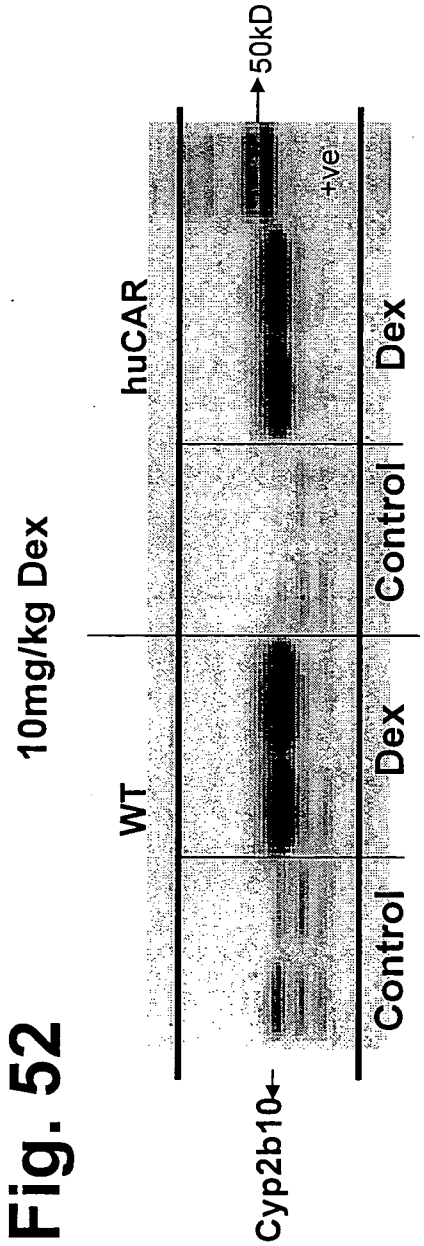

40mg/kg PB
50kD
+ve
huCAR
WT
PB
Control
PB
Control
Cyp2b10
PROD Activity - Phenobarbital
mean pmols resorufin formed/min/mg protein
100
80
60
40
20
0
***
***
WT (Control)
WT (40 mg/kg)
huCAR (Control)
huCAR (40 mg/kg)

Fig. 52


10mg/kg Dex
WT
huCAR
50kD
+ve
Control
Dex
Control
Dex
Cyp2b10

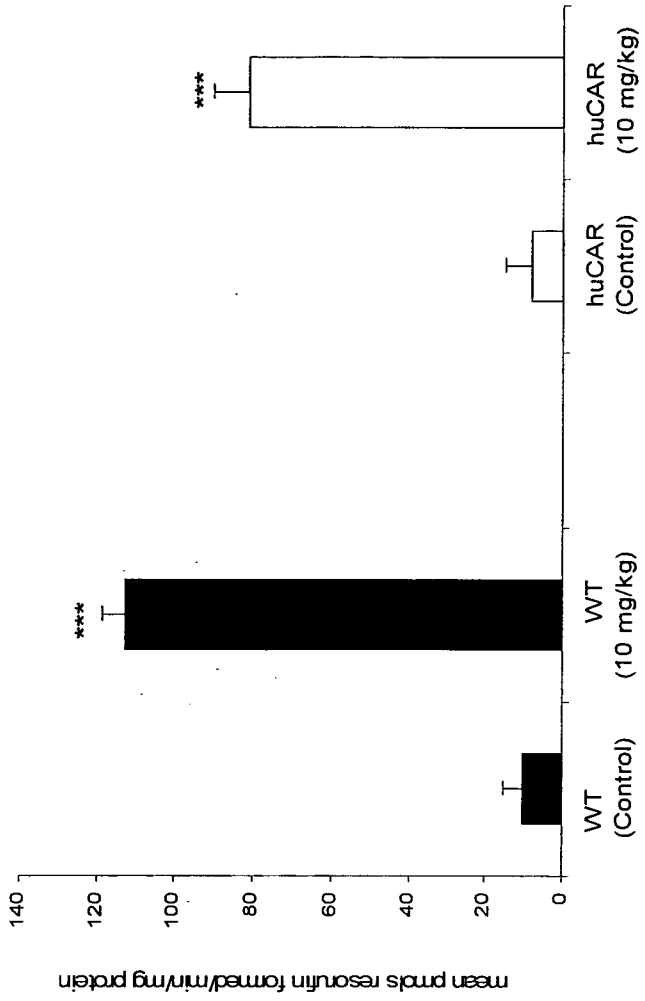

PROD Activity - Dexamethasone
mean pmols resorufin formed/min/mg protein
140
120
100
80
60
40
20
0
***
***
WT (Control)
WT (10 mg/kg)
huCAR (Control)
huCAR (10 mg/kg)

Fig. 53

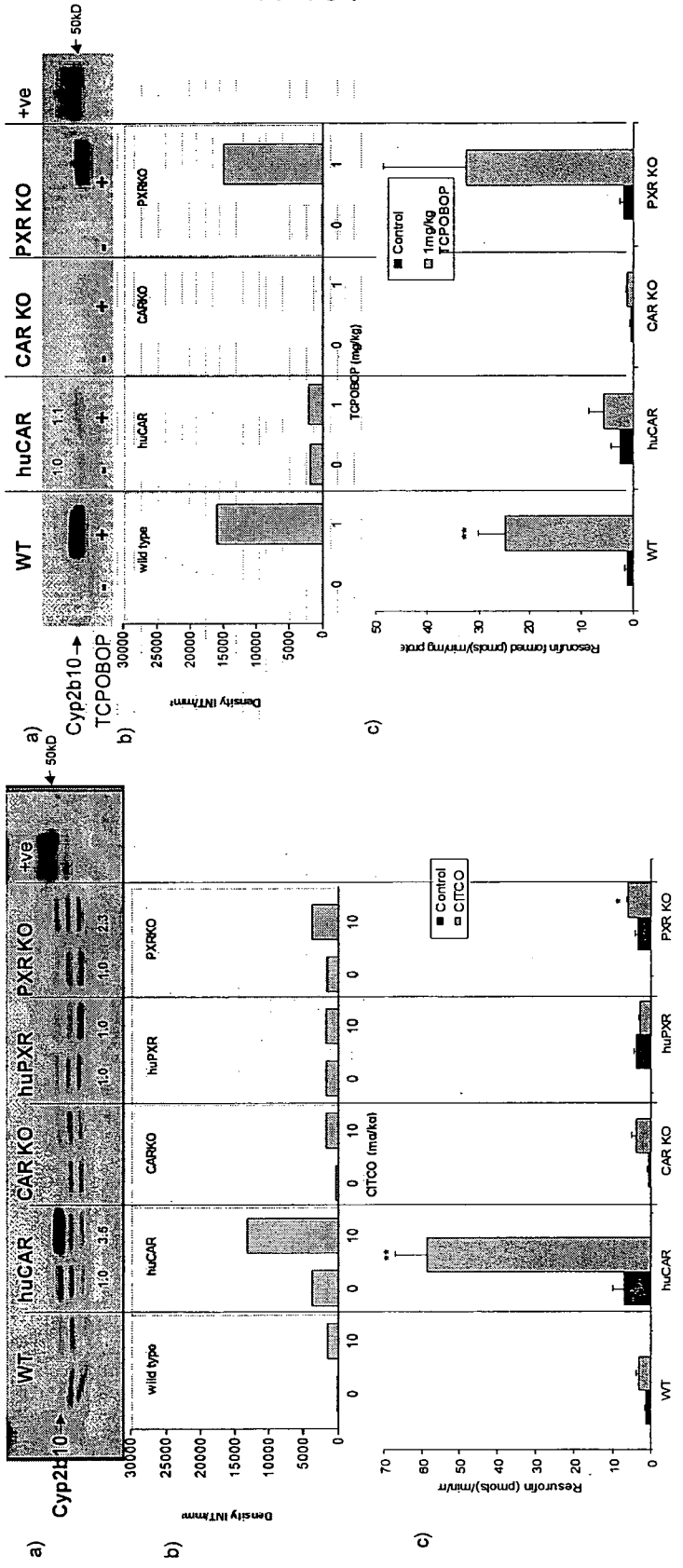
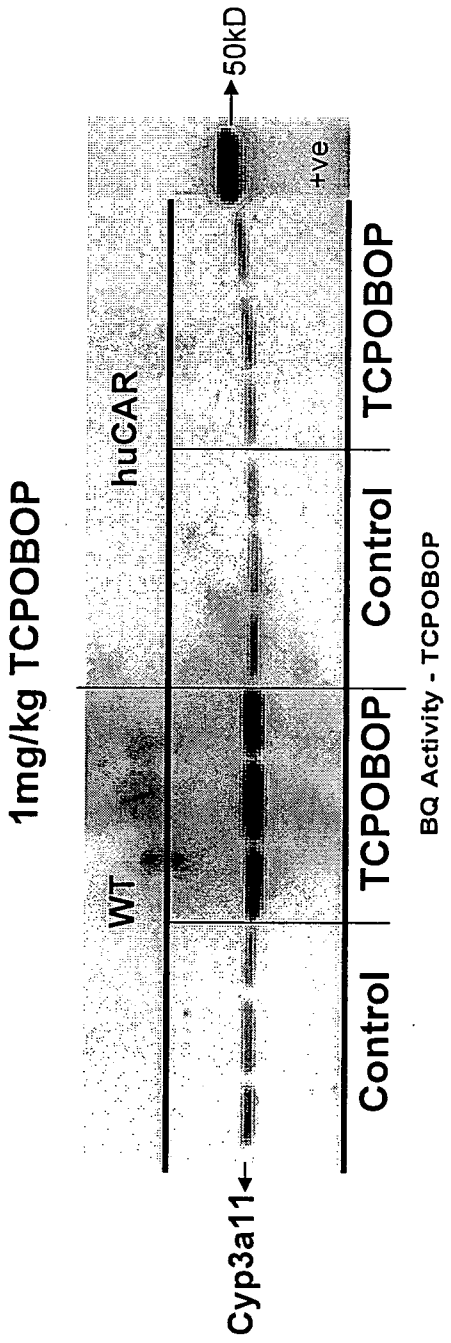

a)
Cyp2b10
TCPOBOP
WT
huCAR
CAR KO
PXR KO
+ve
50kD
b)
Density INTmm²
wild type
huCAR
CARKO
PXRKO
TCPOBOP (mg/kg)
c)
Resorufin formed (pmols)/min/mg prot
Control
1mg/kg TCPOBOP
CITCO (mg/kg)
huPXR

Fig. 54


1mg/kg TCPOBOP
WT
huCAR
Control
TCPOBOP
Control
TCPOBOP
50kD
+ve
Cyp3a11

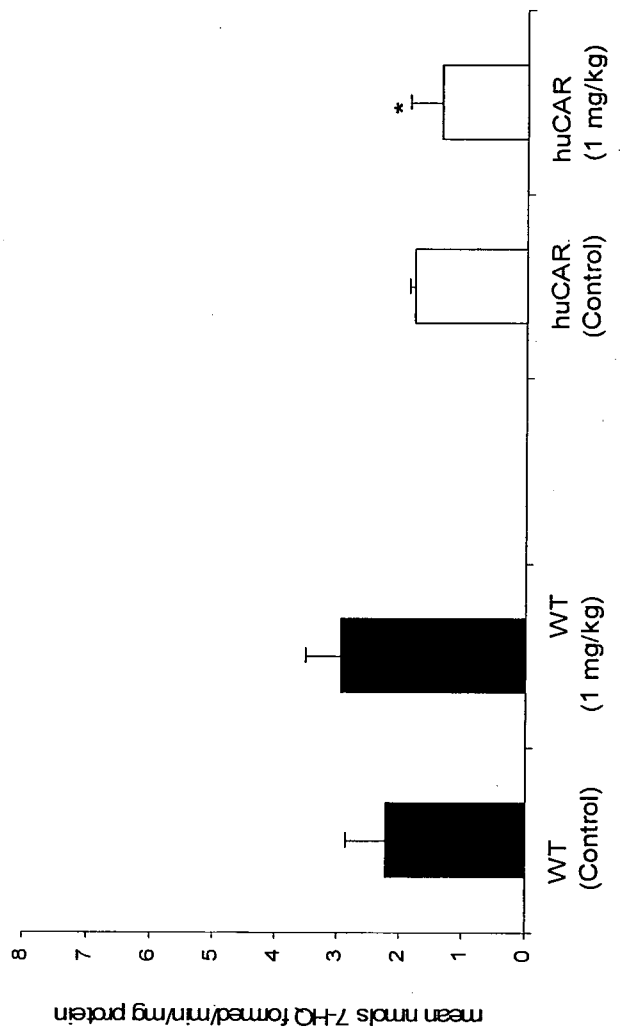

BQ Activity - TCPOBOP
mean nmols 7-HQ formed/min/mg protein
0
1
2
3
4
5
6
7
8
WT (Control)
WT (1 mg/kg)
huCAR (Control)
huCAR (1 mg/kg)
*

Fig. 55

	MOUSE		HUMAN	
	3a11	2b10	3a11	2b10
Phenobarbital	CAR	CAR	CAR	CAR
TCPOBOP	CAR	CAR	-ve	CAR
CITCO	-ve	-ve	CAR	CAR
Rifampicin	PXR	-ve	PXR	-
Dexamethasone	PXR	other	PXR	other
PCN	PXR	PXR		
Clotrimazole	other	CAR		

Fig. 56

	Mouse Lines									
	WT	huPXR	PXR KO	huCAR	CAR KO	WT	huPXR	PXR KO	huCAR	CAR KO
	3a11					2b10				
Rifampicin	+	+++	NC			NC	NC	NC		
PCN	++	NC*				++	NC*			
Dex	++	NC/+	NC	++		+++	+++	+++	+++	
Clotrimazole	+	+				++	++			
TCPOBOP	++	++		NC		+++	+++		NC	
PB	++	++		++		+++	+++		+++	

Table : Effect of inducing agents on PXR and CAR target genes in mouse liver..

+, slight increase in expression compared to vehicle-treated mice of the same strain,
++, medium increase in expression compared to vehicle-treated mice of the same strain
+++, strong increase in expression compared to vehicle-treated mice of the same strain
NC, no change in expression compared to vehicle-treated mice of the same strain
*, only tested 1 and 3 mg/kg PCN

PXR mediated
CAR mediated
Both/other NRs

Fig. 57

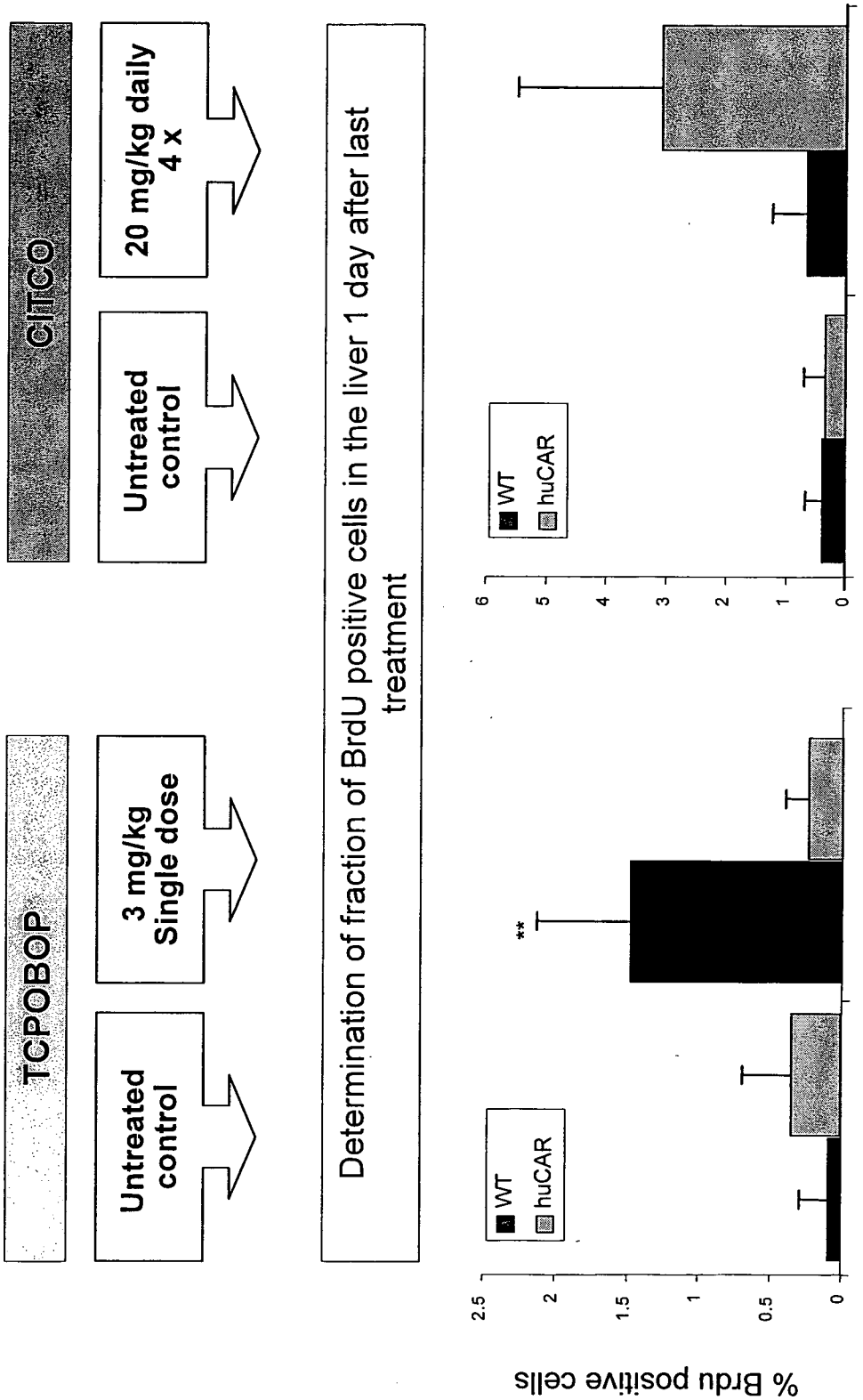

TCPOBOP
CITCO
Untreated control
3 mg/kg Single dose
Untreated control
20 mg/kg daily 4 x
Determination of fraction of BrdU positive cells in the liver 1 day after last treatment
WT
huCAR
**
% Brdu positive cells
2.5
2
1.5
1
0.5
0
6
5
4
3
2
1
0

Fig. 58

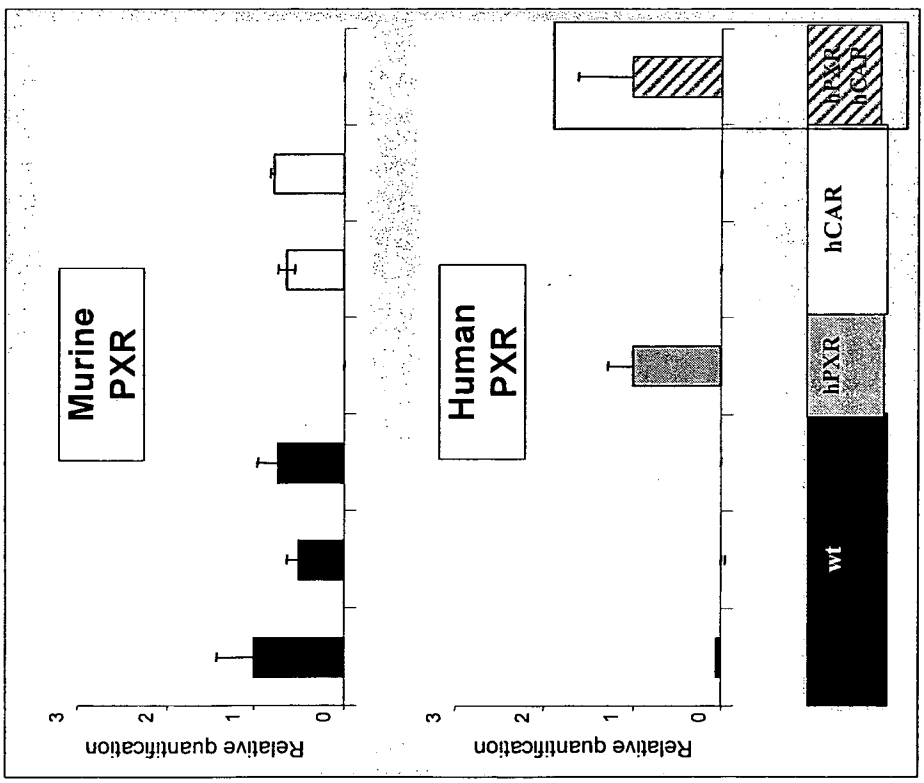
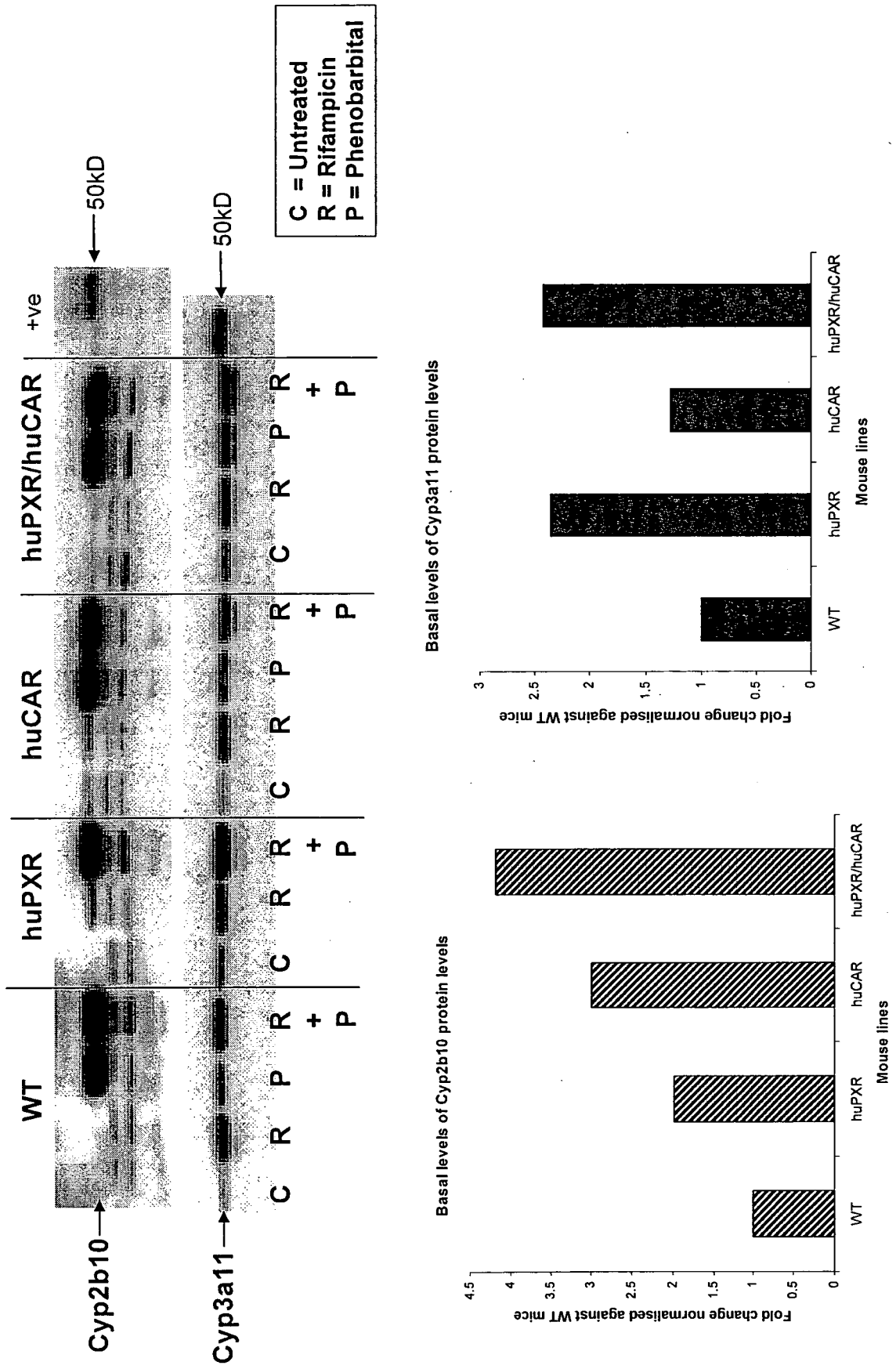

Murine PXR
Human PXR
Murine CAR
Human CAR
Relative quantification
0
1
2
3
wt
hPXR
hCAR
hPXR hCAR

Fig. 59


WT
huPXR
huCAR
huPXR/huCAR
+ve
50kD
50kD
C = Untreated
R = Rifampicin
P = Phenobarbital
Cyp2b10
Cyp3a11
C R P R + P
Basal levels of Cyp2b10 protein levels
Fold change normalised against WT mice
Mouse lines
WT
huPXR
huCAR
huPXR/huCAR
Basal levels of Cyp3a11 protein levels
Fold change normalised against WT mice
Mouse lines

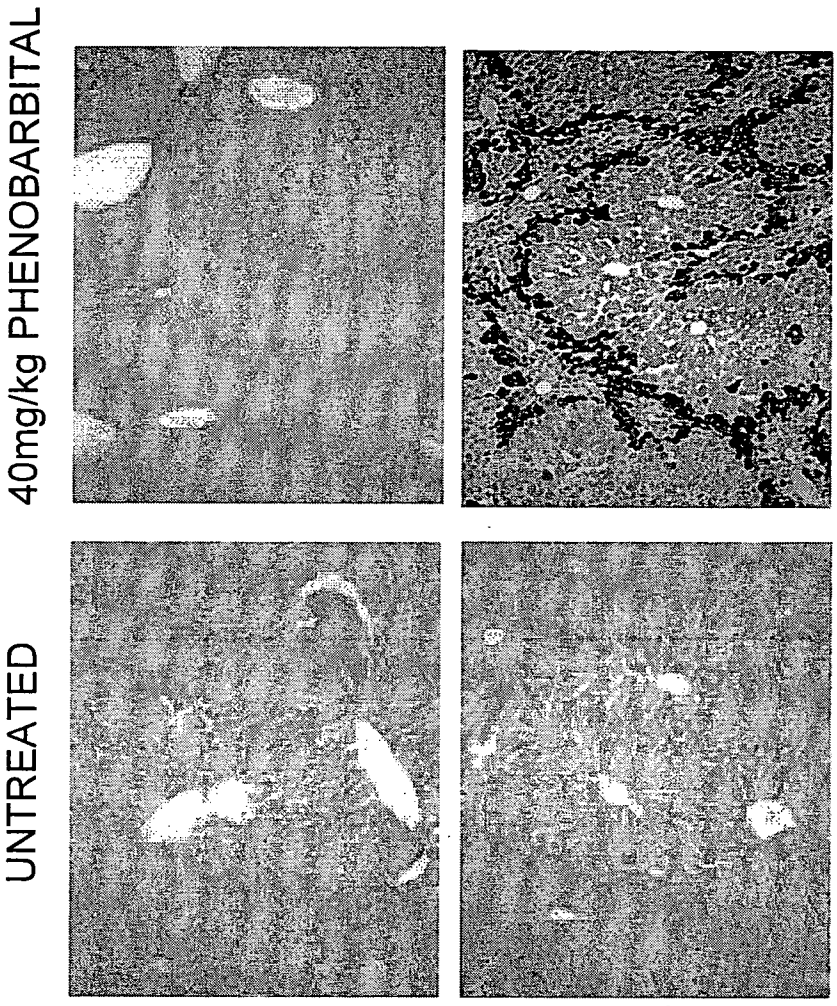

40mg/kg PHENOBARBITAL
UNTREATED
WILDTYPE
CYP2B6 reporter

Fig. 60

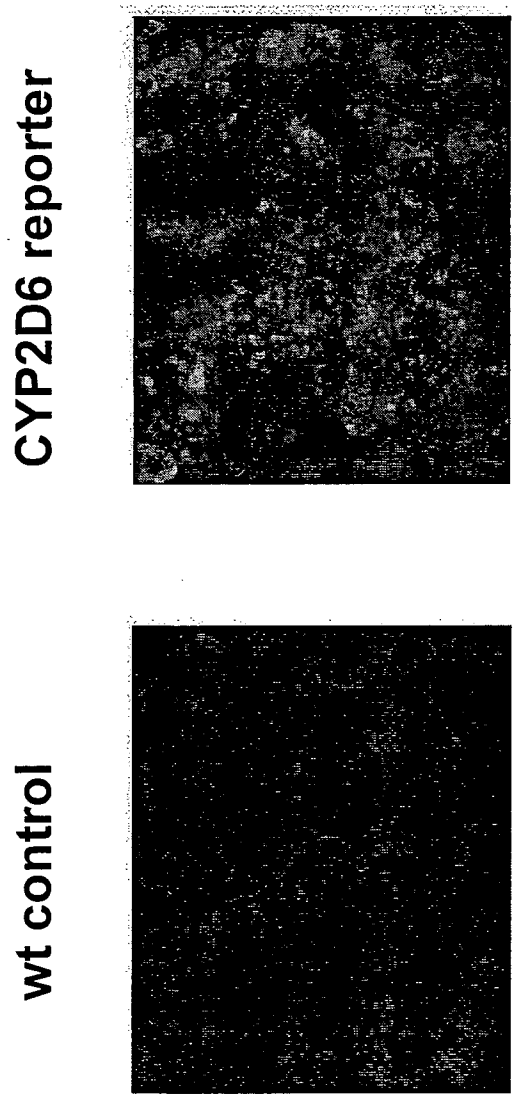
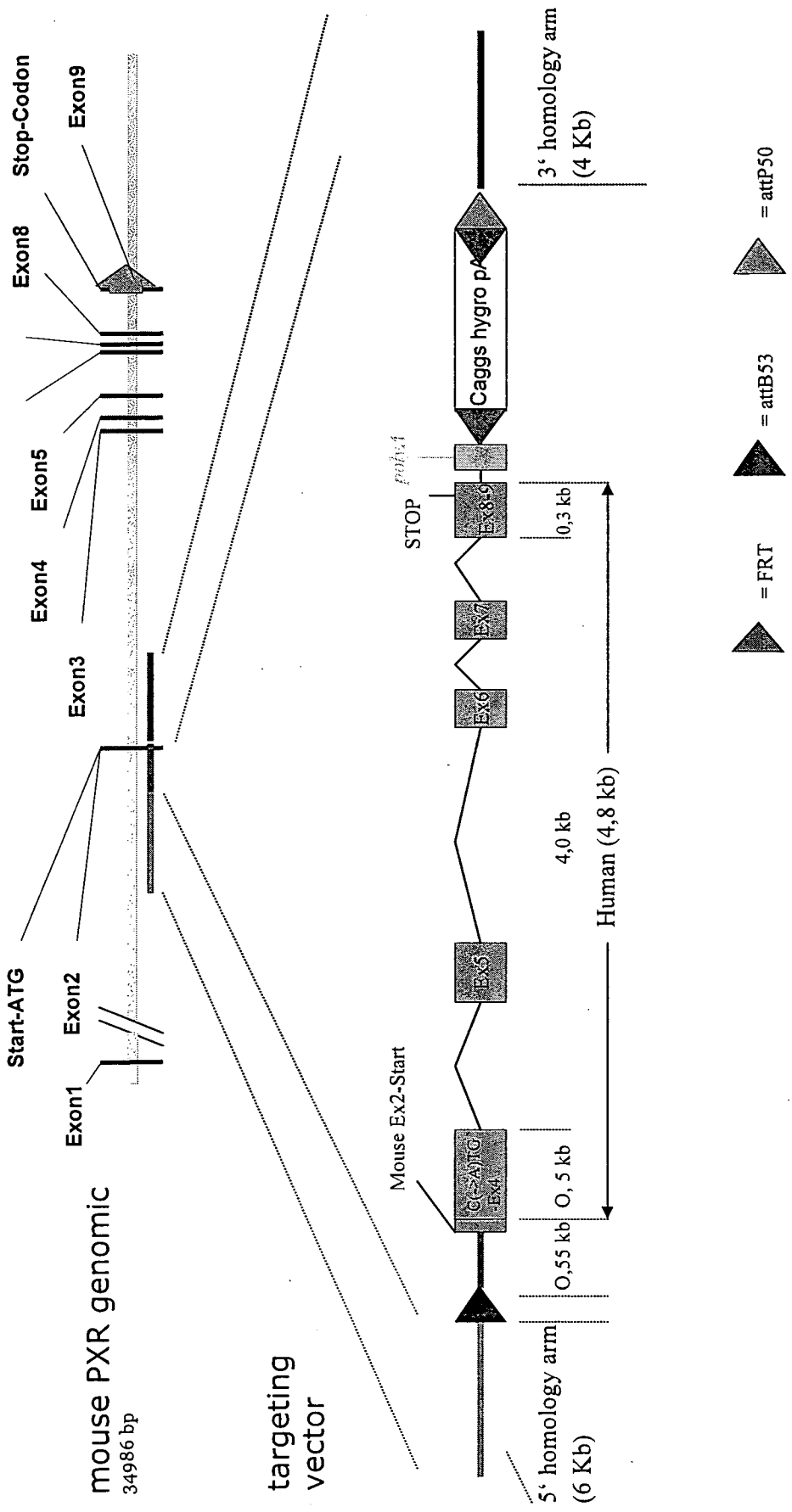


Fig. 61

Fig. 62


mouse PXR genomic
34986 bp
Start-ATG
Exon1
Exon2
Exon3
Exon4
Exon5
Exon6
Exon7
Exon8
Stop-Codon
Exon9
targeting vector
Mouse Ex2-Start
(C->A)TG Ex4
Ex5
Ex6
Ex7
Ex8-9
STOP
Caggs hygro pA
5' homology arm (6 Kb)
3' homology arm (4 Kb)
0,55 kb
0, 5 kb
4,0 kb
0,3 kb
Human (4,8 kb)
= FRT
= attB53
= attP50

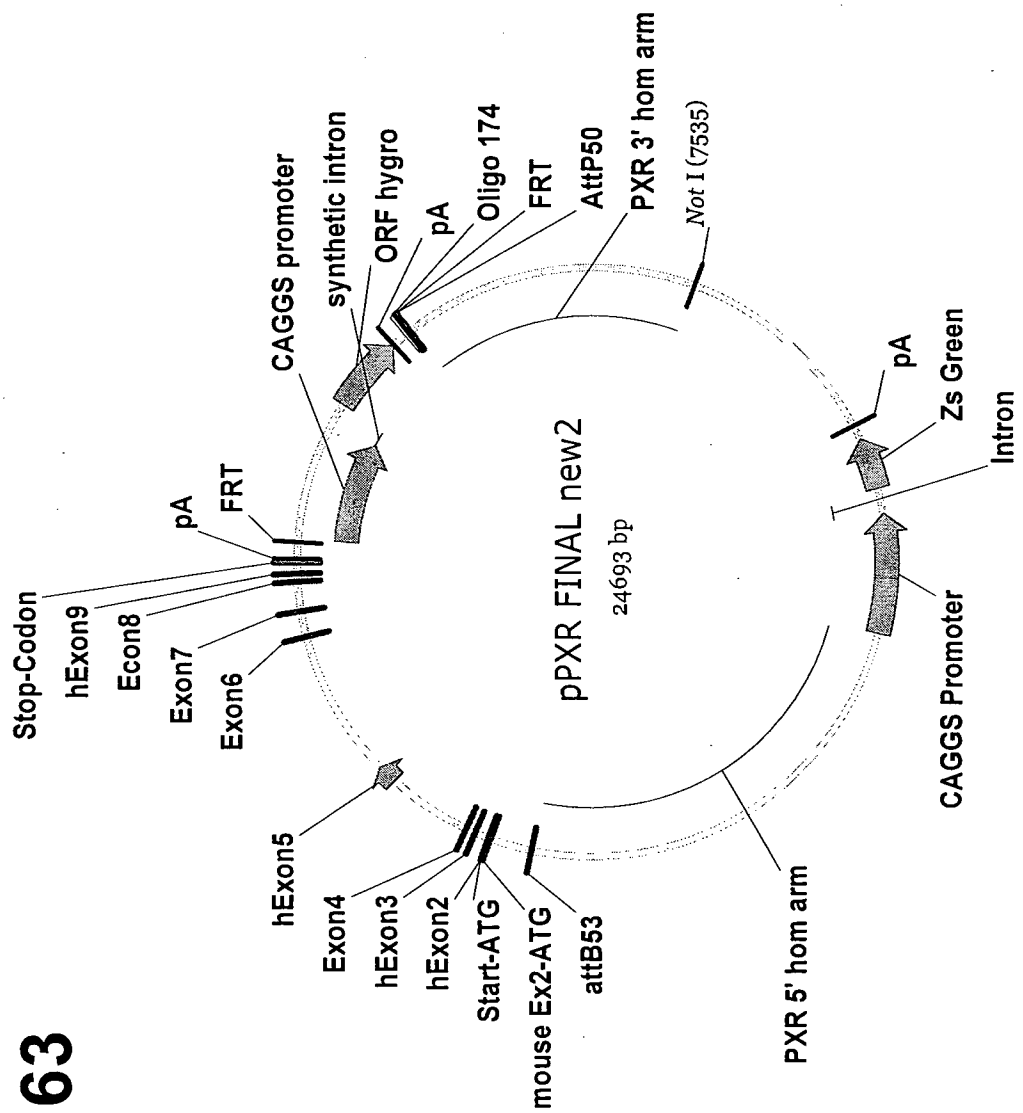
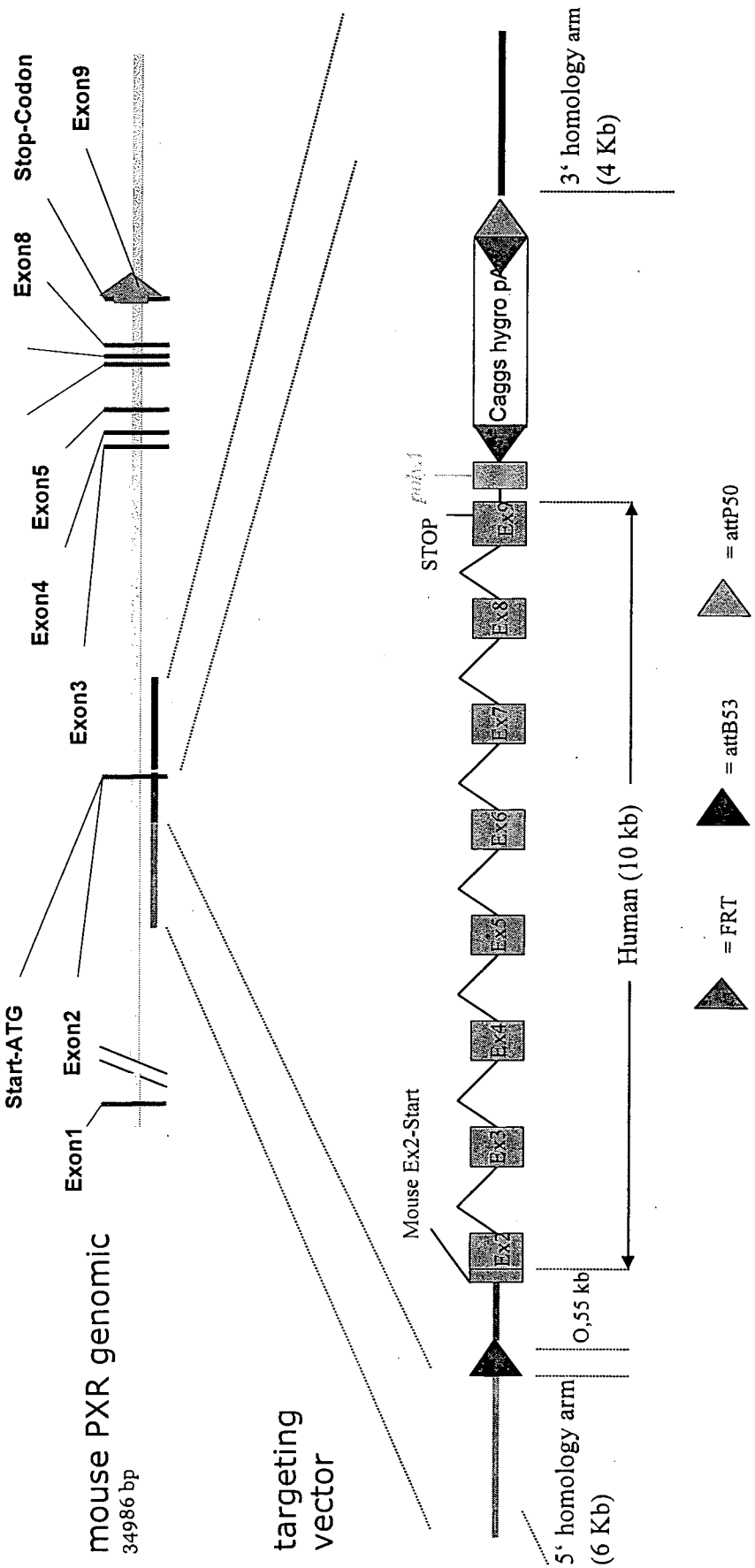

pPXR FINAL new2
24693 bp
Not I (7535)
PXR 3' hom arm
AttP50
FRT
Oligo 174
pA
ORF hygro
synthetic intron
CAGGS promoter
pA
FRT
Stop-Codon
hExon9
Econ8
Exon7
Exon6
hExon5
Exon4
hExon3
hExon2
Start-ATG
mouse Ex2-ATG
attB53
PXR 5' hom arm
CAGGS Promoter
Intron
Zs Green
pA

Fig. 63

Fig. 64


mouse PXR genomic
34986 bp
Start-ATG
Exon1
Exon2
Exon3
Exon4
Exon5
Exon6
Exon7
Exon8
Stop-Codon
Exon9
targeting vector
Mouse Ex2-Start
STOP
Caggs hygro pA
3' homology arm (4 Kb)
5' homology arm (6 Kb)
0,55 kb
Human (10 kb)
= FRT
= attB53
= attP50

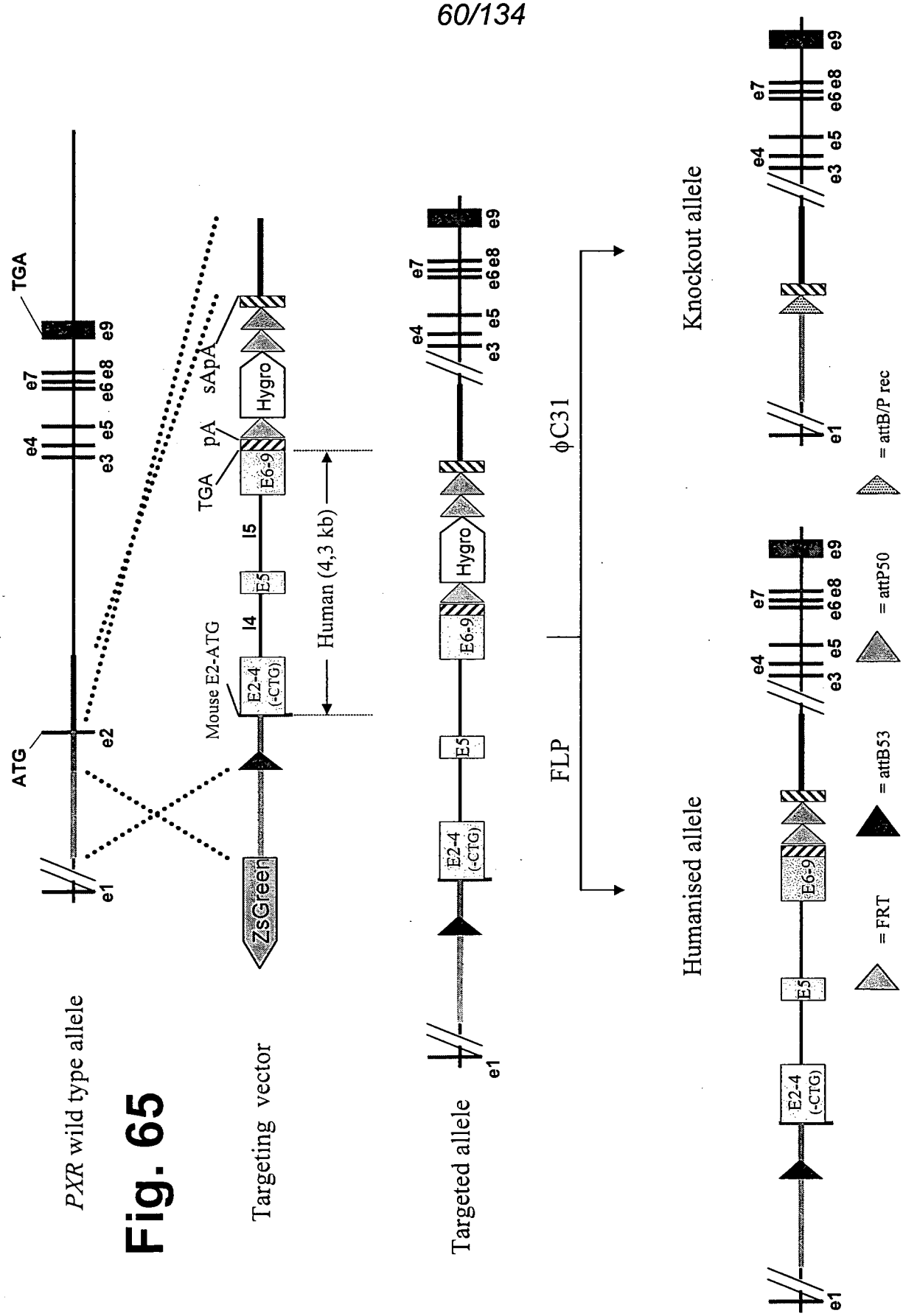

PXR wild type allele
ATG
TGA
e1
e2
e3
e4
e5
e6e8
e7
e9
Targeting vector
Mouse E2-ATG
E2-4 (-CTG)
I4
E5
I5
E6-9
TGA
pA
sApA
Hygro
ZsGreen
Human (4,3 kb)
Targeted allele
FLP
ϕC31
Knockout allele
Humanised allele
= attB/P rec
= attP50
= attB53
= FRT

Fig. 65

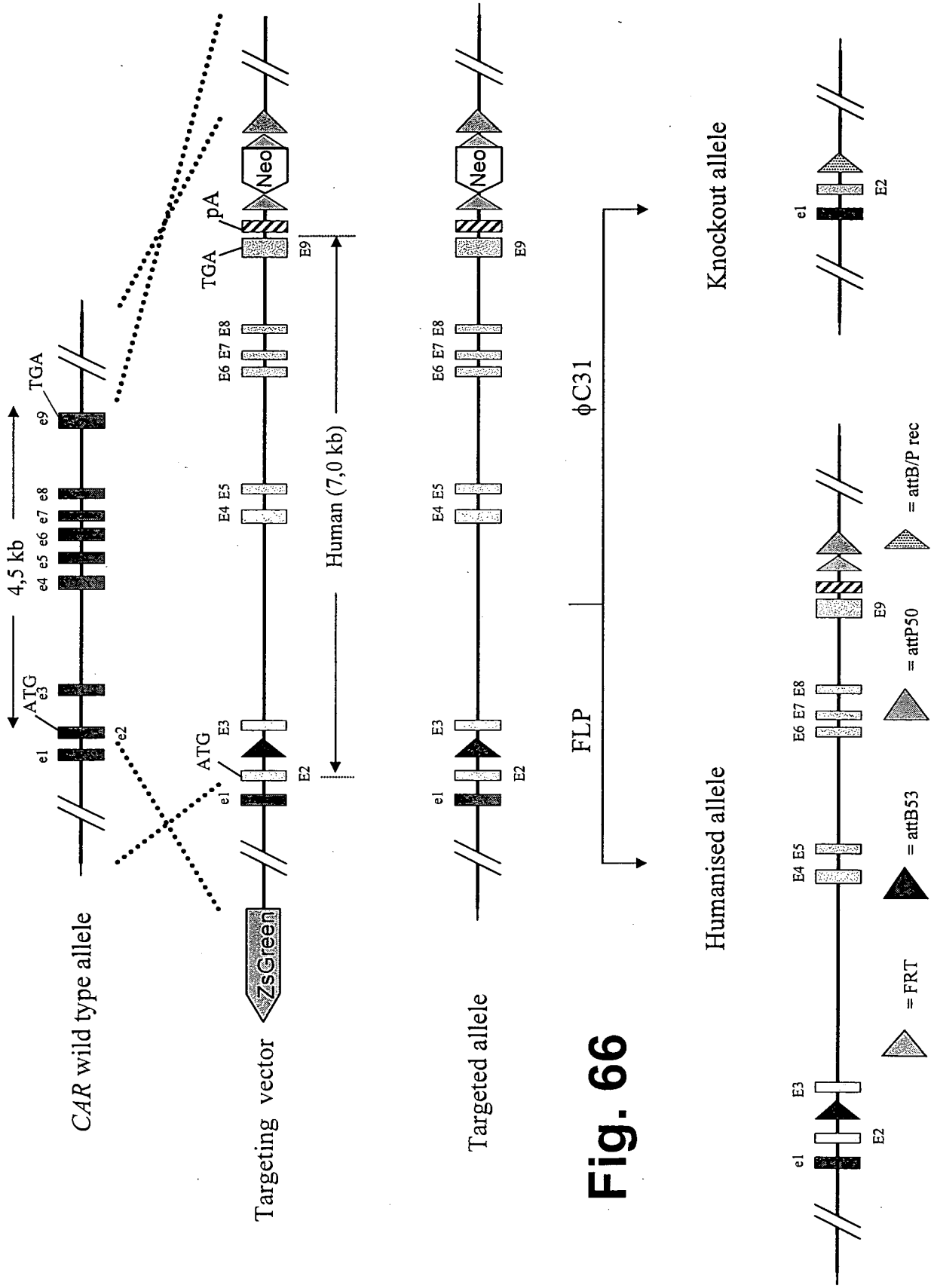

CAR wild type allele
4,5 kb
ATG
TGA
e1
e2
e3
e4 e5 e6 e7 e8
e9
Targeting vector
ATG
TGA
pA
Neo
ZsGreen
E1
E2
E3
E4 E5
E6 E7 E8
E9
Human (7,0 kb)
Targeted allele
FLP
ϕC31
Knockout allele
Humanised allele
= FRT
= attB53
= attP50
= attB/P rec

Fig. 66

Fig. 67

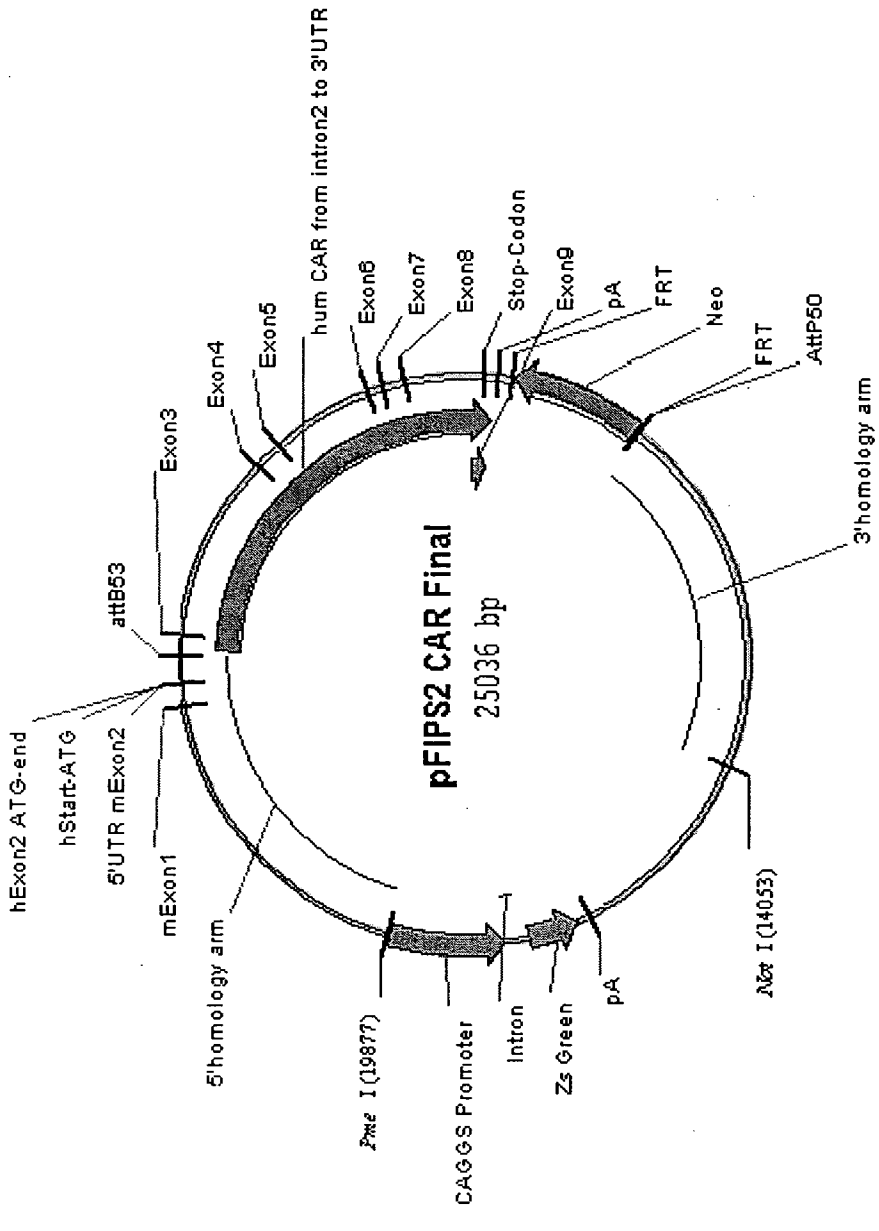

pFIPS2 CAR Final
25036 bp
hExon2 ATG-end
hStart-ATG
5'UTR mExon2
attB53
Exon3
mExon1
Exon4
Exon5
hum CAR from intron2 to 3'UTR
Exon6
Exon7
Exon8
Stop-Codon
Exon9
pA
FRT
Neo
FRT
AttP50
3'homology arm
5'homology arm
Pme I (19877)
CAGGS Promoter
Intron
Zs Green
pA
Not I (14053)

Fig. 68

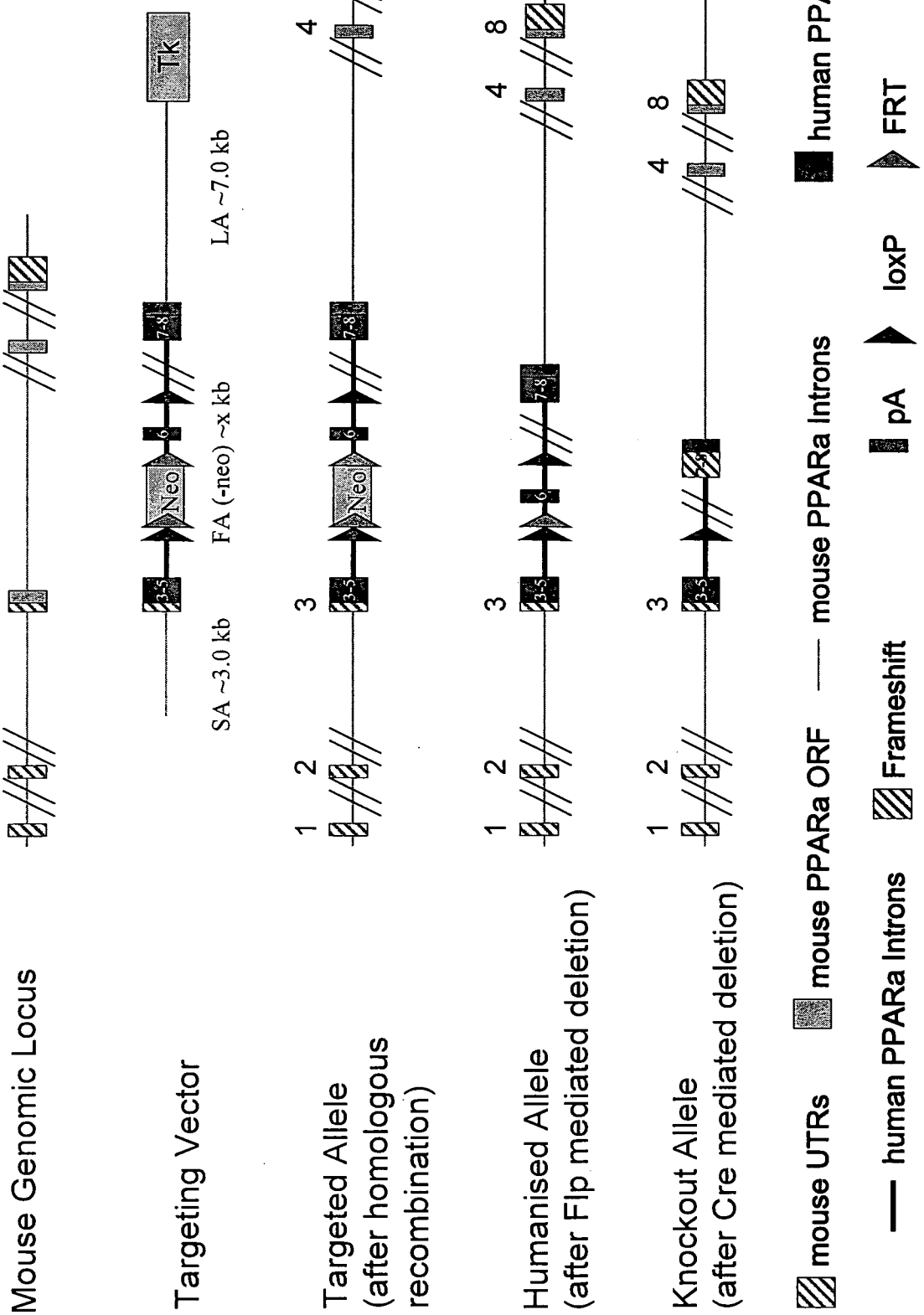

Mouse Genomic Locus
1 2 3 4 8
Targeting Vector
SA ~3.0 kb
Neo
FA (-neo) ~x kb
TK
LA ~7.0 kb
Targeted Allele
(after homologous
recombination)
Humanised Allele
(after Flp mediated deletion)
Knockout Allele
(after Cre mediated deletion)
mouse UTRs
mouse PPARa ORF
human PPARa Introns
Frameshift
mouse PPARa Introns
pA
loxP
human PPARa ORF
FRT

Fig. 69

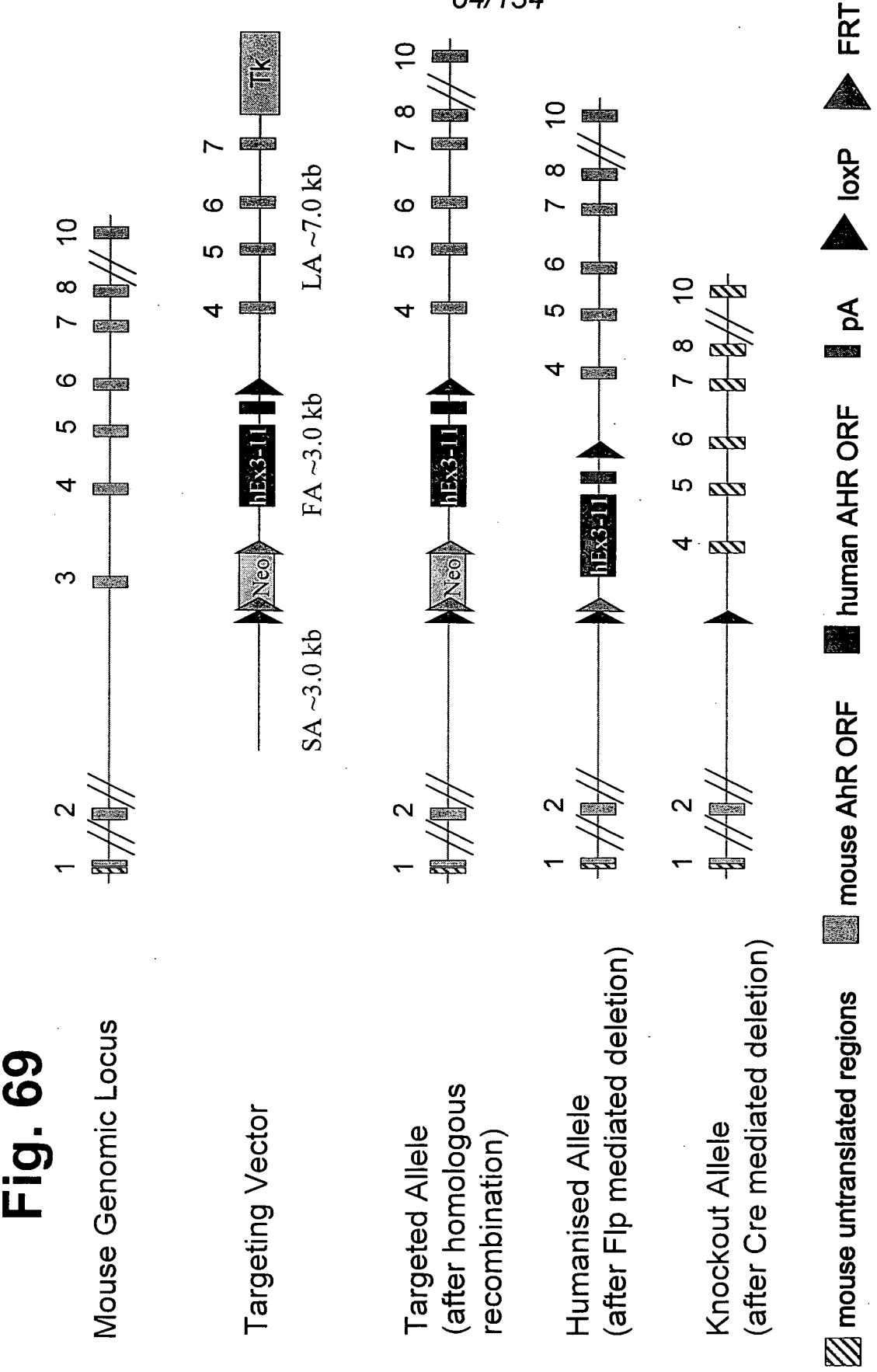

Mouse Genomic Locus
1 2 3 4 5 6 7 8 10
Targeting Vector
SA ~3.0 kb
Neo
hEx3-11
FA ~3.0 kb
4 5 6 7
Tk
LA ~7.0 kb
Targeted Allele
(after homologous
recombination)
1 2
4 5 6 7 8 10
Humanised Allele
(after Flp mediated deletion)
Knockout Allele
(after Cre mediated deletion)
mouse untranslated regions
mouse AhR ORF
human AHR ORF
pA
loxP
FRT
Frameshift

Fig. 70

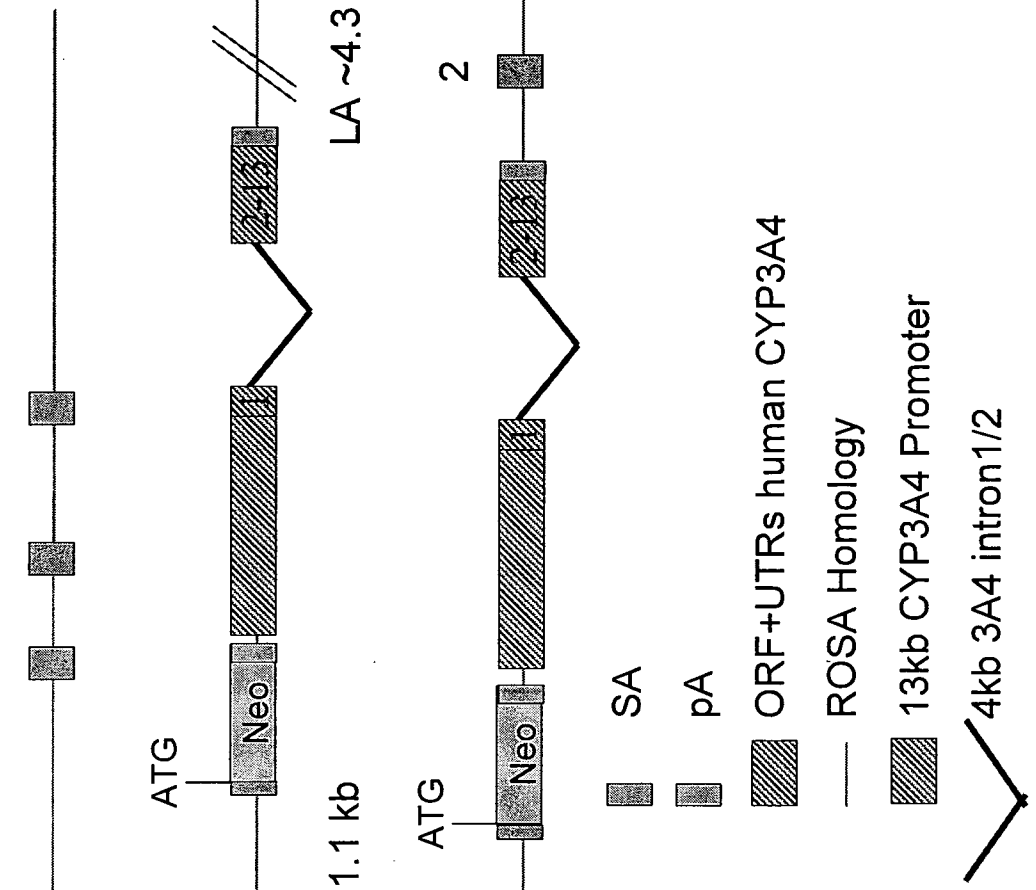

Mouse ROSA 26 Locus
1
2
3
Targeting Vector
ATG
Neo
SA ~1.1 kb
LA ~4.3 kb
Targeted Allele
(after homologous recombination)
ATG
Neo
SA
pA
ORF+UTRs human CYP3A4
ROSA Homology
13kb CYP3A4 Promoter
4kb 3A4 intron1/2

Fig. 71

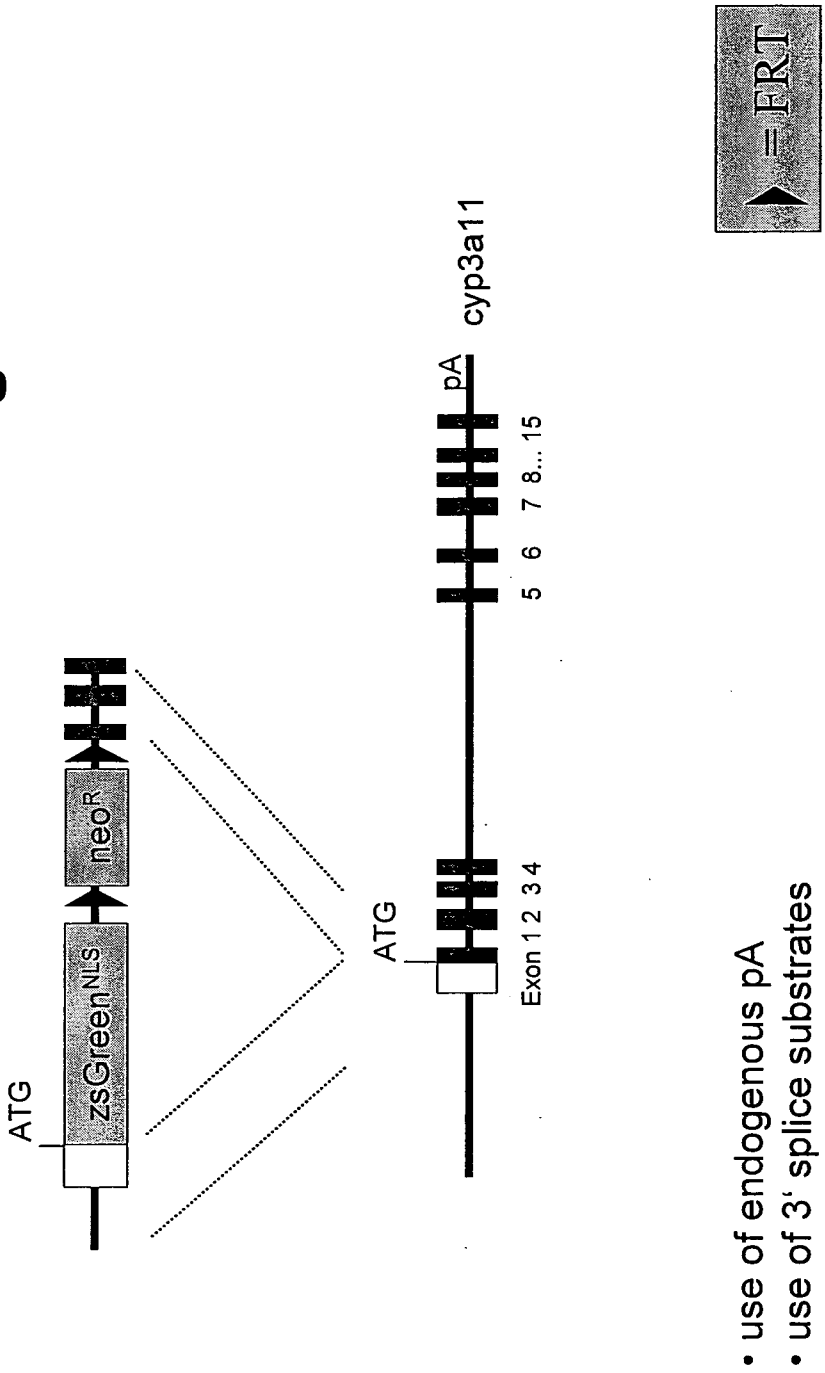

ATG
zsGreen NLS
neo R
ATG
Exon 1 2 3 4
5 6 7 8... 15
pA
cyp3a11
= FRT
• use of endogenous pA
• use of 3' splice substrates

Fig. 72 A

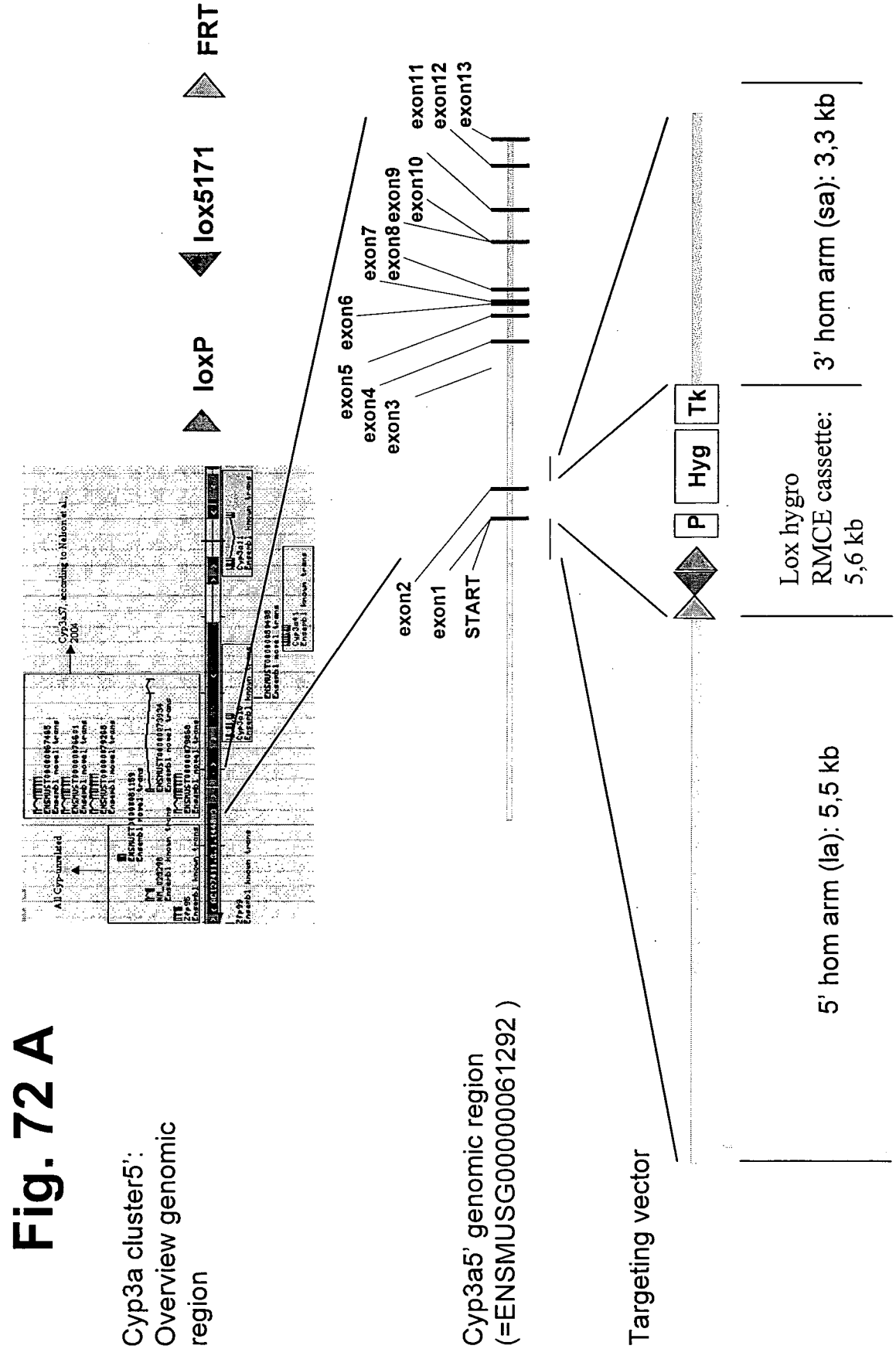

Cyp3a cluster5':
Overview genomic region
loxP
lox5171
FRT
Cyp3a5' genomic region
(=ENSMUSG00000061292)
exon1
exon2
START
exon3
exon4
exon5
exon6
exon7
exon8
exon9
exon10
exon11
exon12
exon13
Targeting vector
P
Hyg
Tk
Lox hygro
RMCE cassette:
5,6 kb
5' hom arm (la): 5,5 kb
3' hom arm (sa): 3,3 kb

Fig. 72 B

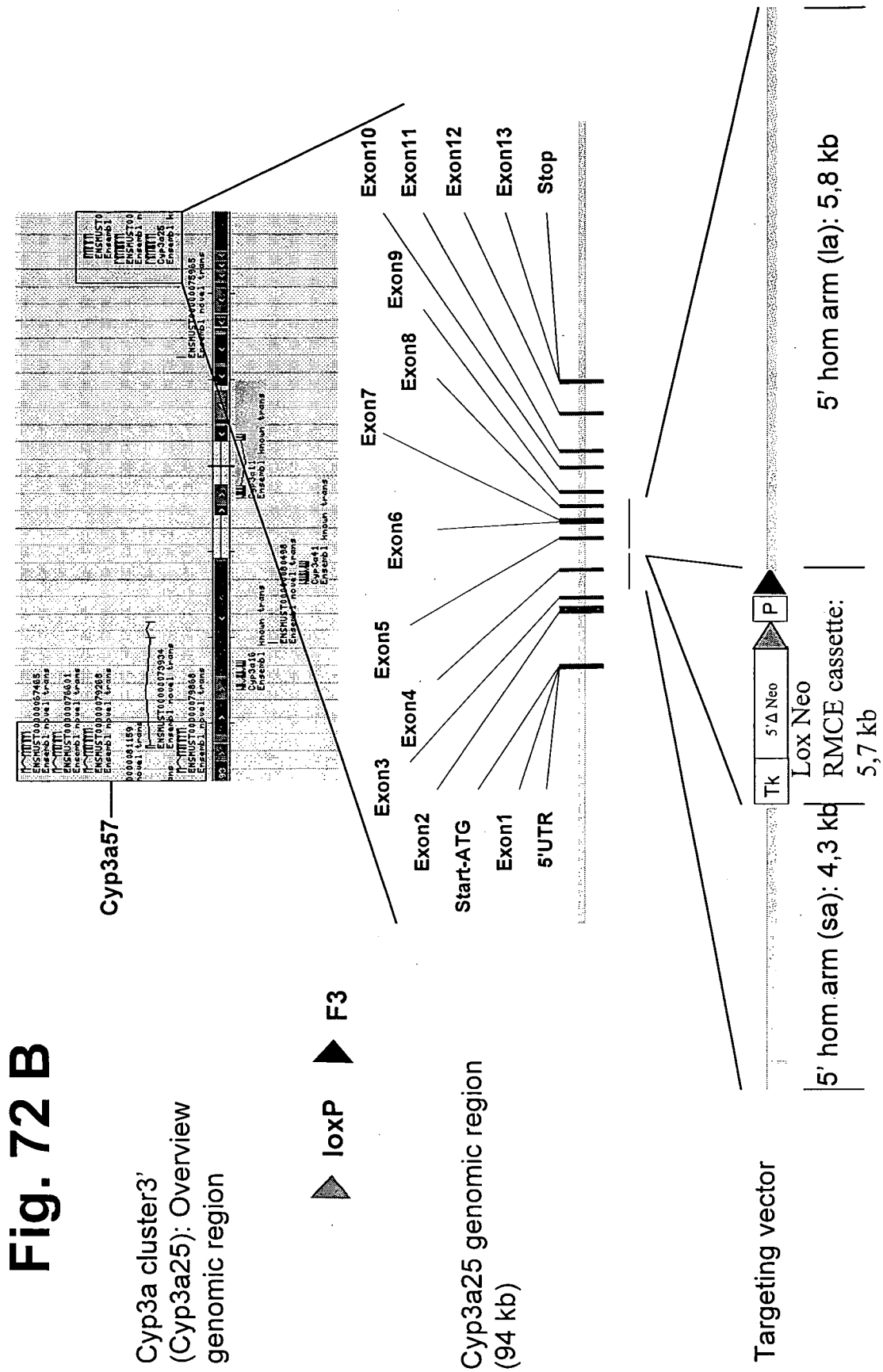

Cyp3a cluster3' (Cyp3a25): Overview genomic region
Cyp3a57
loxP
F3
Cyp3a25 genomic region (94 kb)
Exon2
Exon3
Exon4
Exon5
Exon6
Exon7
Exon8
Exon9
Exon10
Exon11
Exon12
Exon13
Stop
Start-ATG
Exon1
5'UTR
Targeting vector
Tk
5'Δ Neo
P
Lox Neo
RMCE cassette: 5,7 kb
5' hom arm (sa): 4,3 kb
5' hom arm (la): 5,8 kb

Fig. 72 C

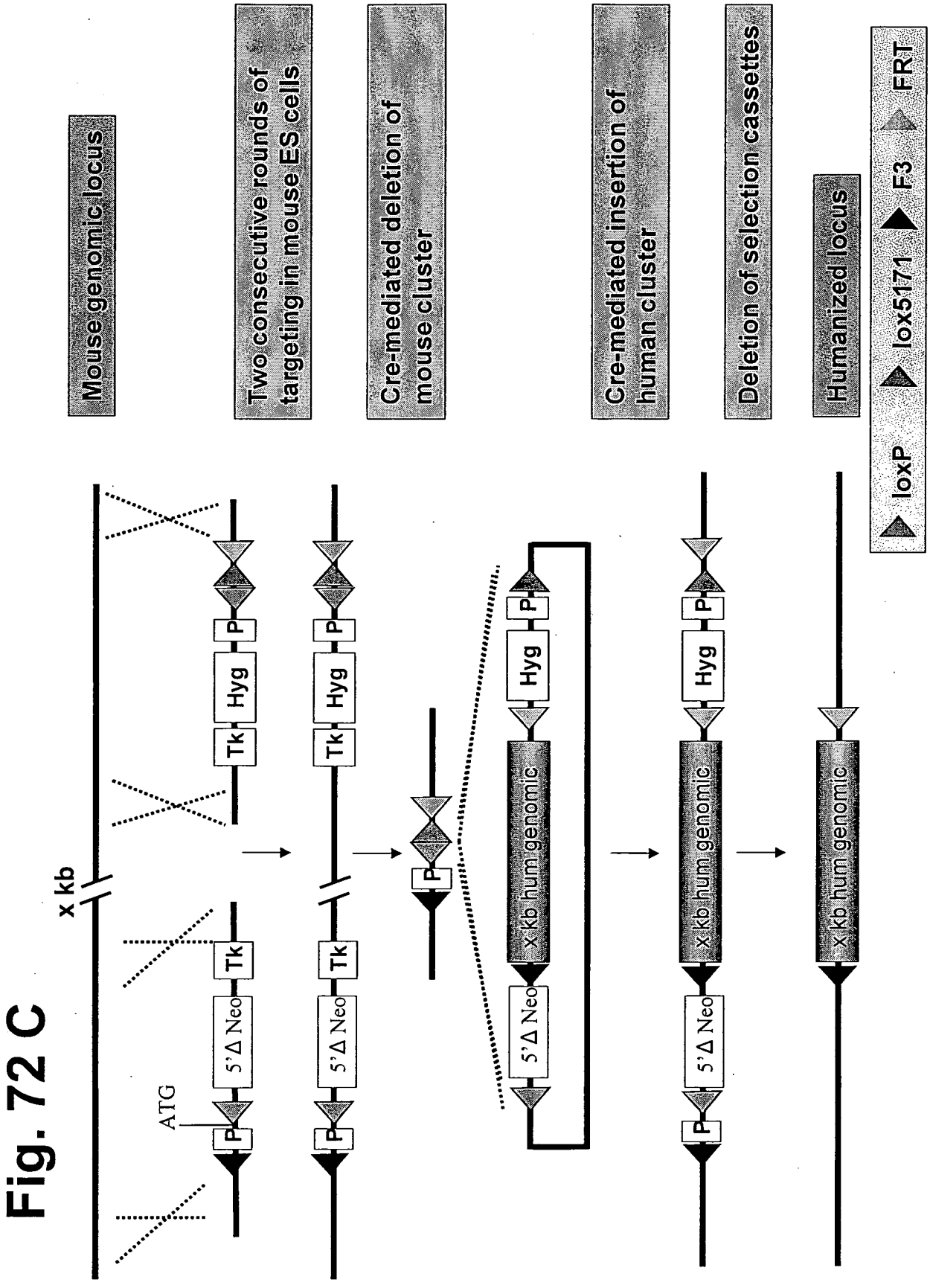
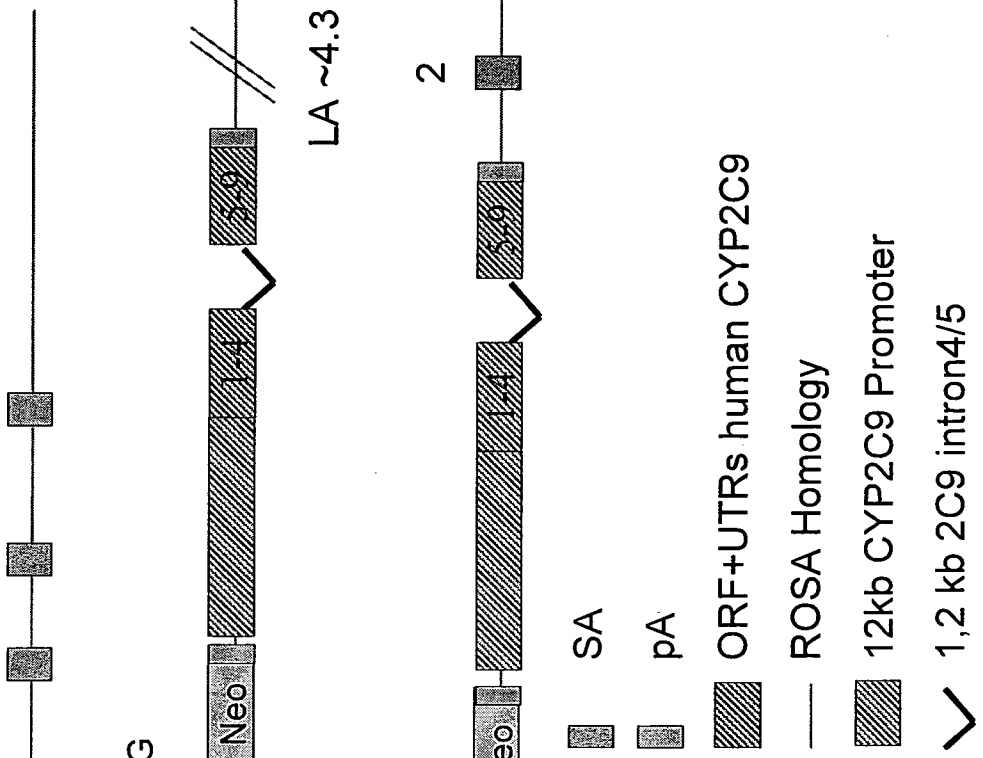

Mouse genomic locus
Two consecutive rounds of targeting in mouse ES cells
Cre-mediated deletion of mouse cluster
Cre-mediated insertion of human cluster
Deletion of selection cassettes
Humanized locus
loxP
lox5171
F3
FRT
x kb
ATG
P
5'Δ Neo
Tk
Hyg
x kb hum genomic

Fig. 73


Mouse ROSA 26 Locus
1
2
3
Targeting Vector
ATG
Neo
1,4
5,6
SA ~1.1 kb
LA ~4.3 kb
Targeted Allele
(after homologous recombination)
1
ATG
Neo
1,4
5,6
2
3
SA
pA
ORF+UTRs human CYP2C9
ROSA Homology
12kb CYP2C9 Promoter
1,2 kb 2C9 intron4/5

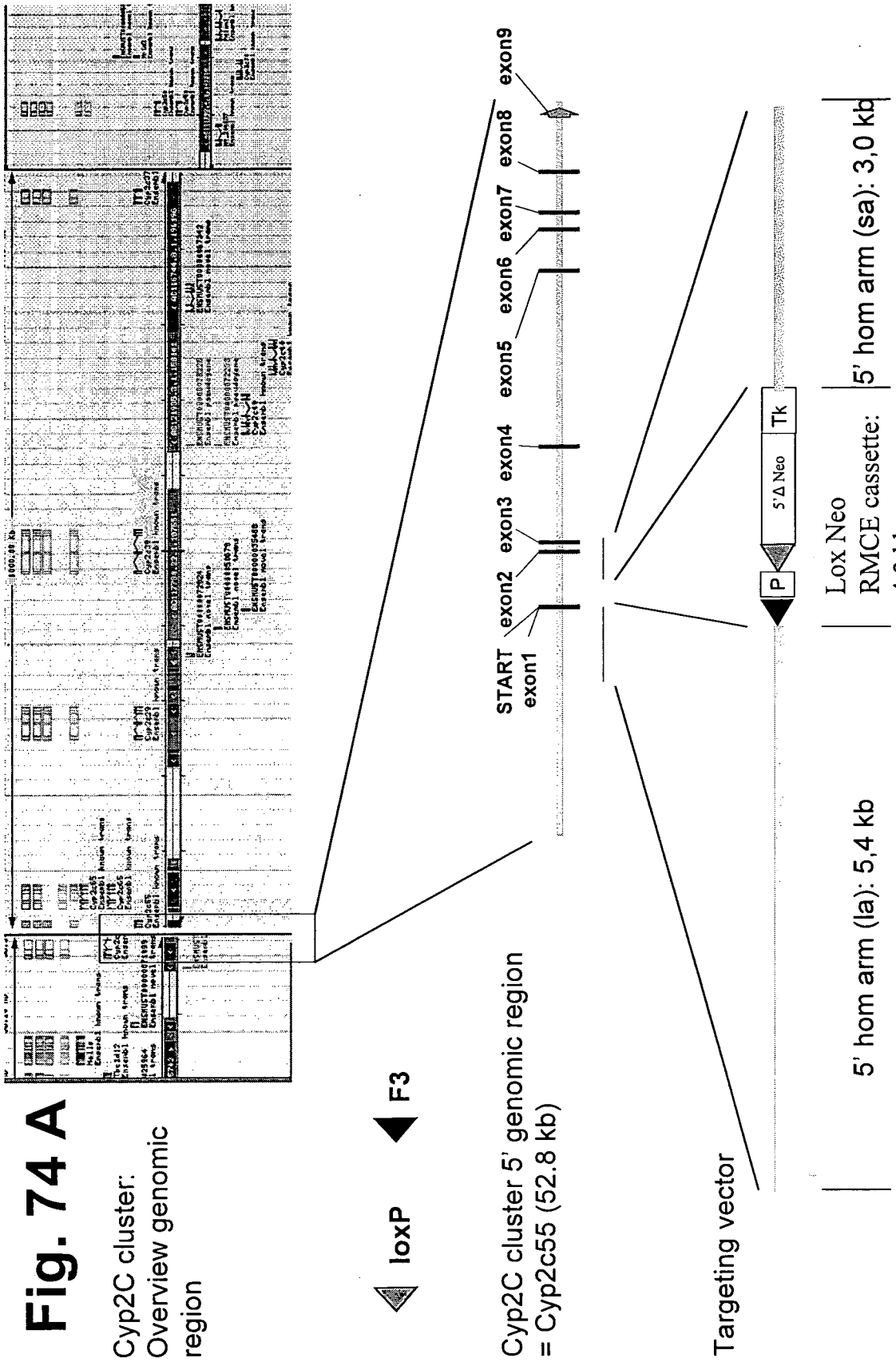


Fig. 74 A
Cyp2C cluster:
Overview genomic region
loxP
F3
Cyp2C cluster 5' genomic region
= Cyp2c55 (52.8 kb)
START
exon1
exon2
exon3
exon4
exon5
exon6
exon7
exon8
exon9
Targeting vector
P
5'Δ Neo
Tk
Lox Neo
RMCE cassette:
4,3 kb
5' hom arm (la): 5,4 kb
5' hom arm (sa): 3,0 kb

Fig. 74 B

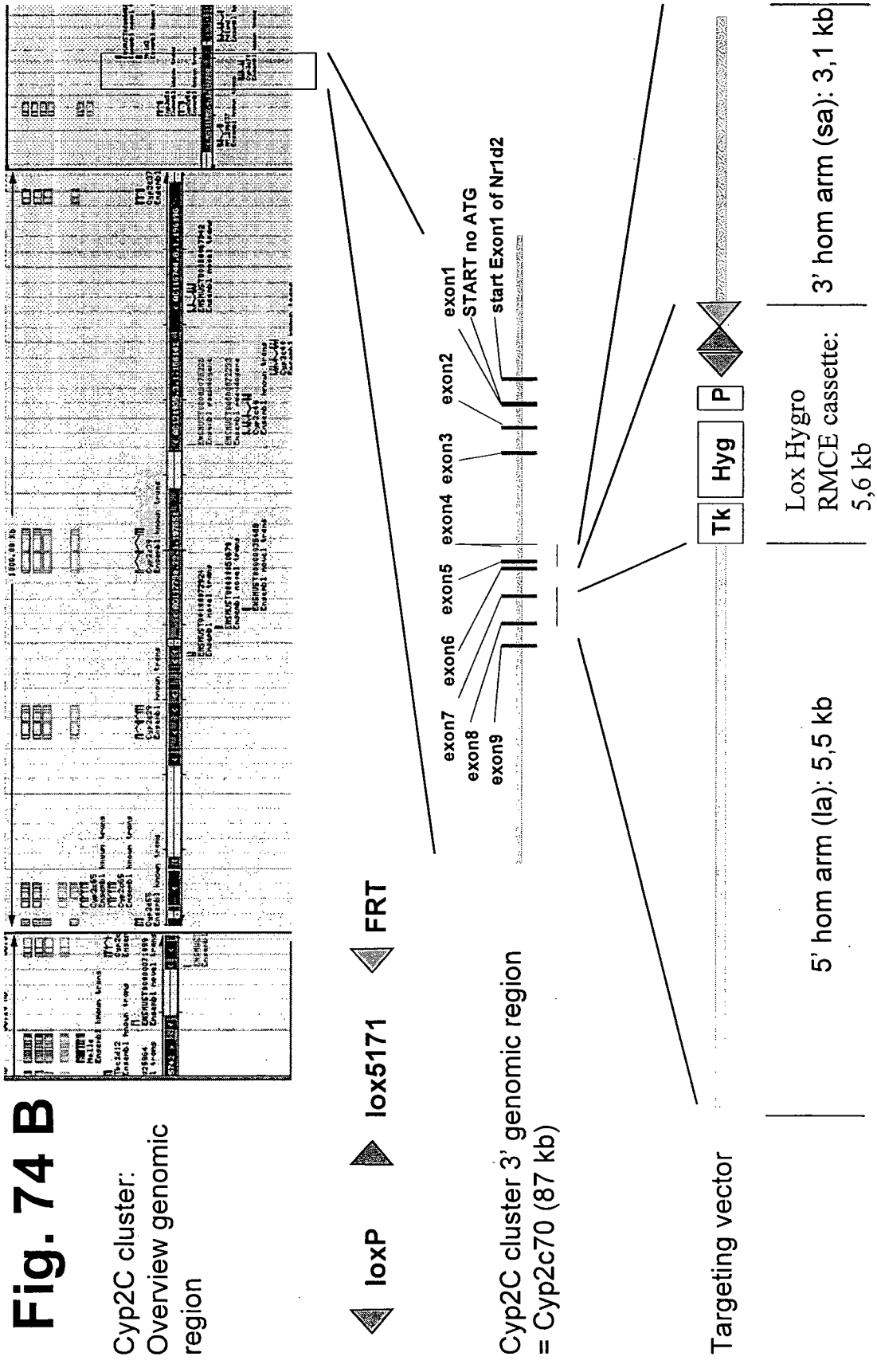

Cyp2C cluster:
Overview genomic region
loxP
lox5171
FRT
Cyp2C cluster 3' genomic region
= Cyp2c70 (87 kb)
exon1
START no ATG
start Exon1 of Nr1d2
exon2
exon3
exon4
exon5
exon6
exon7
exon8
exon9
Targeting vector
Tk
Hyg
P
Lox Hygro
RMCE cassette:
5,6 kb
5' hom arm (la): 5,5 kb
3' hom arm (sa): 3,1 kb

Fig. 74 C

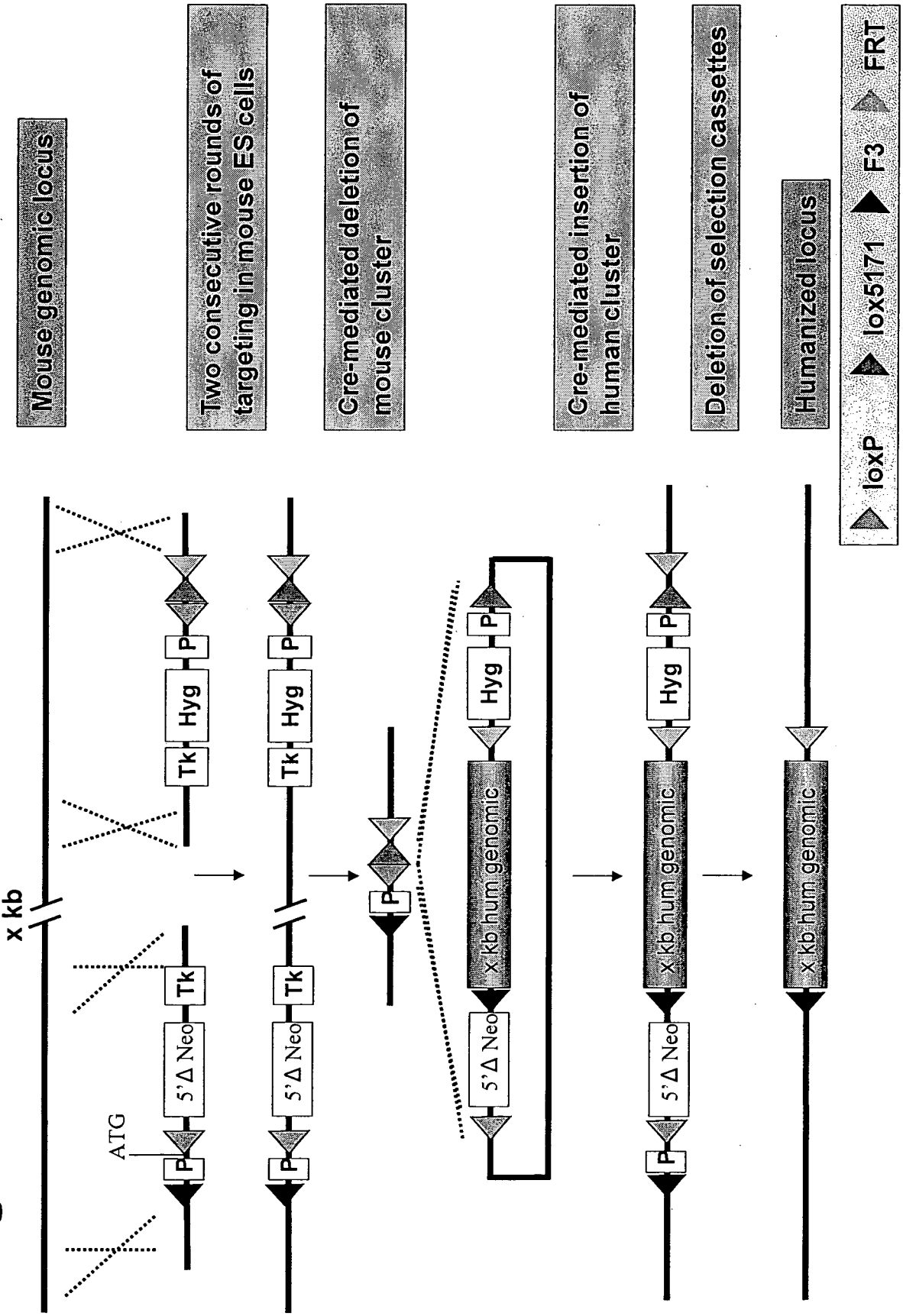

Mouse genomic locus
Two consecutive rounds of targeting in mouse ES cells
Cre-mediated deletion of mouse cluster
Cre-mediated insertion of human cluster
Deletion of selection cassettes
Humanized locus
loxP
lox5171
F3
FRT
x kb
ATG
Tk
5'Δ Neo
P
Hyg
x kb hum genomic

Fig. 75 A

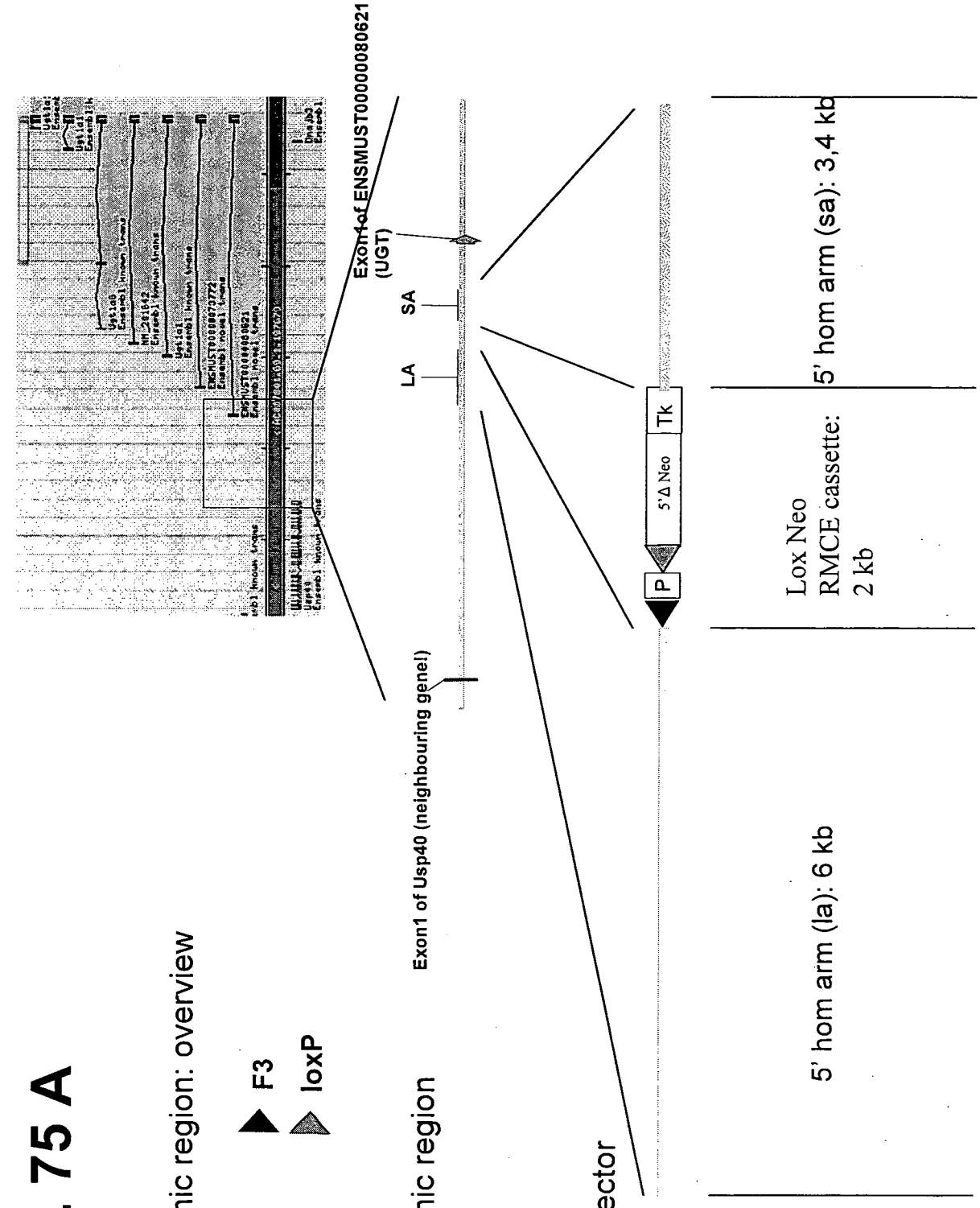

UGT genomic region: overview
F3
loxP
Exon1 of ENSMUST00000080621 (UGT)
SA
LA
Exon1 of Usp40 (neighbouring gene!)
UGT genomic region (65.3 kb)
Targeting vector
P
5'Δ Neo
Tk
Lox Neo RMCE cassette: 2 kb
5' hom arm (sa): 3,4 kb
5' hom arm (la): 6 kb

Fig. 75 B

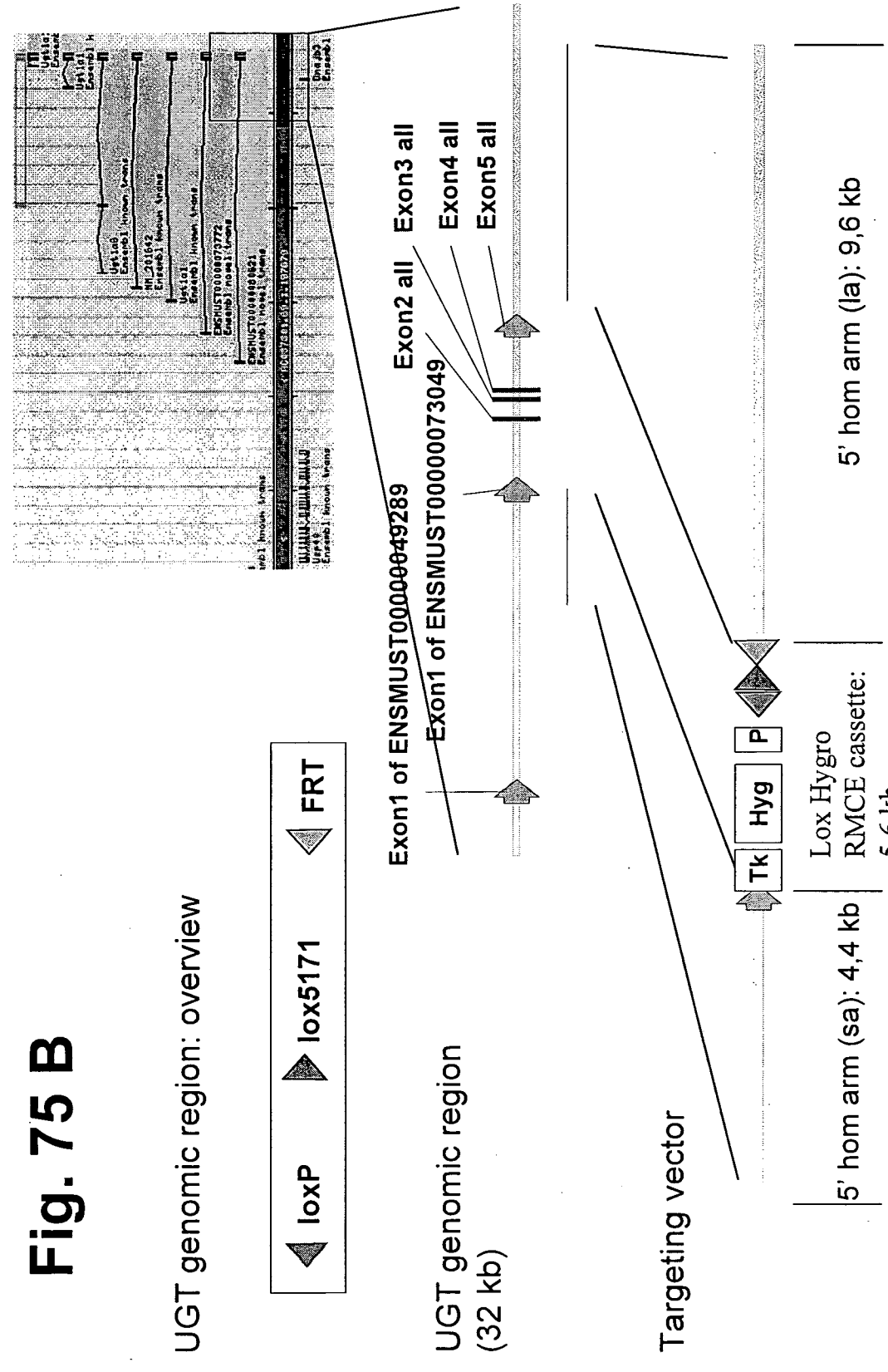

UGT genomic region: overview
loxP
lox5171
FRT
UGT genomic region
(32 kb)
Exon1 of ENSMUST00000049289
Exon1 of ENSMUST00000073049
Exon2 all
Exon3 all
Exon4 all
Exon5 all
Targeting vector
Tk
Hygro
P
Lox Hygro
RMCE cassette:
5,6 kb
5' hom arm (sa): 4,4 kb
5' hom arm (la): 9,6 kb

Fig. 75 C

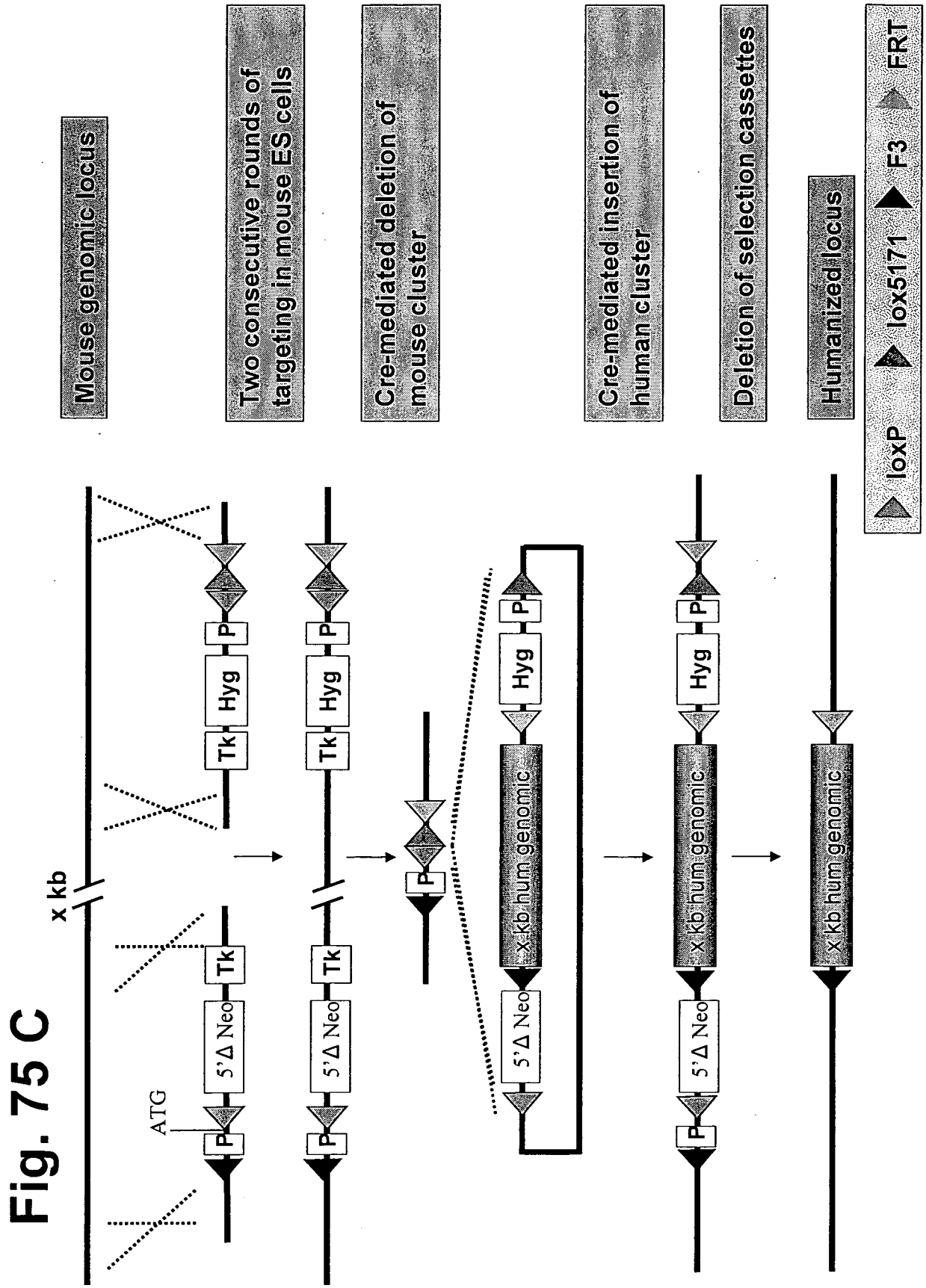

Mouse genomic locus
Two consecutive rounds of targeting in mouse ES cells
Cre-mediated deletion of mouse cluster
Cre-mediated insertion of human cluster
Deletion of selection cassettes
Humanized locus
loxP
lox5171
F3
FRT
x kb
ATG
P
5'Δ Neo
Tk
Hyg
x kb hum genomic

Fig. 76

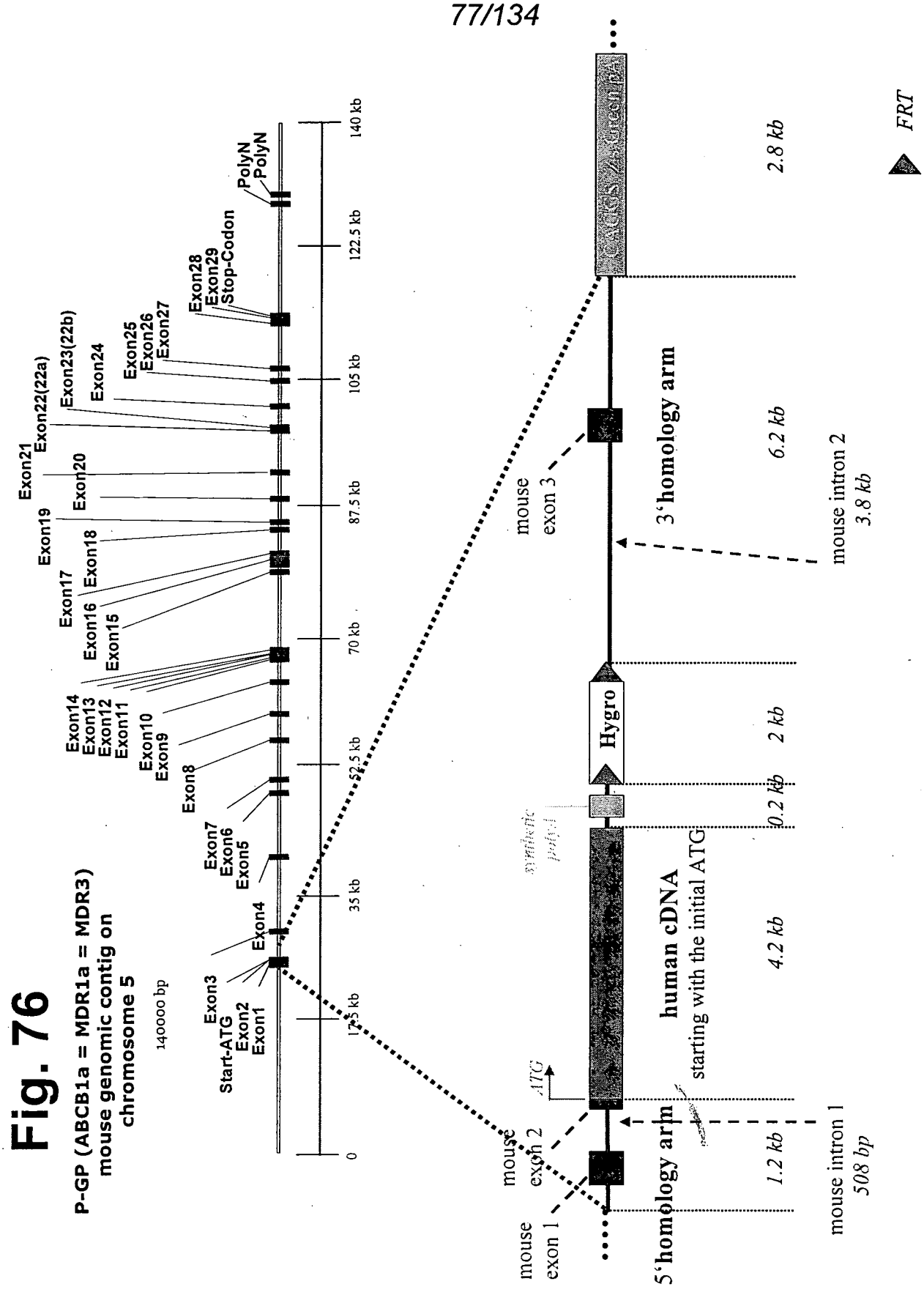
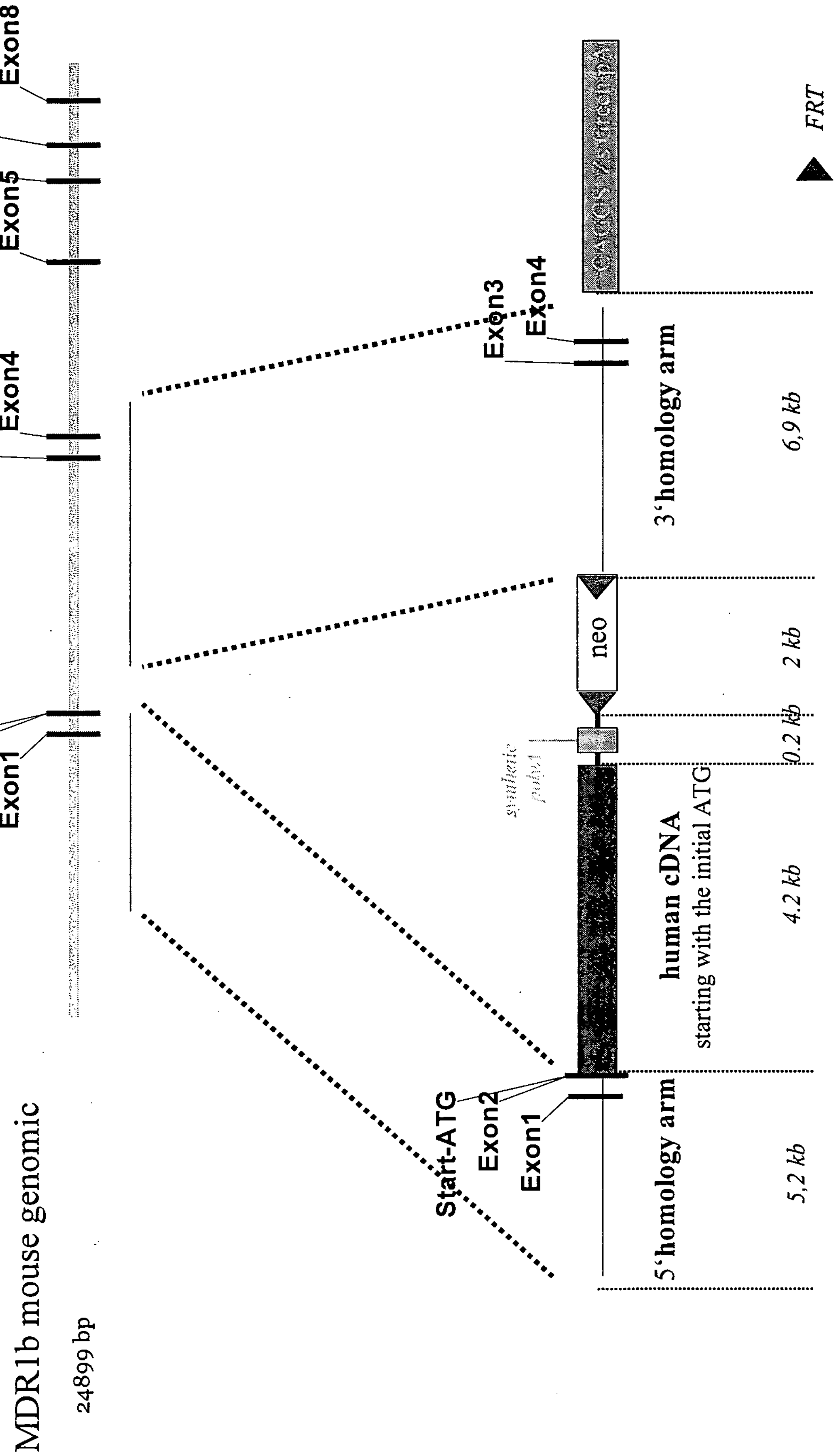

P-GP (ABCB1a = MDR1a = MDR3)
mouse genomic contig on chromosome 5
140000 bp
0
35 kb
70 kb
87.5 kb
105 kb
122.5 kb
140 kb
Exon1
Exon2
Start-ATG
Exon3
Exon4
Exon5
Exon6
Exon7
Exon8
Exon9
Exon10
Exon11
Exon12
Exon13
Exon14
Exon15
Exon16
Exon17
Exon18
Exon19
Exon20
Exon21
Exon22(22a)
Exon23(22b)
Exon24
Exon25
Exon26
Exon27
Exon28
Exon29
Stop-Codon
PolyN
PolyN
mouse exon 1
mouse exon 2
ATG
synthetic polyA
Hygro
mouse exon 3
3'homology arm
mouse intron 2
3.8 kb
2.8 kb
6.2 kb
2 kb
0.2 kb
4.2 kb
human cDNA
starting with the initial ATG
5'homology arm
1.2 kb
mouse intron 1
508 bp
FRT

Fig. 77


MDR1b mouse genomic
24899 bp
Start-ATG
Exon2
Exon1
Exon3
Exon4
Exon5
Exon6
Exon7
Exon8
5'homology arm
human cDNA
starting with the initial ATG
neo
3'homology arm
5,2 kb
4.2 kb
0.2 kb
2 kb
6,9 kb
FRT

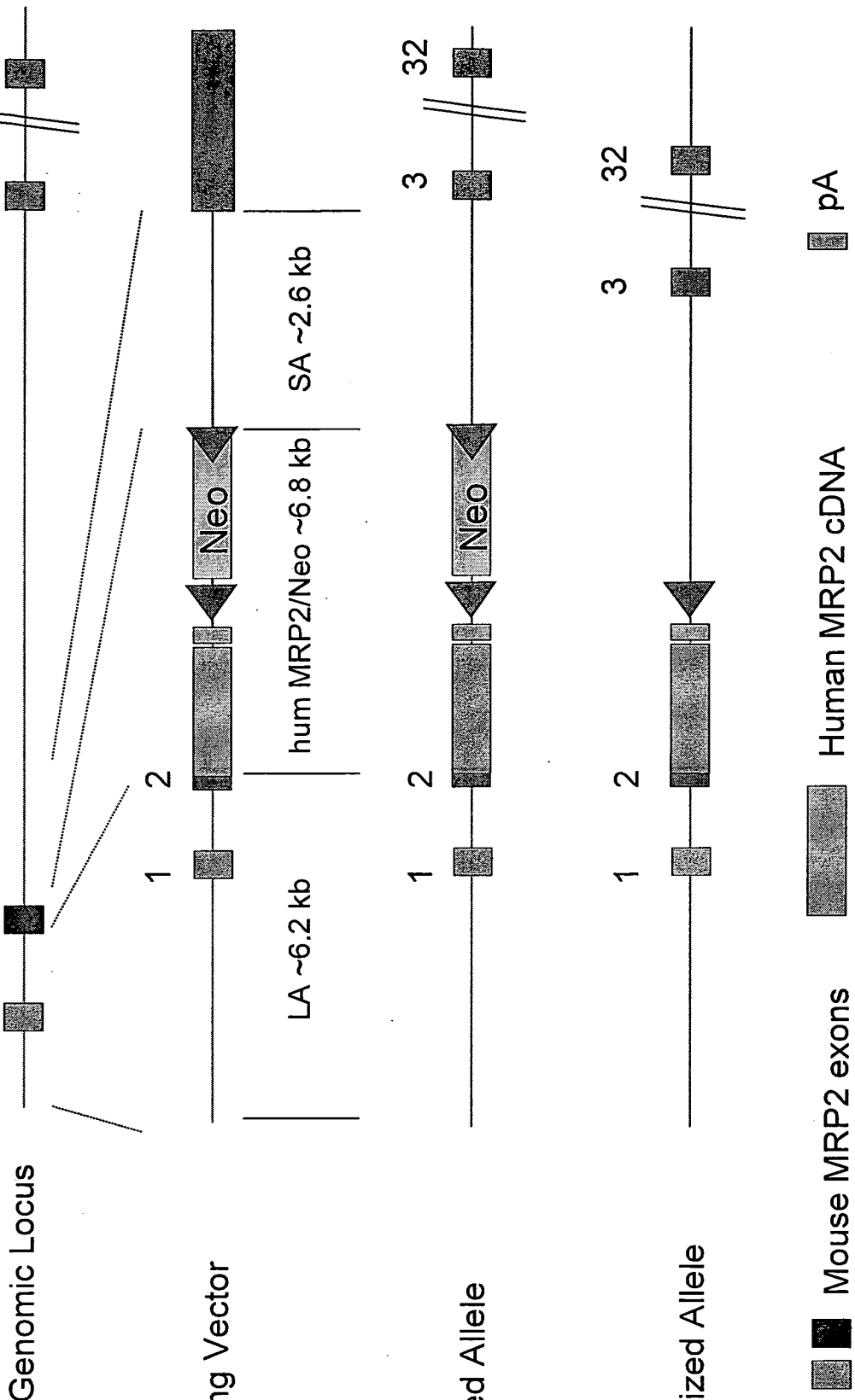

Mouse Genomic Locus
1
2
3
32
Targeting Vector
LA ~6.2 kb
hum MRP2/Neo ~6.8 kb
SA ~2.6 kb
Neo
Targeted Allele
Humanized Allele
Mouse MRP2 exons
Human MRP2 cDNA
pA

Fig. 78

Fig. 79

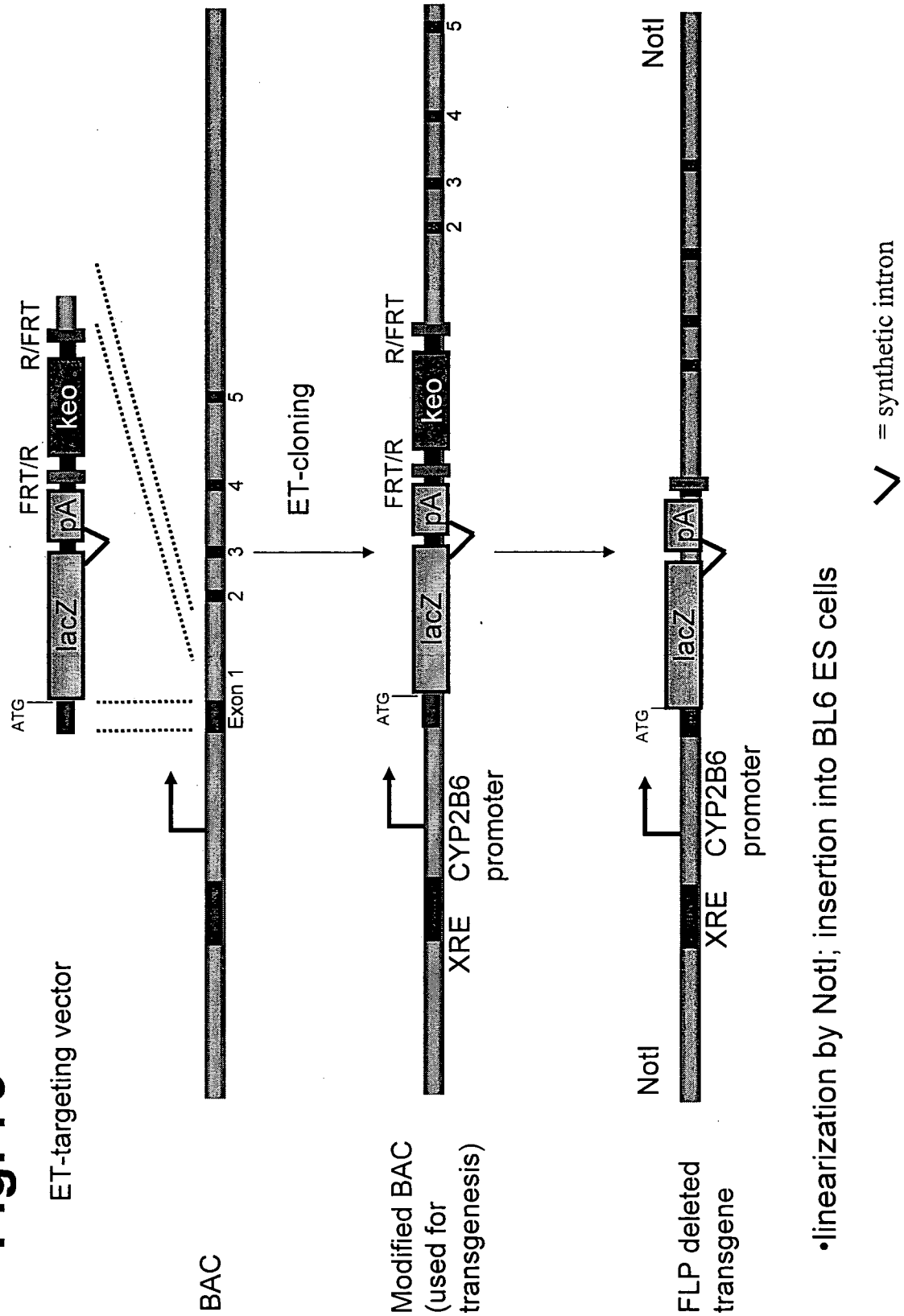

ET-targeting vector
FRT/R
keo
R/FRT
pA
lacZ
ATG
BAC
Exon 1
2
3
4
5
ET-cloning
Modified BAC
(used for
transgenesis)
XRE
CYP2B6
promoter
NotI
FLP deleted
transgene
•linearization by NotI; insertion into BL6 ES cells
= synthetic intron

Fig. 80

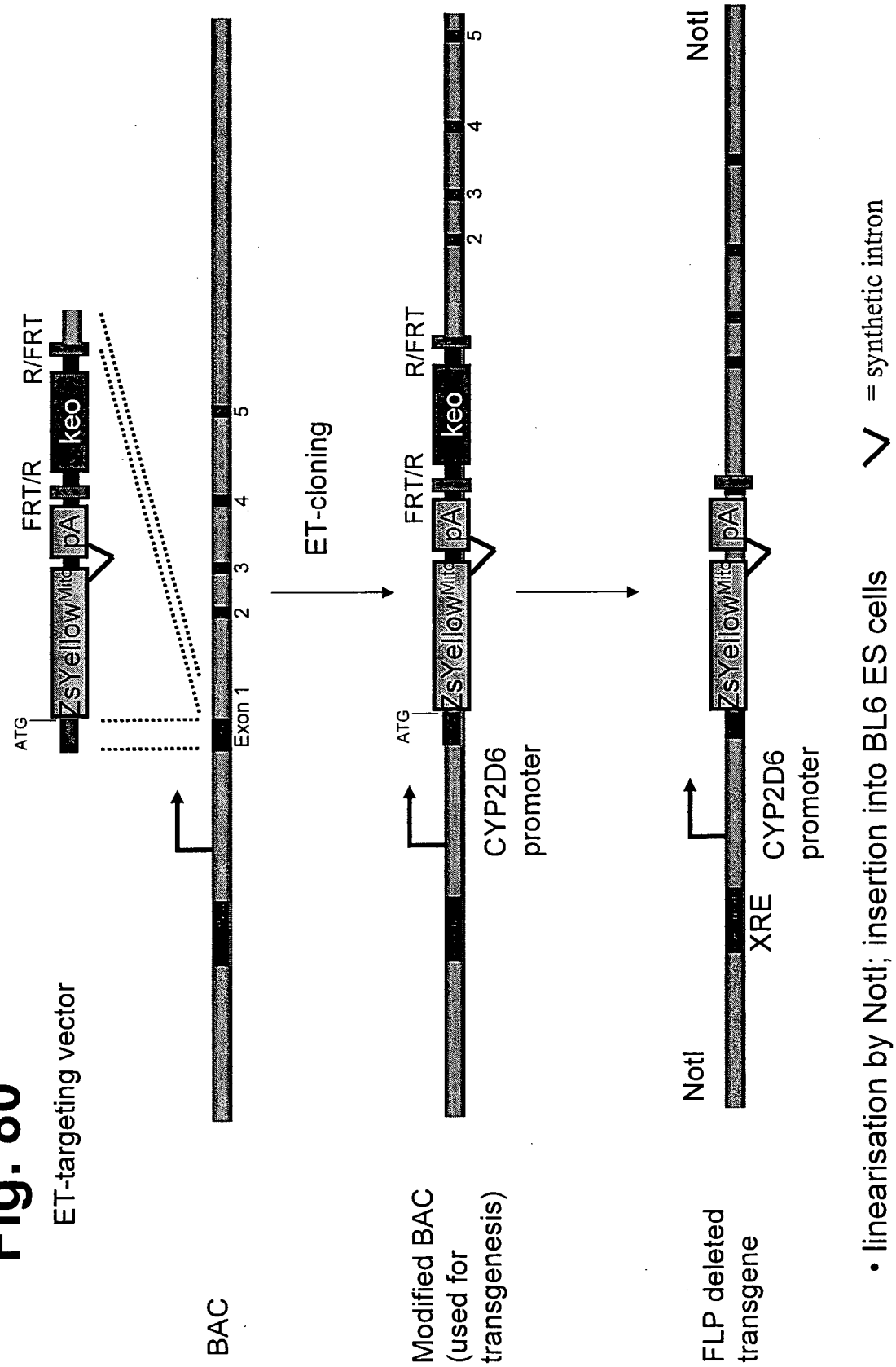
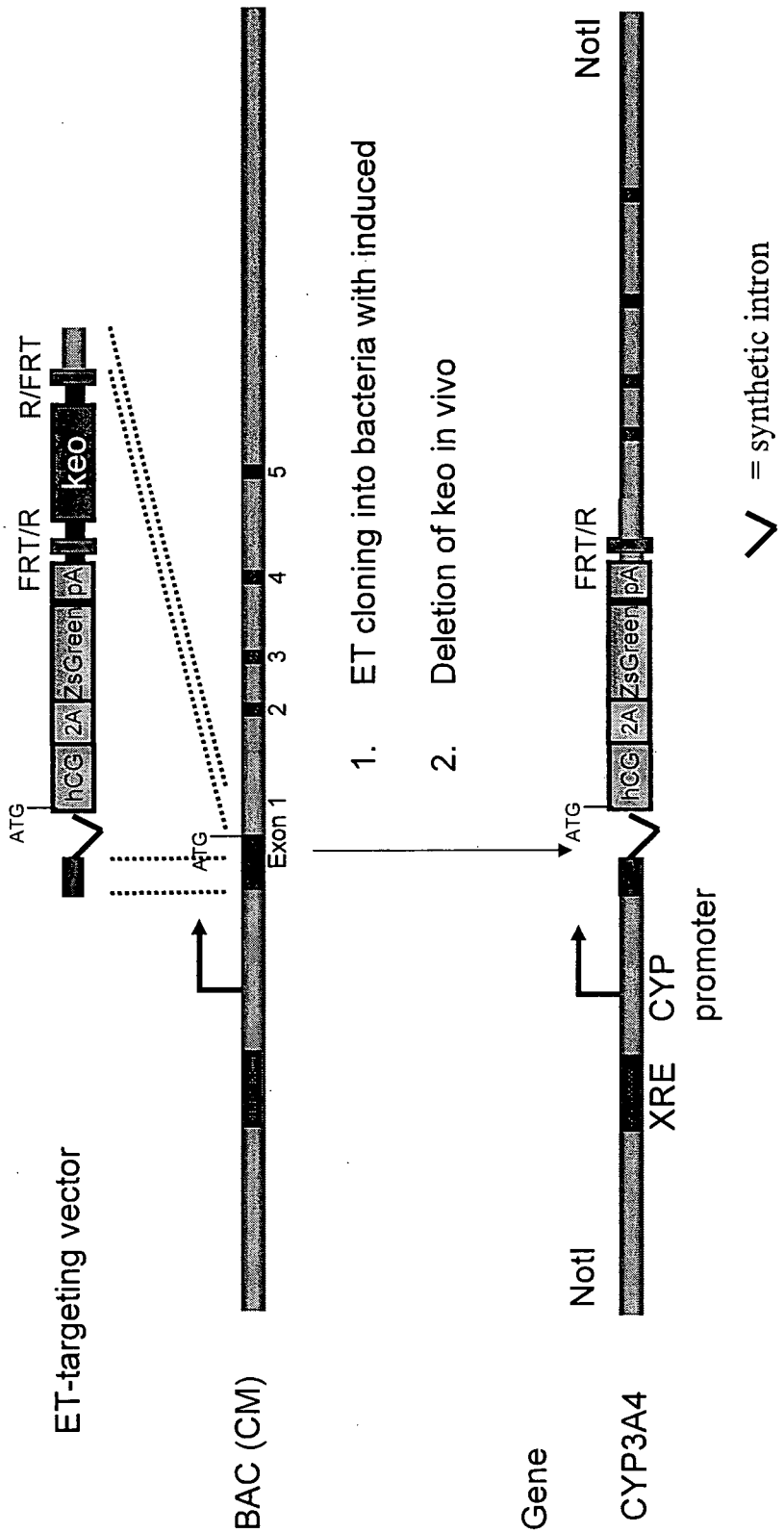

ET-targeting vector
R/FRT
FRT/R
keo
pA
ZsYellow
ATG
Exon 1
BAC
ET-cloning
Modified BAC
(used for
transgenesis)
CYP2D6
promoter
NotI
XRE
FLP deleted
transgene
= synthetic intron
• linearisation by NotI; insertion into BL6 ES cells

Fig. 81



• linearization by NotI; insertion into BL6 ES cells

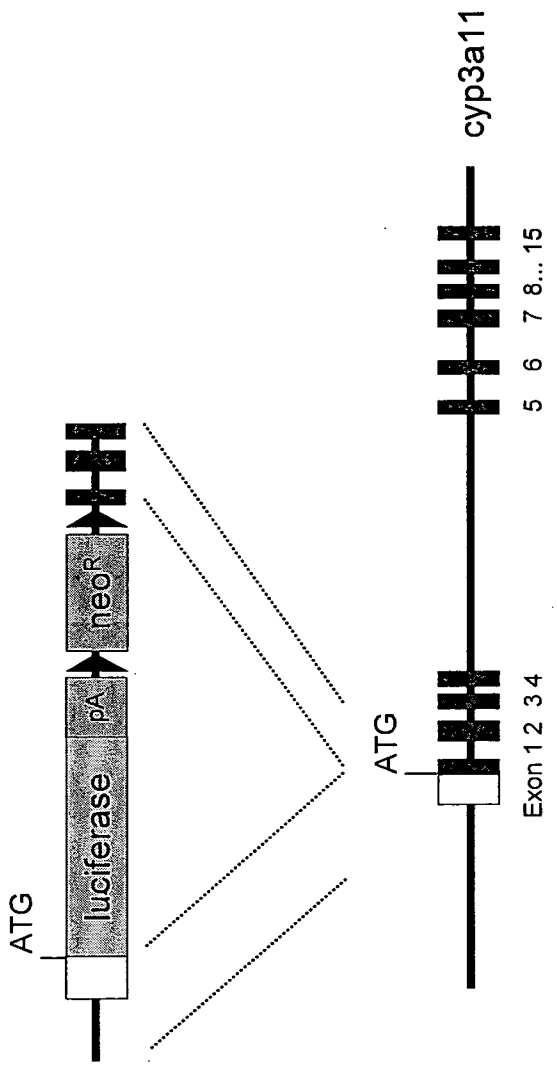


Fig. 82

Fig. 83

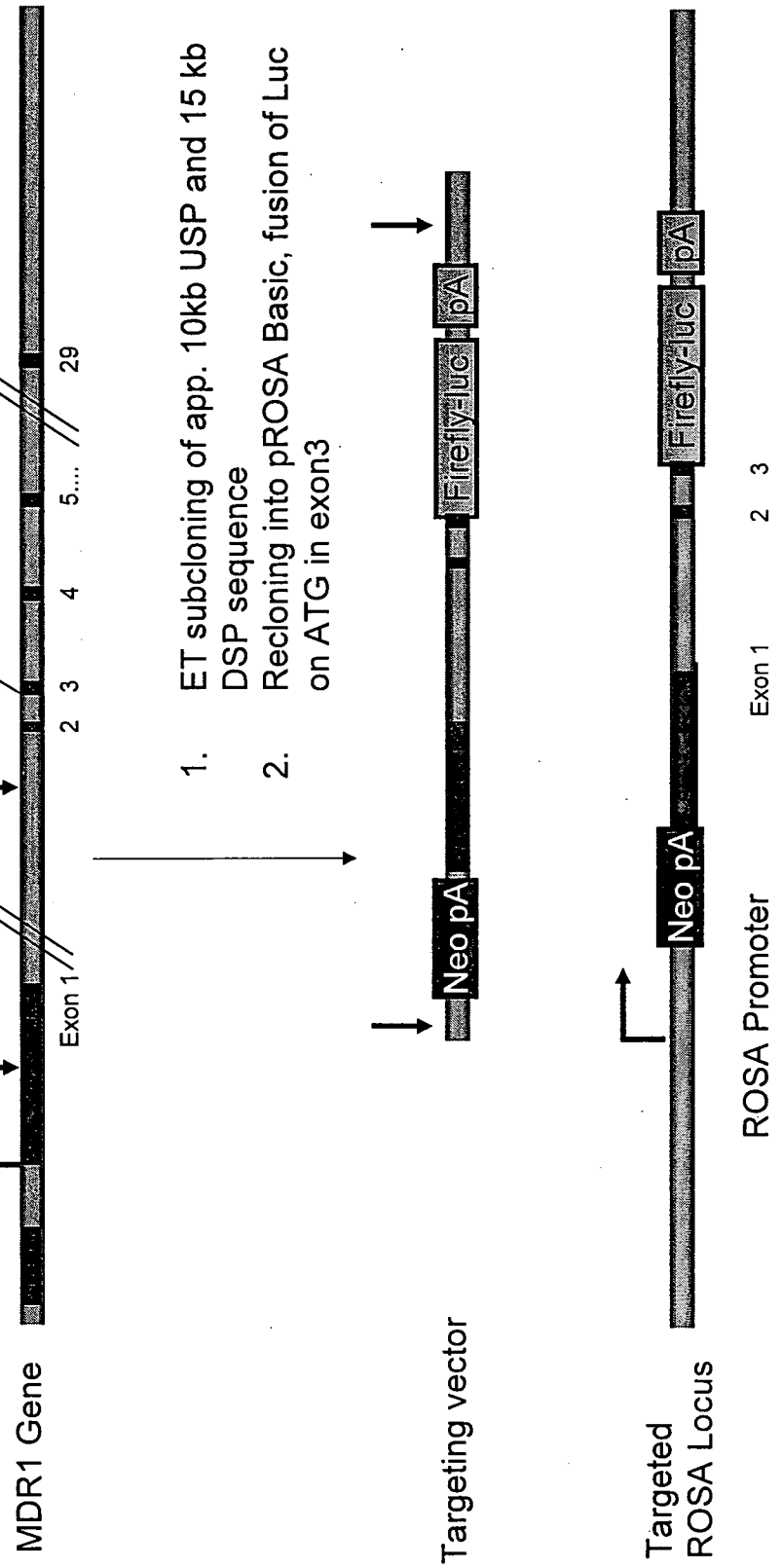

MDR1 Gene
USP
DSP
ATG
Exon 1
2 3 4 5.... 29
1. ET subcloning of app. 10kb USP and 15 kb DSP sequence
2. Recloning into pROSA Basic, fusion of Luc on ATG in exon3
Targeting vector
Neo pA
Firefly-luc
pA
Targeted ROSA Locus
ROSA Promoter
Exon 1
2 3
Neo pA
Firefly-luc
pA

FIG. 84

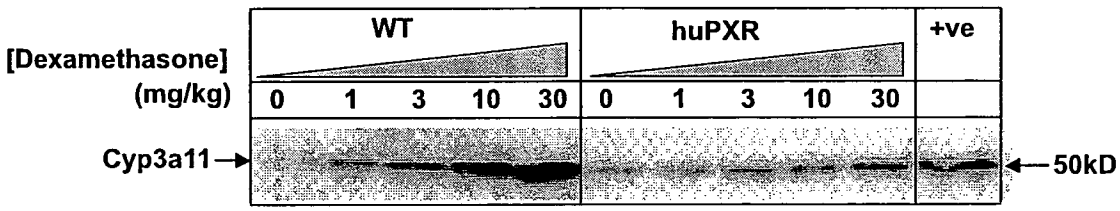
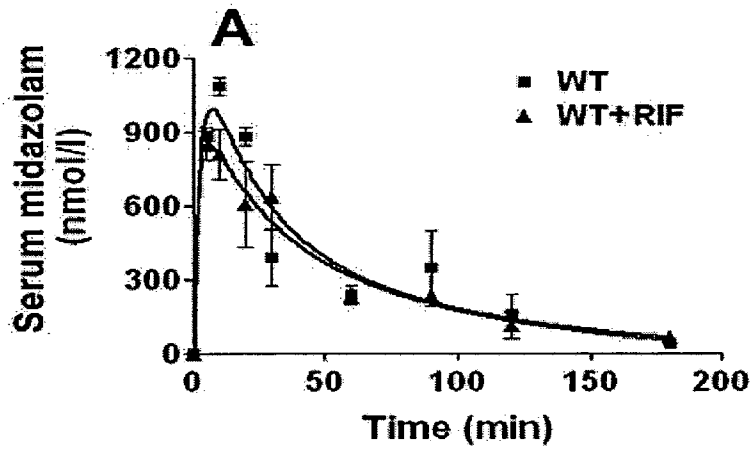

WT
huPXR
+ve
[Dexamethasone]
(mg/kg)
0 1 3 10 30 0 1 3 10 30
Cyp3a11
50kD

FIG. 93


A
Serum midazolam (nmol/l)
1200
900
600
300
0
0 50 100 150 200
Time (min)
WT
WT+RIF

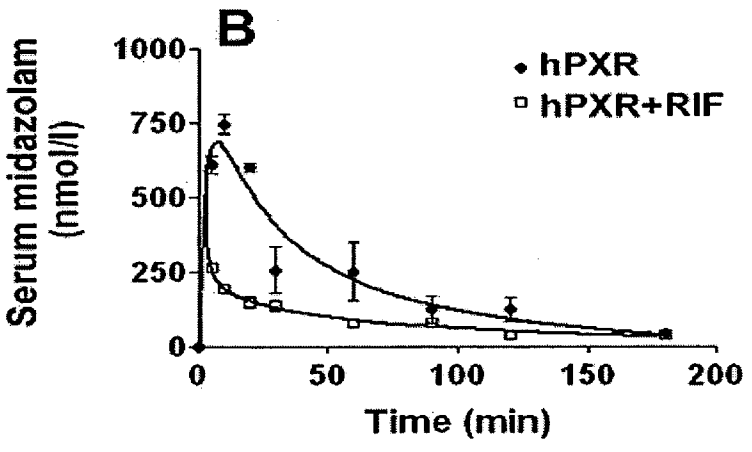
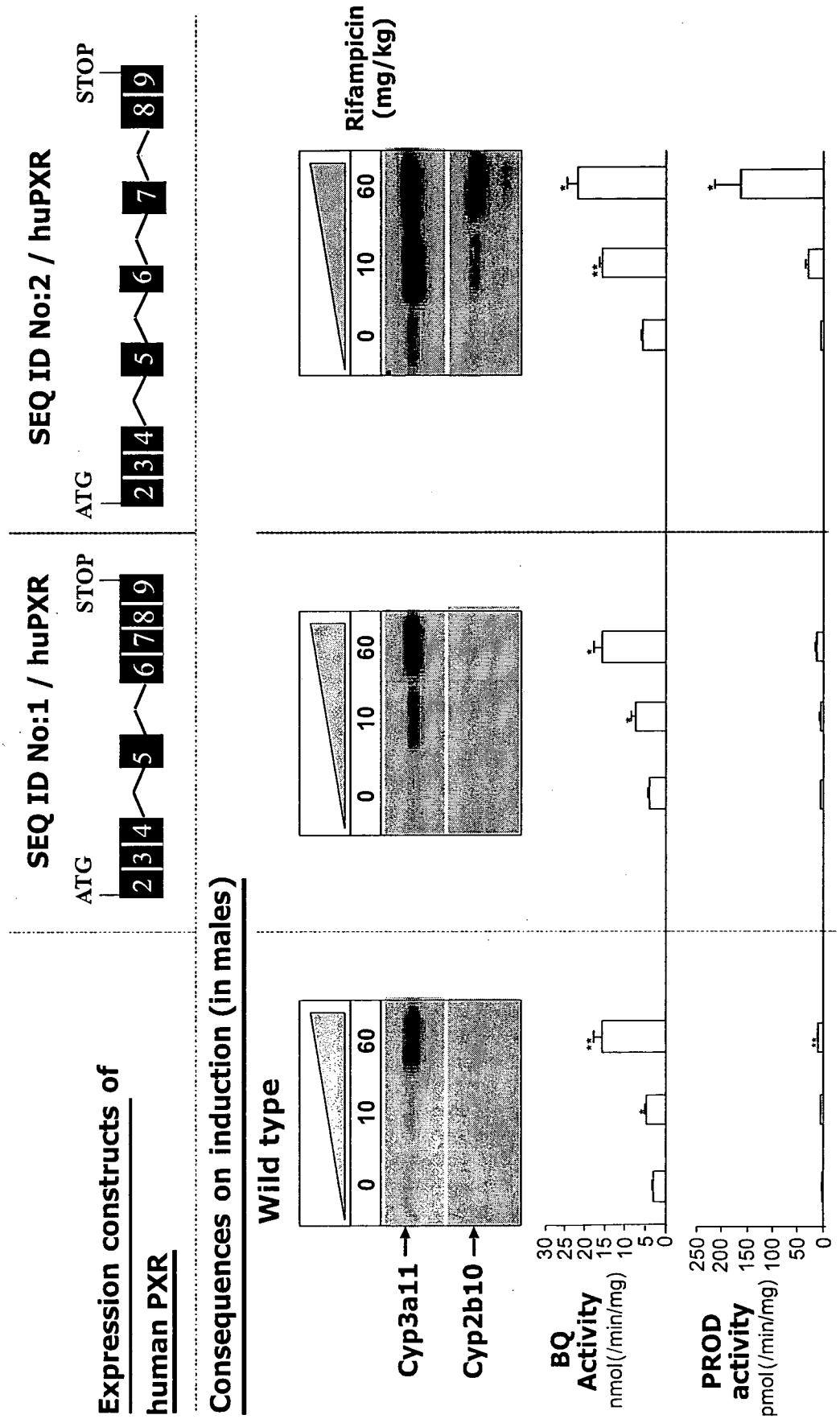

B
Serum midazolam (nmol/l)
1000
750
500
250
0
0 50 100 150 200
Time (min)
hPXR
hPXR+RIF

FIG. 85


Expression constructs of human PXR
SEQ ID No:1 / huPXR
ATG
STOP
2 3 4 5 6 7 8 9
SEQ ID No:2 / huPXR
ATG
STOP
2 3 4 5 6 7 8 9
Consequences on induction (in males)
Wild type
Rifampicin (mg/kg)
0 10 60
Cyp3a11
Cyp2b10
BQ Activity nmol(/min/mg)
30 25 20 15 10 5 0
PROD activity pmol(/min/mg)
250 200 150 100 50 0

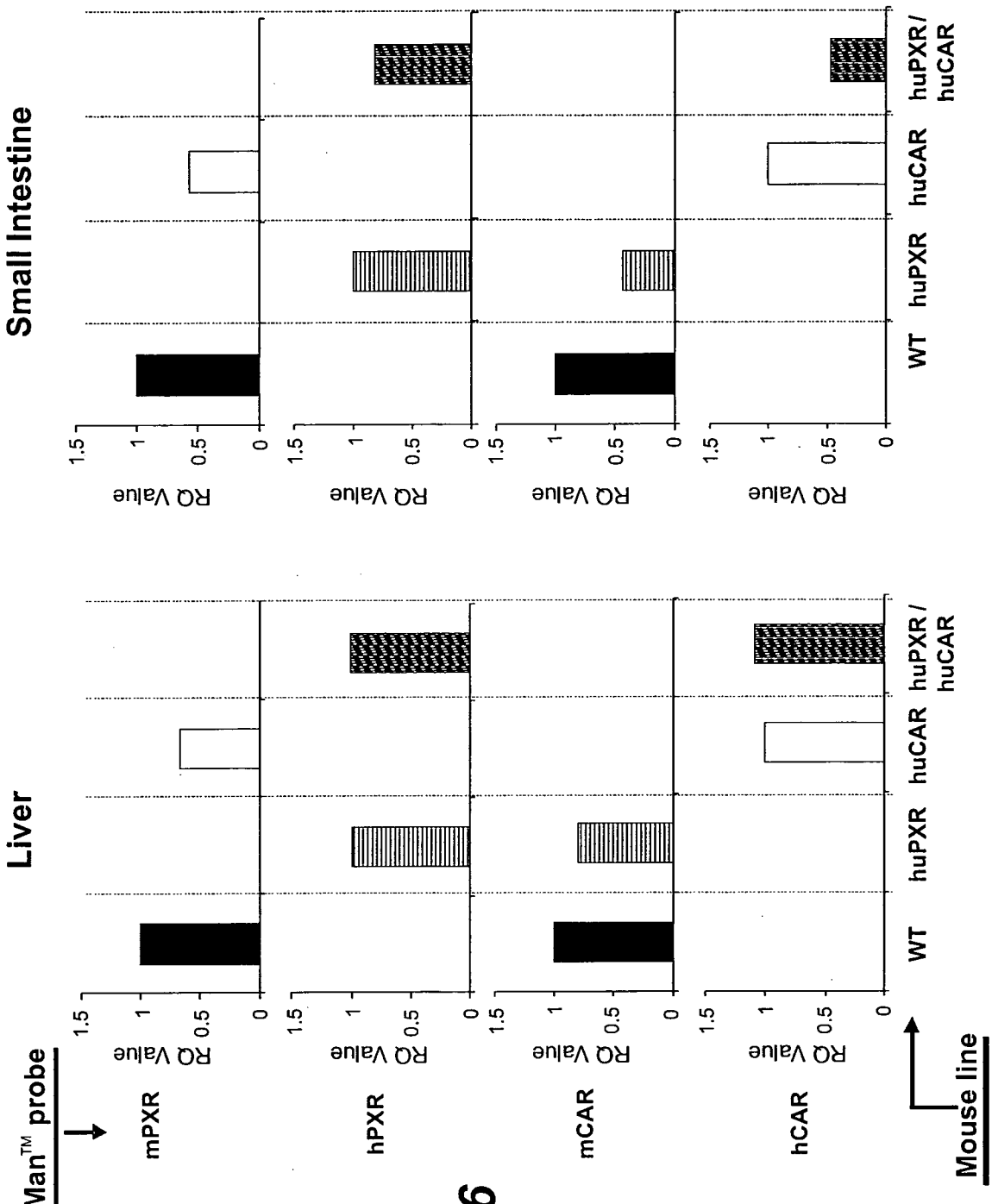

Liver
Small Intestine
TaqMan™ probe
mPXR
hPXR
mCAR
hCAR
RQ Value
1.5
1
0.5
0
WT
huPXR
huCAR
huPXR / huCAR
Mouse line

FIG. 86

FIG. 87

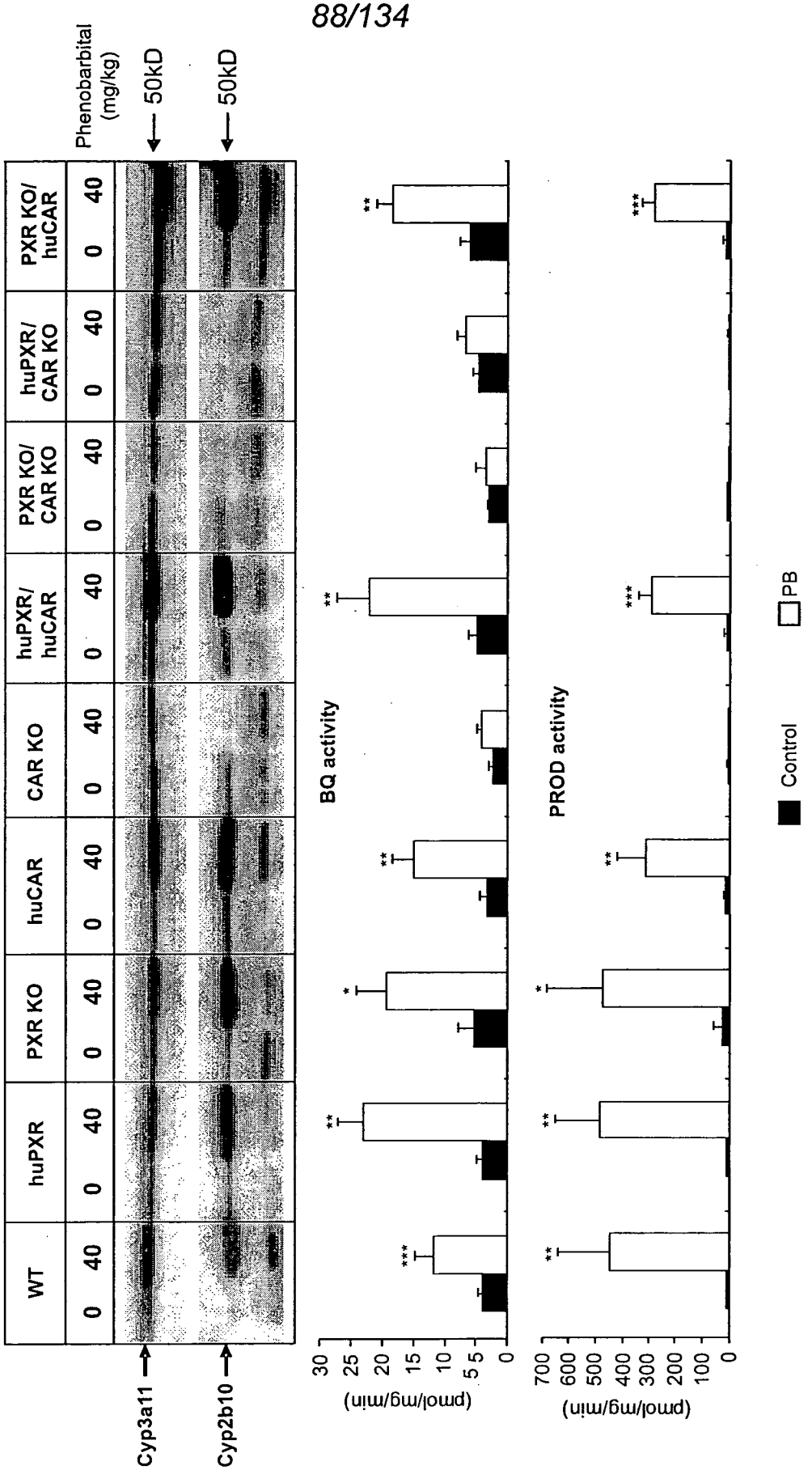

WT
huPXR
PXR KO
huCAR
CAR KO
huPXR/ huCAR
PXR KO/ CAR KO
huPXR/ CAR KO
PXR KO/ huCAR
0 40 0 40 0 40 0 40 0 40 0 40 0 40 0 40 0 40
Phenobarbital (mg/kg)
50kD
50kD
Cyp3a11
Cyp2b10
BQ activity
PROD activity
(pmol/mg/min)
Control
PB

FIG. 88

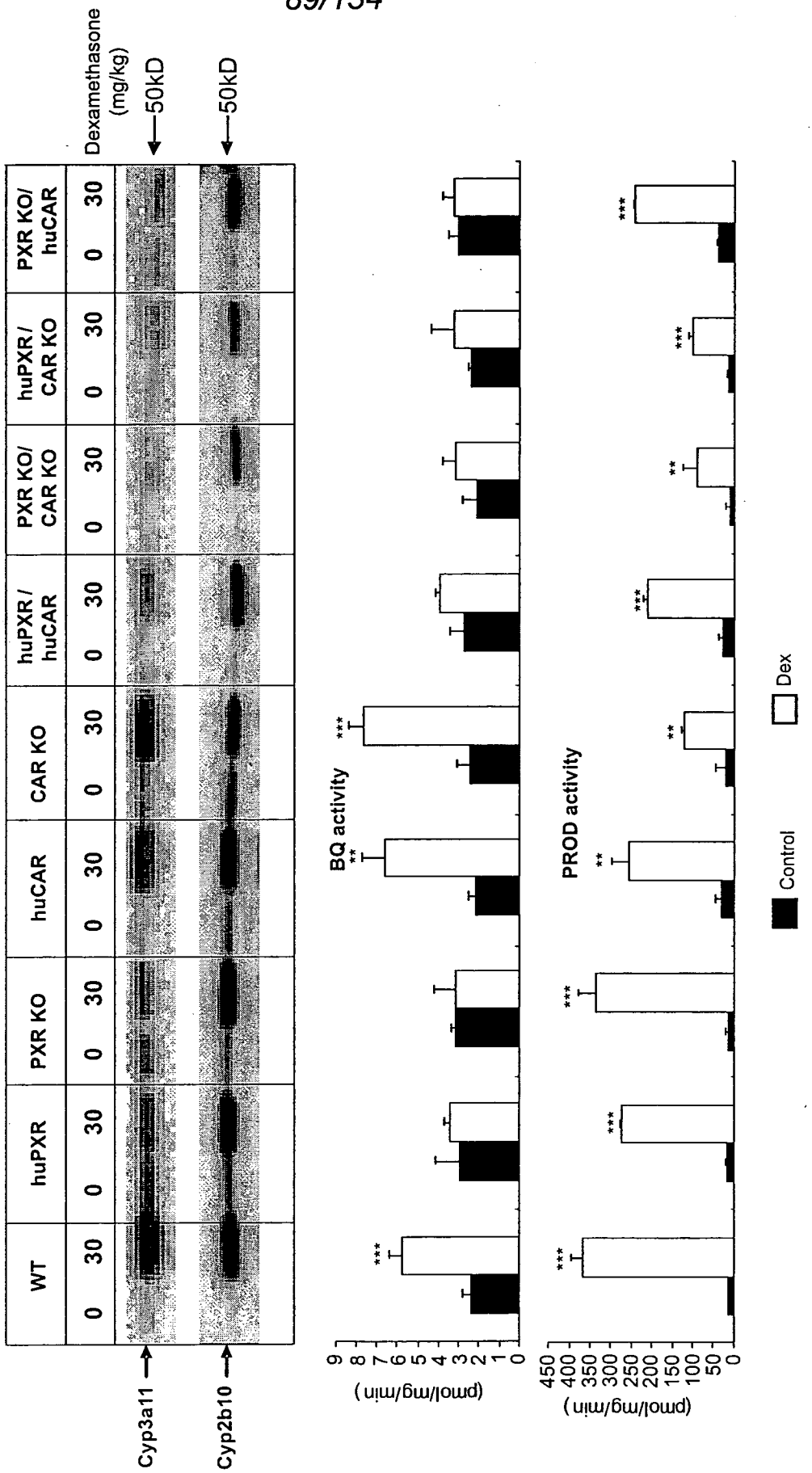

WT
huPXR
PXR KO
huCAR
CAR KO
huPXR / huCAR
PXR KO/ CAR KO
huPXR / CAR KO
PXR KO/ huCAR
0 30 0 30 0 30 0 30 0 30 0 30 0 30 0 30 0 30
Dexamethasone (mg/kg)
50kD
50kD
Cyp3a11
Cyp2b10
BQ activity
(pmol/mg/min)
PROD activity
(pmol/mg/min)
Control
Dex

FIG. 89

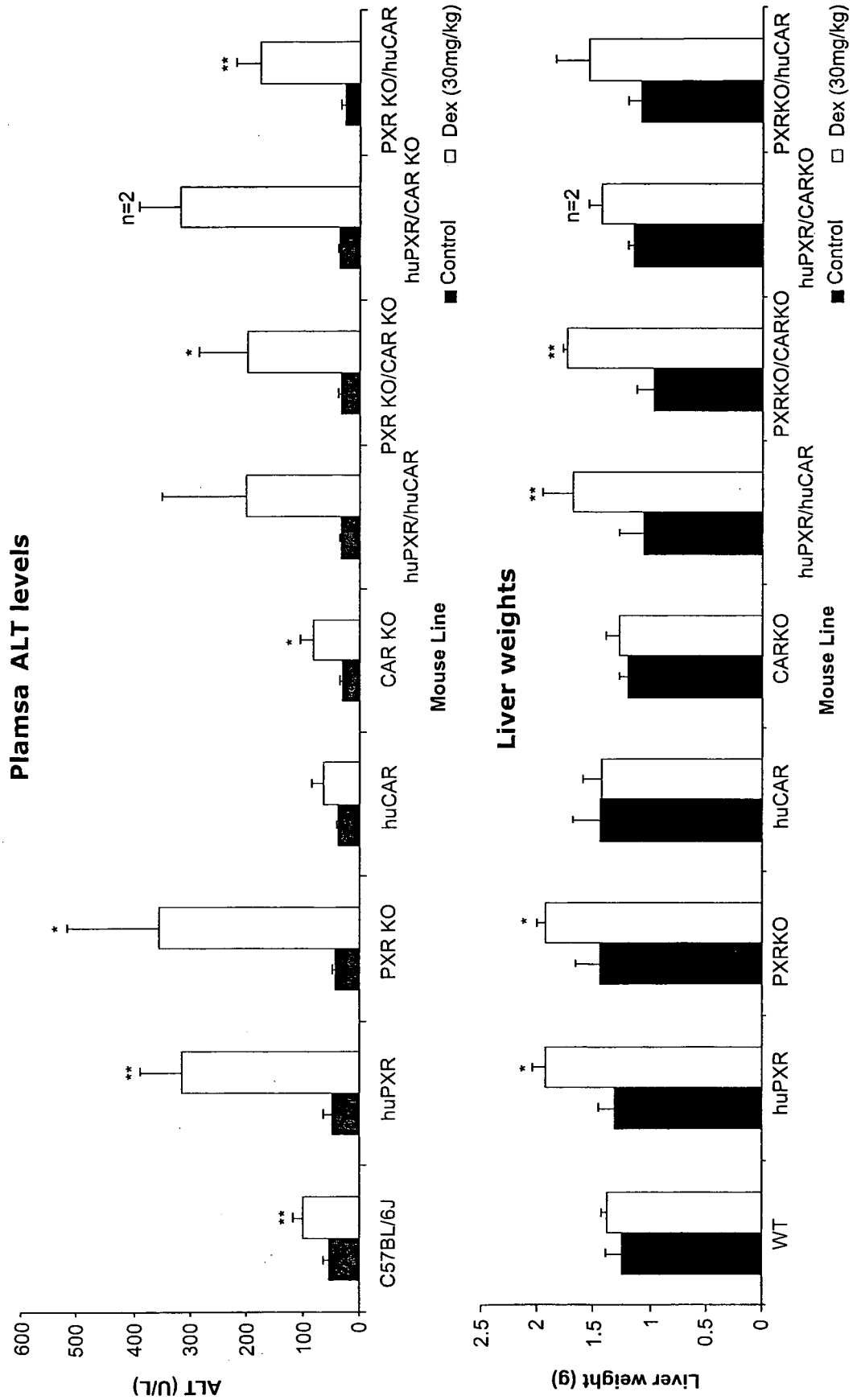

Plamsa ALT levels
ALT (U/L)
0
100
200
300
400
500
600
C57BL/6J
huPXR
PXR KO
huCAR
CAR KO
huPXR/huCAR
PXR KO/CAR KO
huPXR/CAR KO
PXR KO/huCAR
n=2
Control
Dex (30mg/kg)
Mouse Line
Liver weights
Liver weight (g)
0
0.5
1
1.5
2
2.5
WT
huPXR
PXRKO
huCAR
CARKO
huPXR/huCAR
PXRKO/CARKO
huPXR/CARKO
PXRKO/huCAR
n=2
Control
Dex (30mg/kg)
Mouse Line

FIG. 90

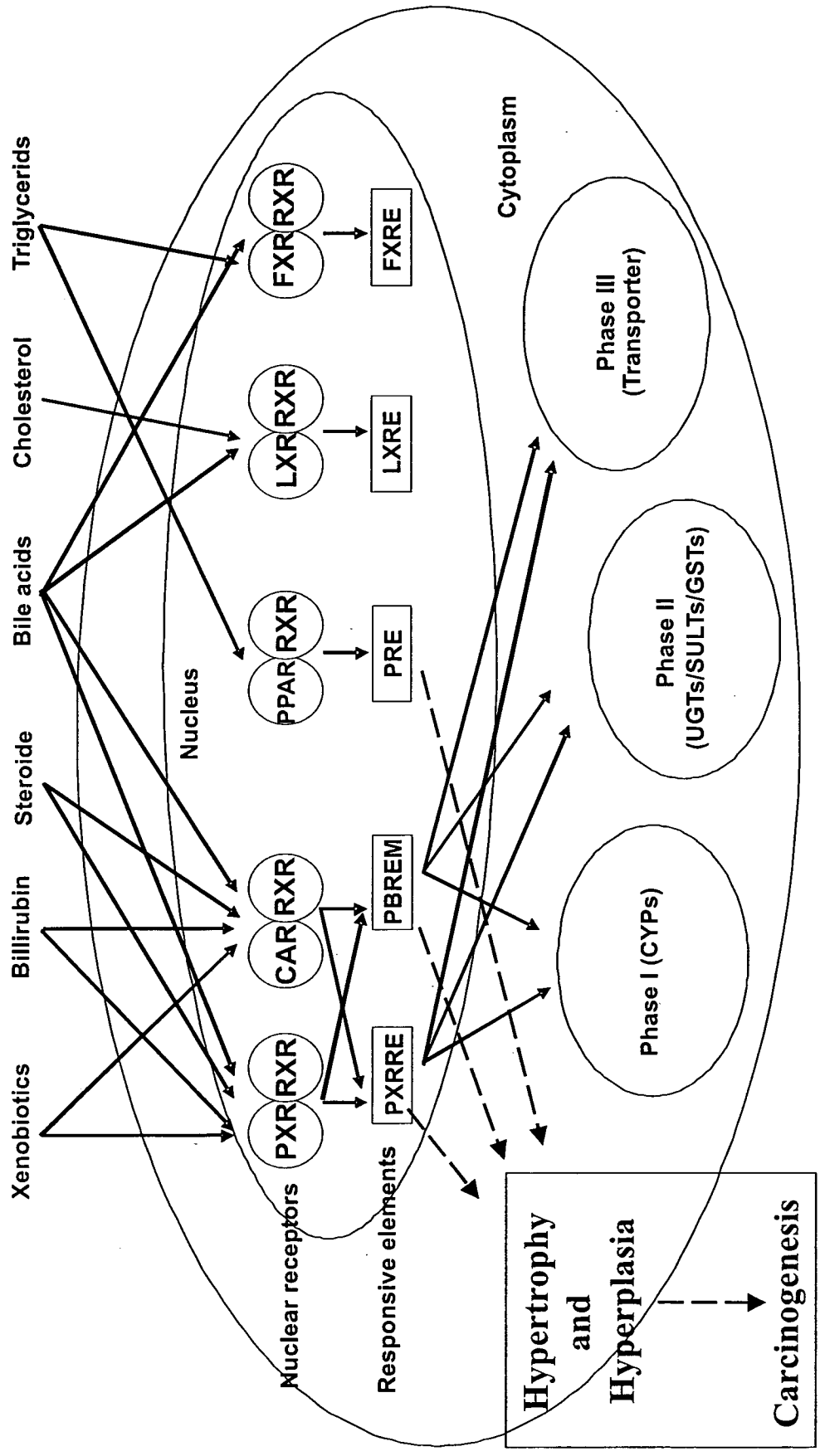

Xenobiotics
Billirubin
Steroide
Bile acids
Cholesterol
Triglycerids
Nucleus
Cytoplasm
PXR
RXR
CAR
RXR
PPAR
RXR
LXR
RXR
FXR
RXR
PXRRE
PBREM
PRE
LXRE
FXRE
Nuclear receptors
Responsive elements
Phase I (CYPs)
Phase II (UGTs/SULTs/GSTs)
Phase III (Transporter)
Hypertrophy and Hyperplasia
Carcinogenesis

FIG. 91

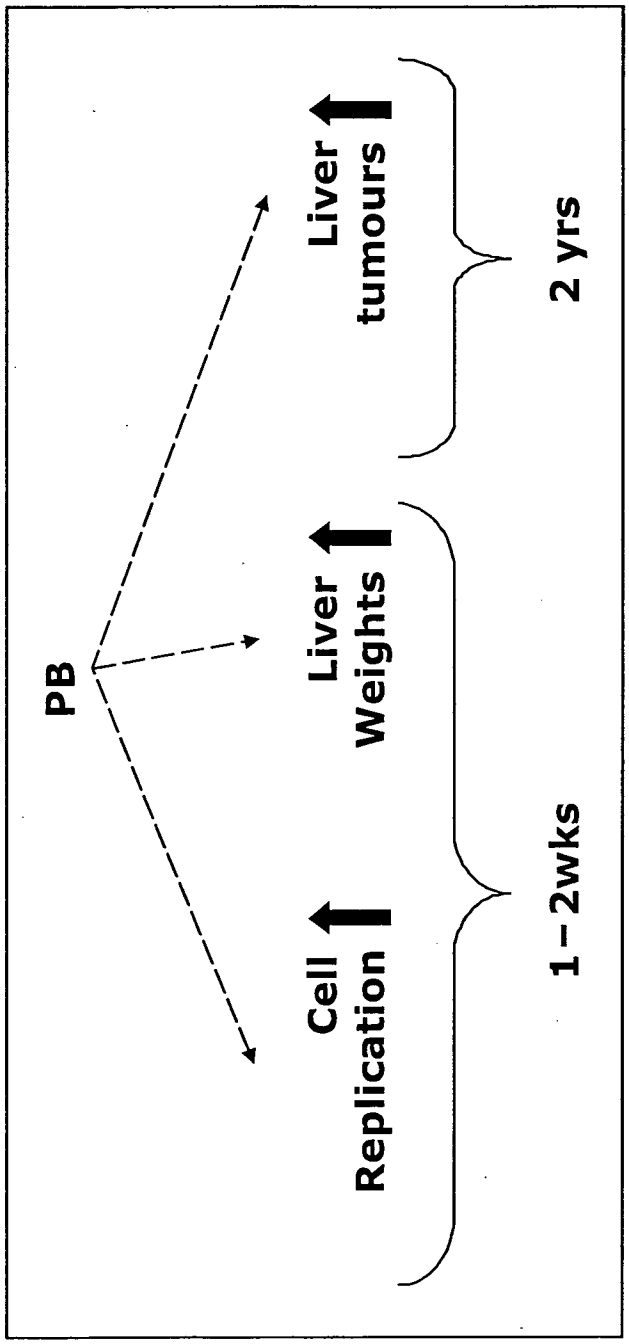

PB causes liver cancer in mice and rats but not in humans
PB
Cell Replication
Liver Weights
Liver tumours
1–2wks
2 yrs
Are hyperplastic responses to chemicals observed in animals relevant to humans?

FIG. 92

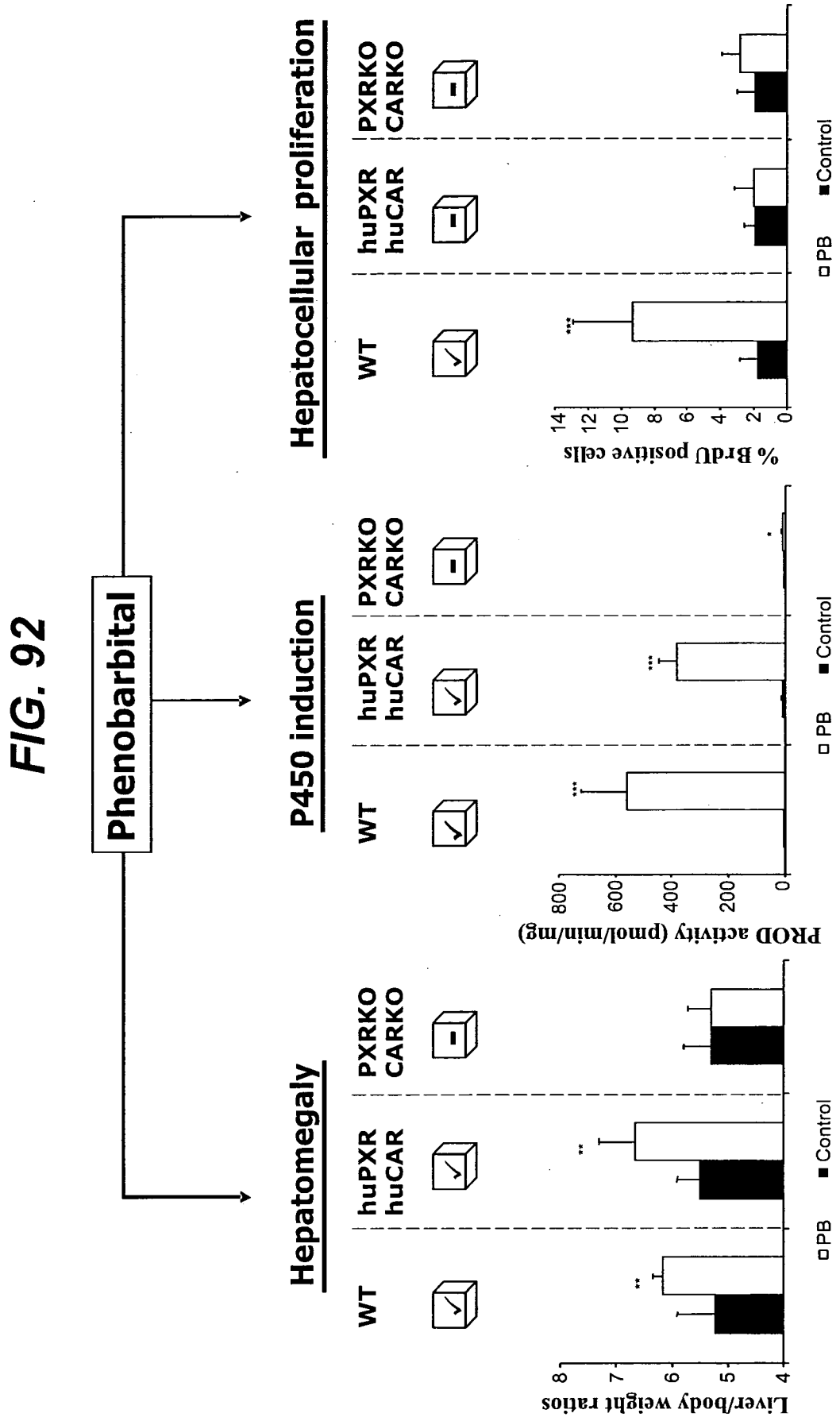

Phenobarbital
Hepatomegaly
P450 induction
Hepatocellular proliferation
WT
huPXR huCAR
PXRKO CARKO
PB
Control
Liver/body weight ratios
PROD activity (pmol/min/mg)
% BrdU positive cells

FIG. 94

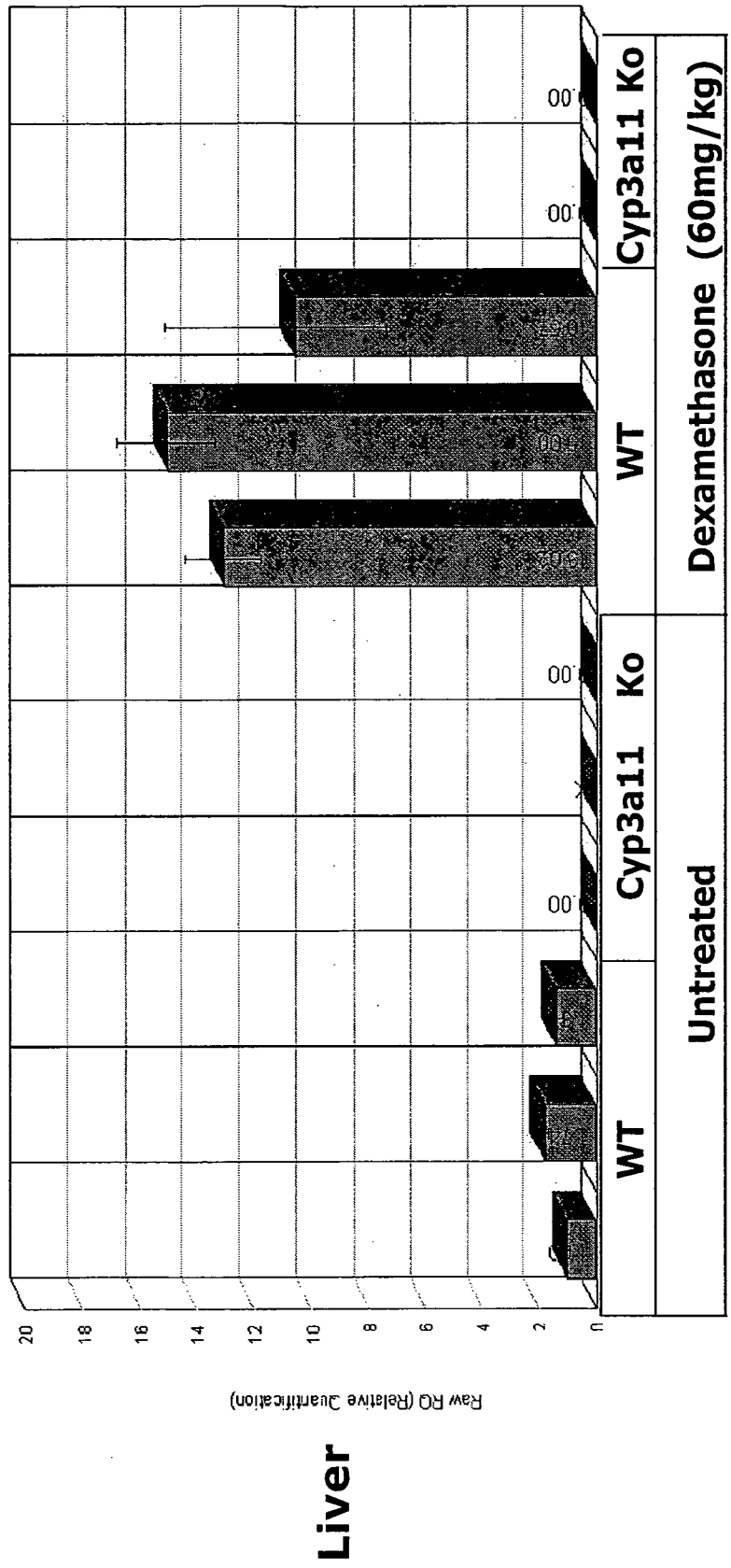

Cyp3a11 mRNA expression
Liver
Raw RQ (Relative Quantification)
0
2
4
6
8
10
12
14
16
18
20
WT
Cyp3a11 Ko
WT
Cyp3a11 Ko
Untreated
Dexamethasone (60mg/kg)

FIG. 95

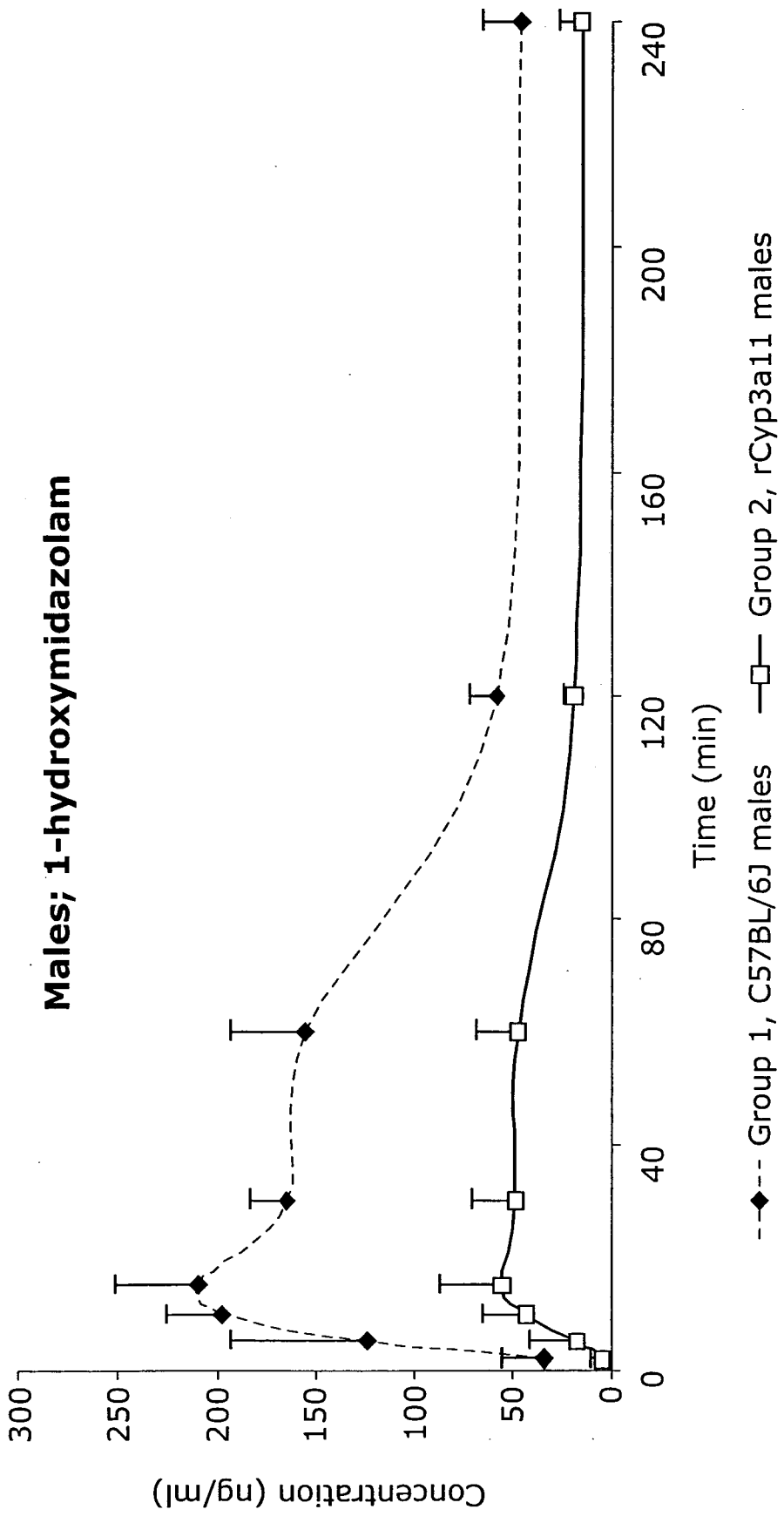

Males; 1-hydroxymidazolam
Concentration (ng/ml)
300
250
200
150
100
50
0
0
40
80
120
160
200
240
Time (min)
Group 1, C57BL/6J males
Group 2, rCyp3a11 males

FIG. 96

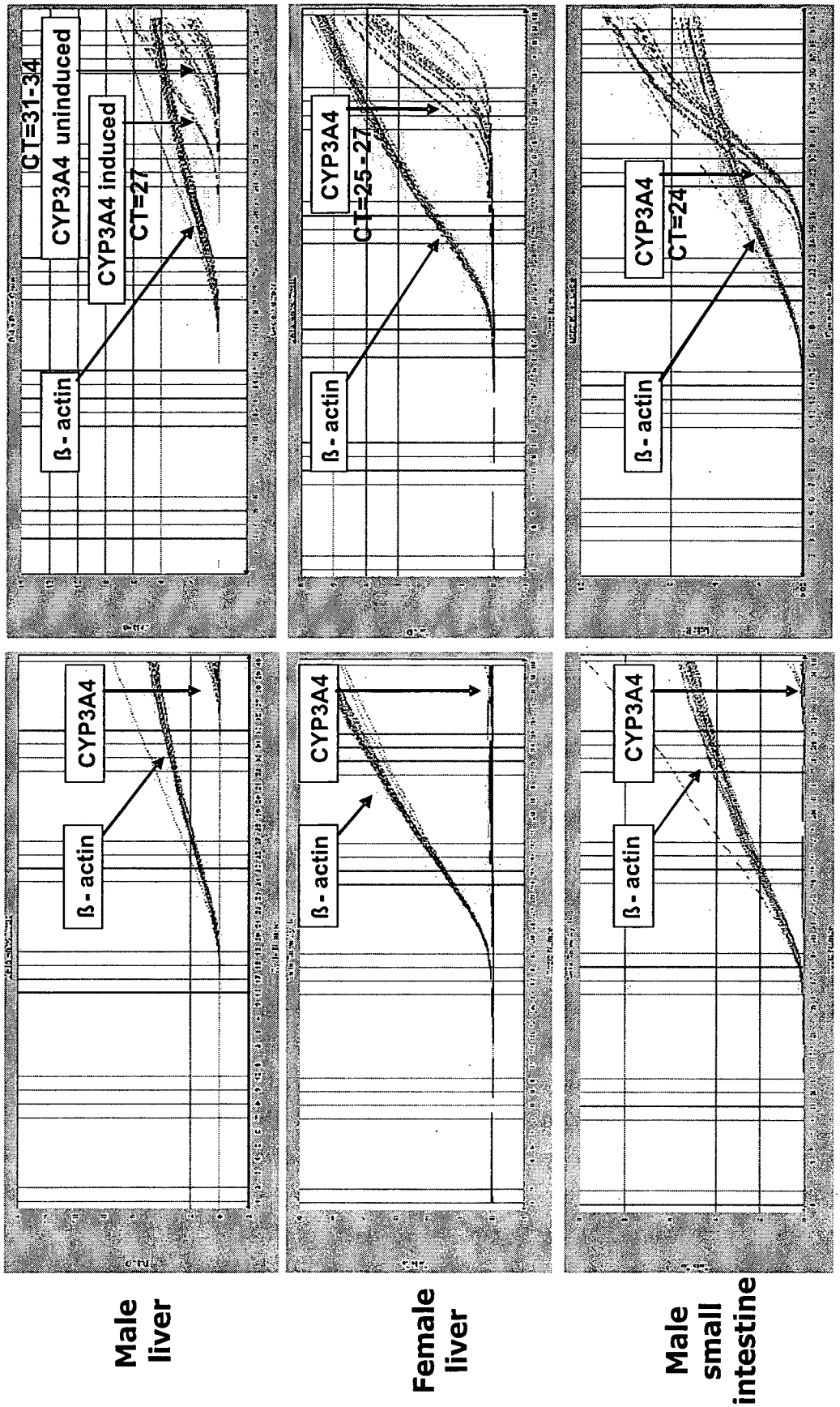

WT
huCYP3A4/huPXR
Male liver
Female liver
Male small intestine
ß - actin
CYP3A4
CYP3A4 uninduced
CYP3A4 induced
CT=31-34
CT=27
CT=25-27
CT=24

FIG. 97

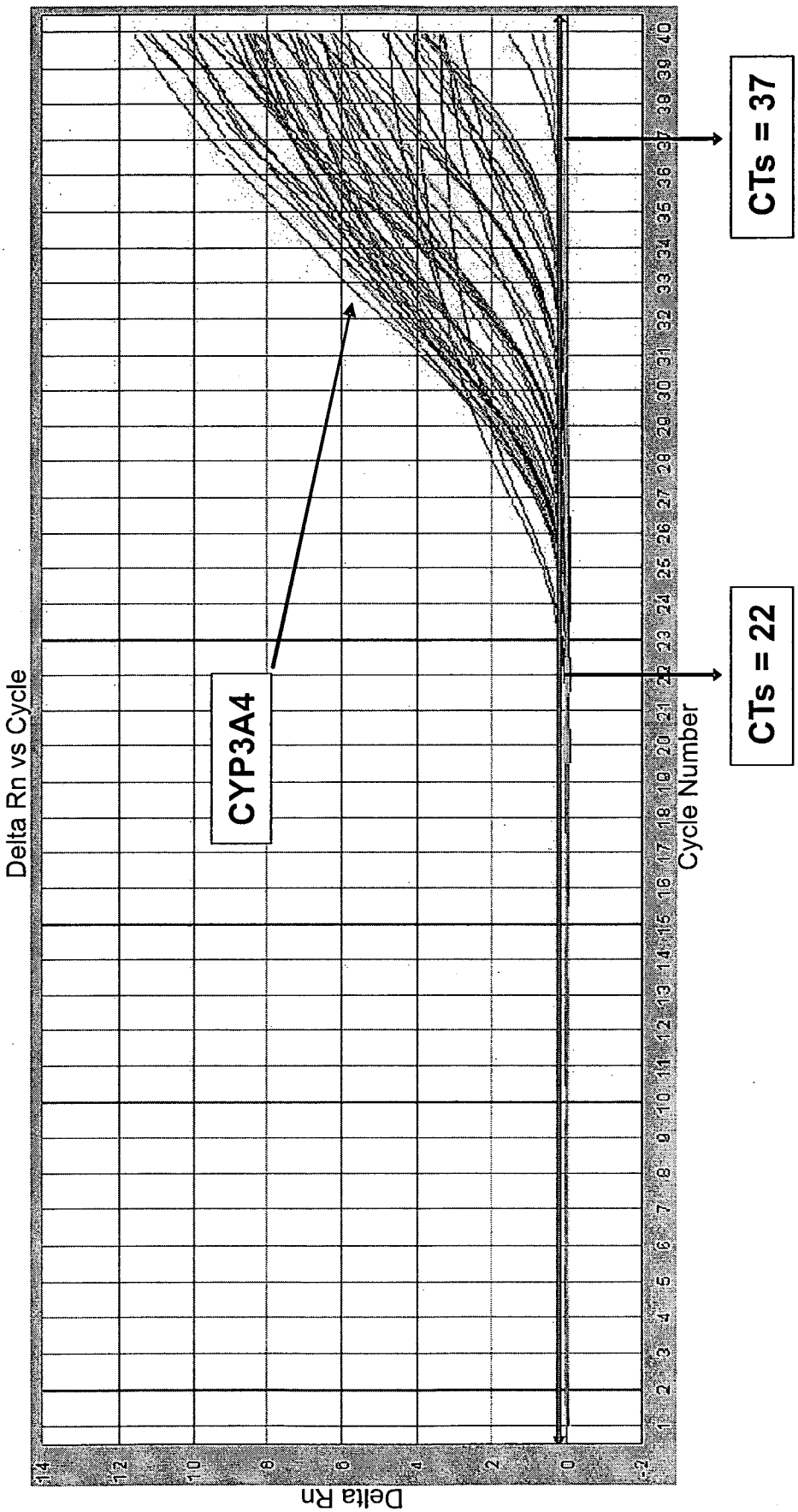


FIG. 98

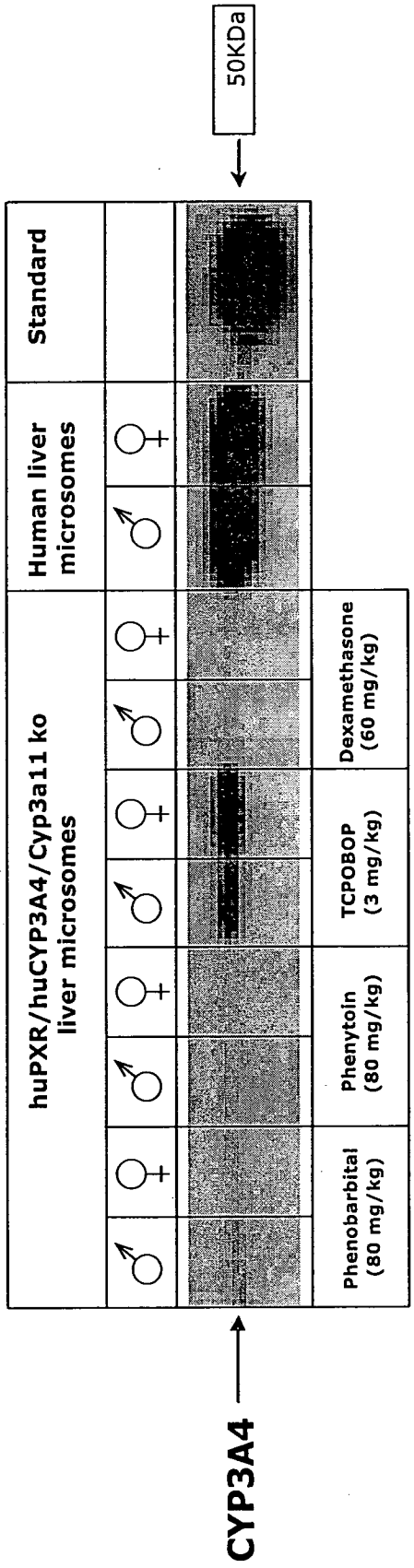


FIG. 103

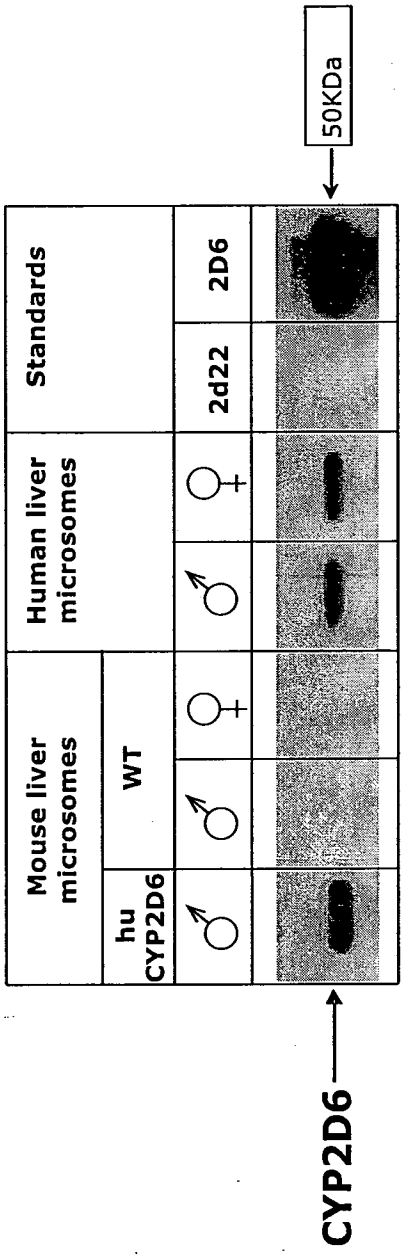


FIG. 99

PHASE I ENZYMES	Fold change relative to WT mice	
Cytochrome P450s	Cyp3a cluster KO (control)	Cyp3a cluster KO (RIF)
Cyp2a4/5	2.8	3.4
Cyp2b13		5.5
Cyp2b10		7.2
Cyp2b9		3.2
Cyp2c55	4.7	
Cyp2c37		1.9
Cyp2c38		
Cyp2c44		
Cyp2c50		
Cyp2c54		2.7
Cyp2c68		
Cyp2c70		
Cyp2c66		
Cyp2c29		1.5
Cyp3a11	-100.0	-96.2
Cyp3a25	-100.0	-85.7
Cyp3a13	-1.4	
Cyp3a16	-100.0	-92.6
Cyp4a10	2.0	1.3
Cyp4a13	-14.9	
Cyp4a12b		3.3

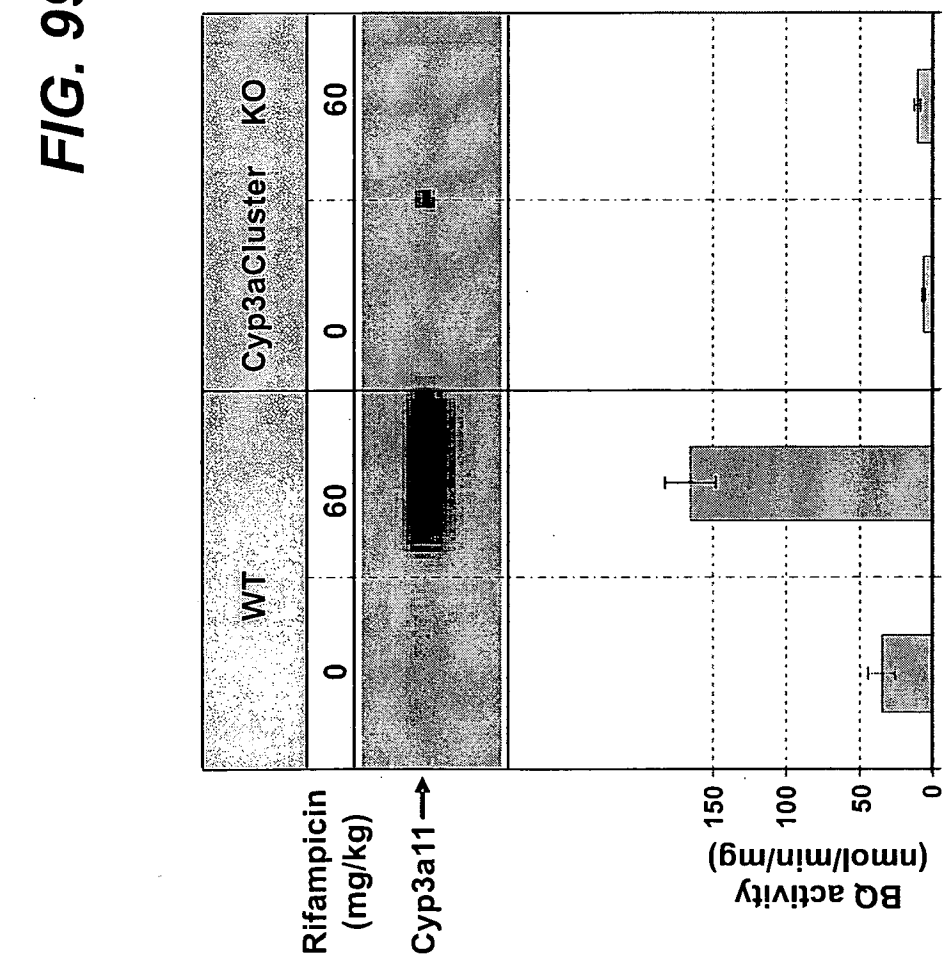


FIG. 100

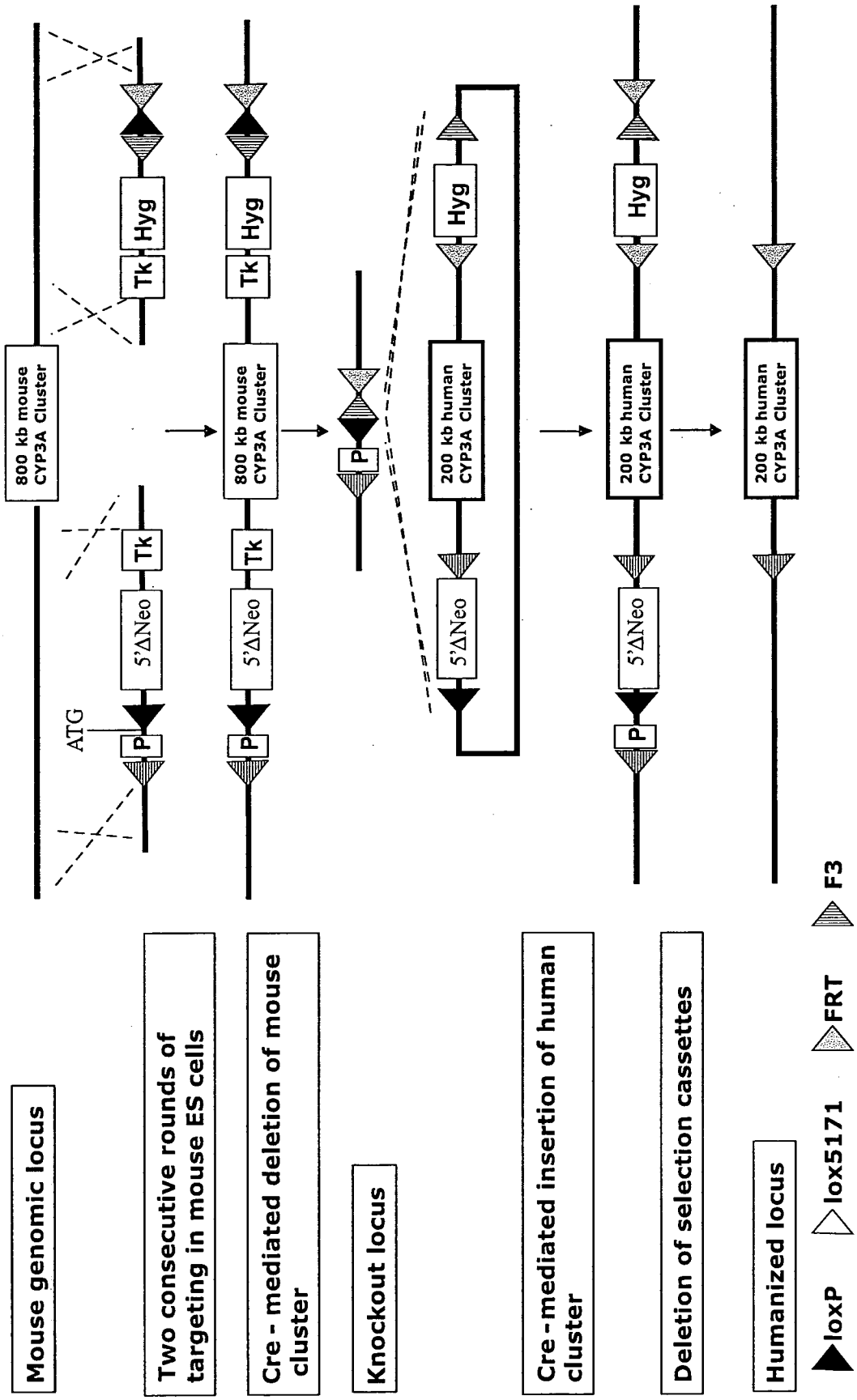

Mouse genomic locus
Two consecutive rounds of targeting in mouse ES cells
Cre - mediated deletion of mouse cluster
Knockout locus
Cre - mediated insertion of human cluster
Deletion of selection cassettes
Humanized locus
loxP
lox5171
FRT
F3
800 kb mouse CYP3A Cluster
ATG
P
5'ΔNeo
Tk
Hyg
200 kb human CYP3A Cluster

FIG. 101

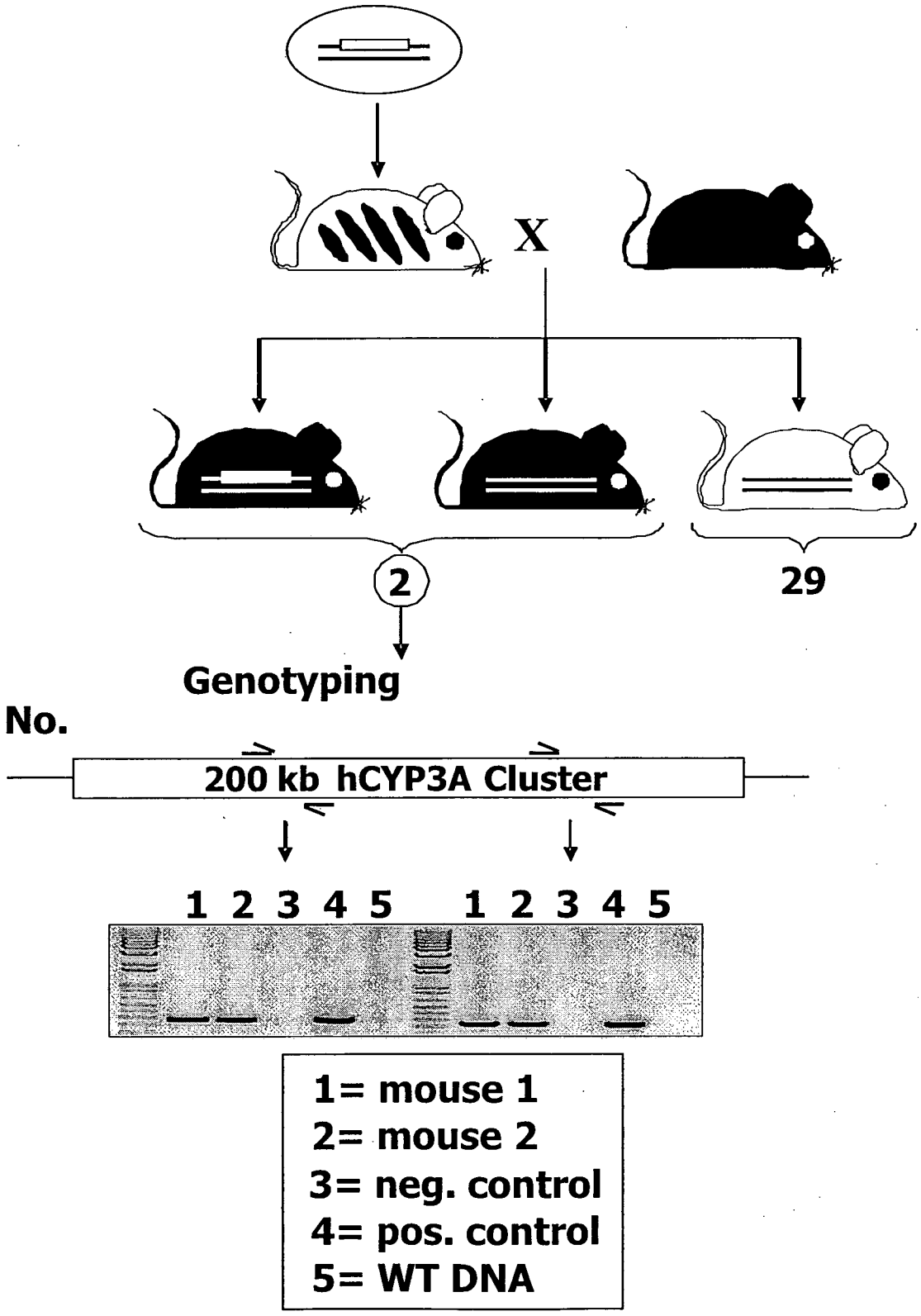

X
2
29
Genotyping
No.
200 kb hCYP3A Cluster
1 2 3 4 5 1 2 3 4 5
1= mouse 1
2= mouse 2
3= neg. control
4= pos. control
5= WT DNA

FIG. 102

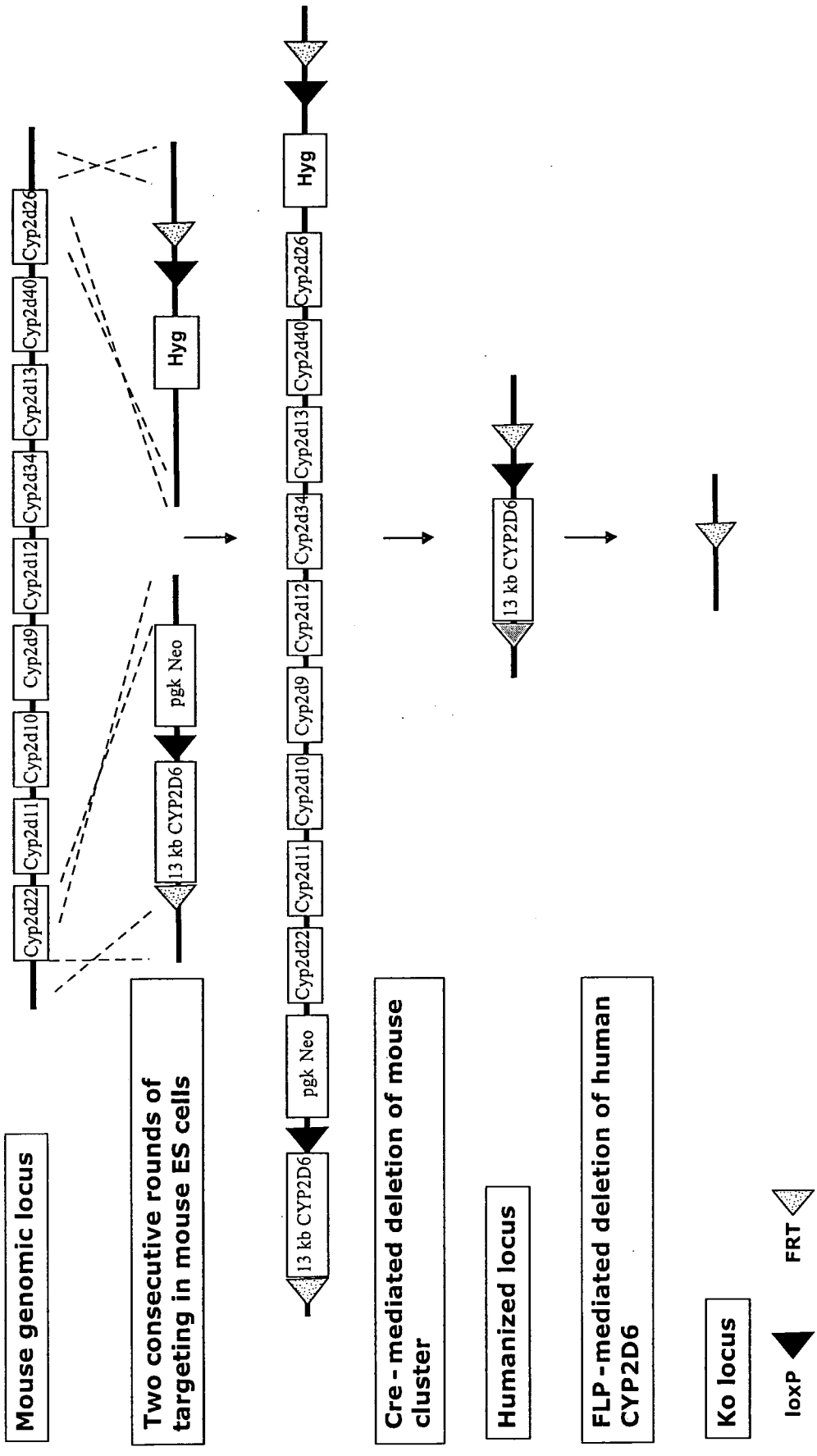

Mouse genomic locus
Cyp2d22
Cyp2d11
Cyp2d10
Cyp2d9
Cyp2d12
Cyp2d34
Cyp2d13
Cyp2d40
Cyp2d26
Two consecutive rounds of targeting in mouse ES cells
13 kb CYP2D6
pgk Neo
Hyg
Cre - mediated deletion of mouse cluster
Humanized locus
FLP - mediated deletion of human CYP2D6
Ko locus
loxP
FRT

FIG. 104

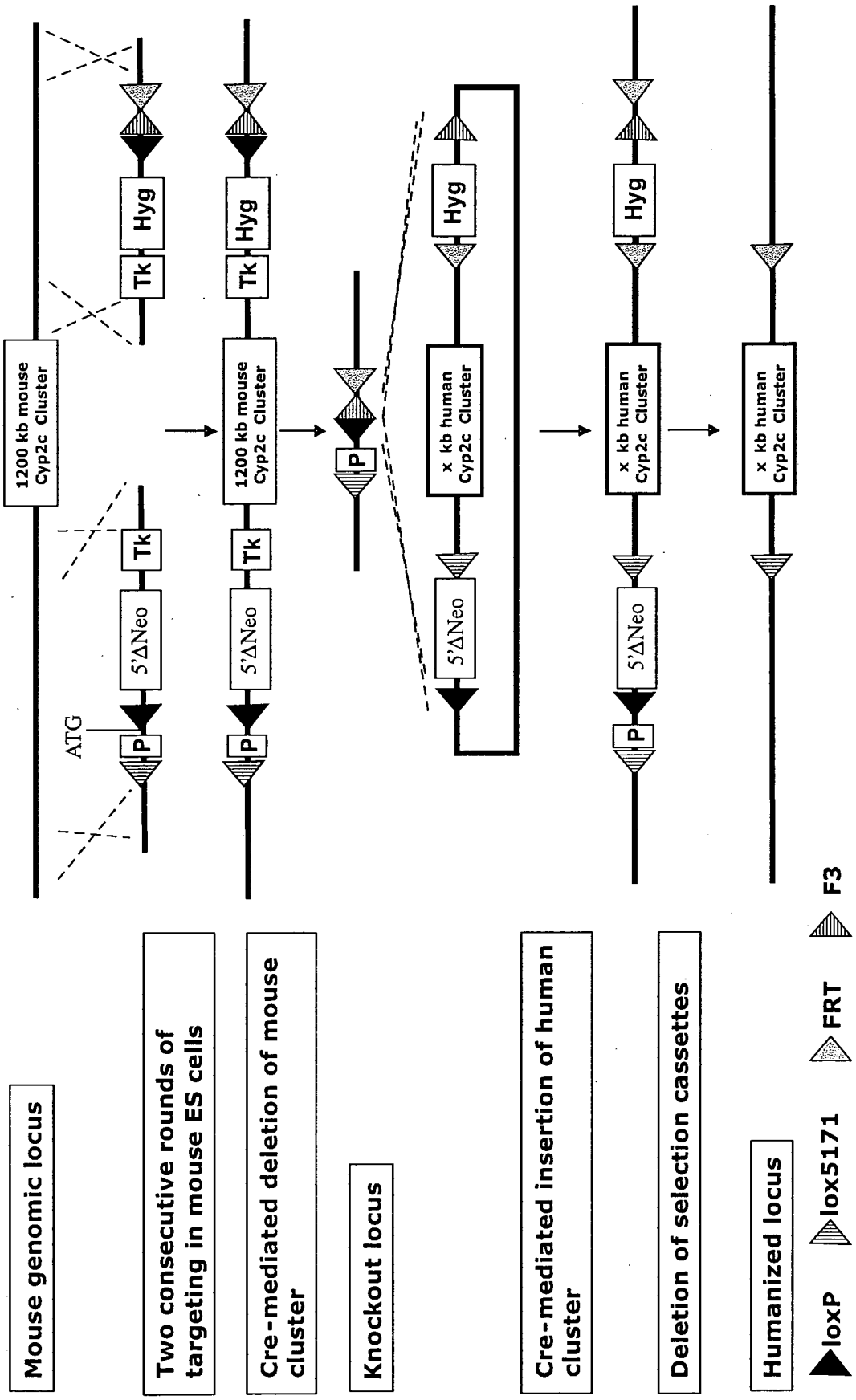

Mouse genomic locus
1200 kb mouse Cyp2c Cluster
ATG
P
5'ΔNeo
Tk
Hyg
Two consecutive rounds of targeting in mouse ES cells
Cre-mediated deletion of mouse cluster
Knockout locus
x kb human Cyp2c Cluster
Cre-mediated insertion of human cluster
Deletion of selection cassettes
Humanized locus
loxP
lox5171
FRT
F3

FIG. 105

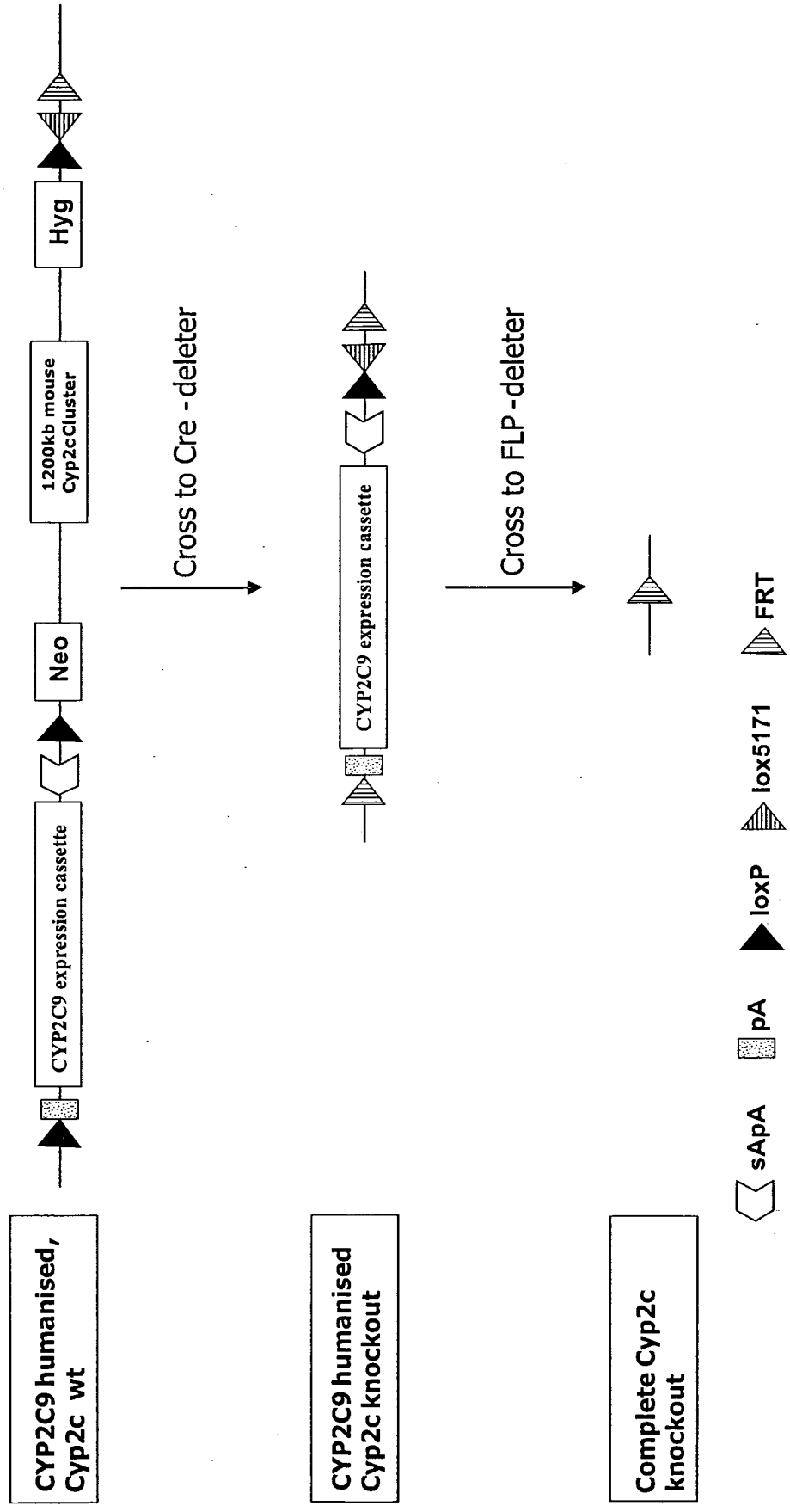
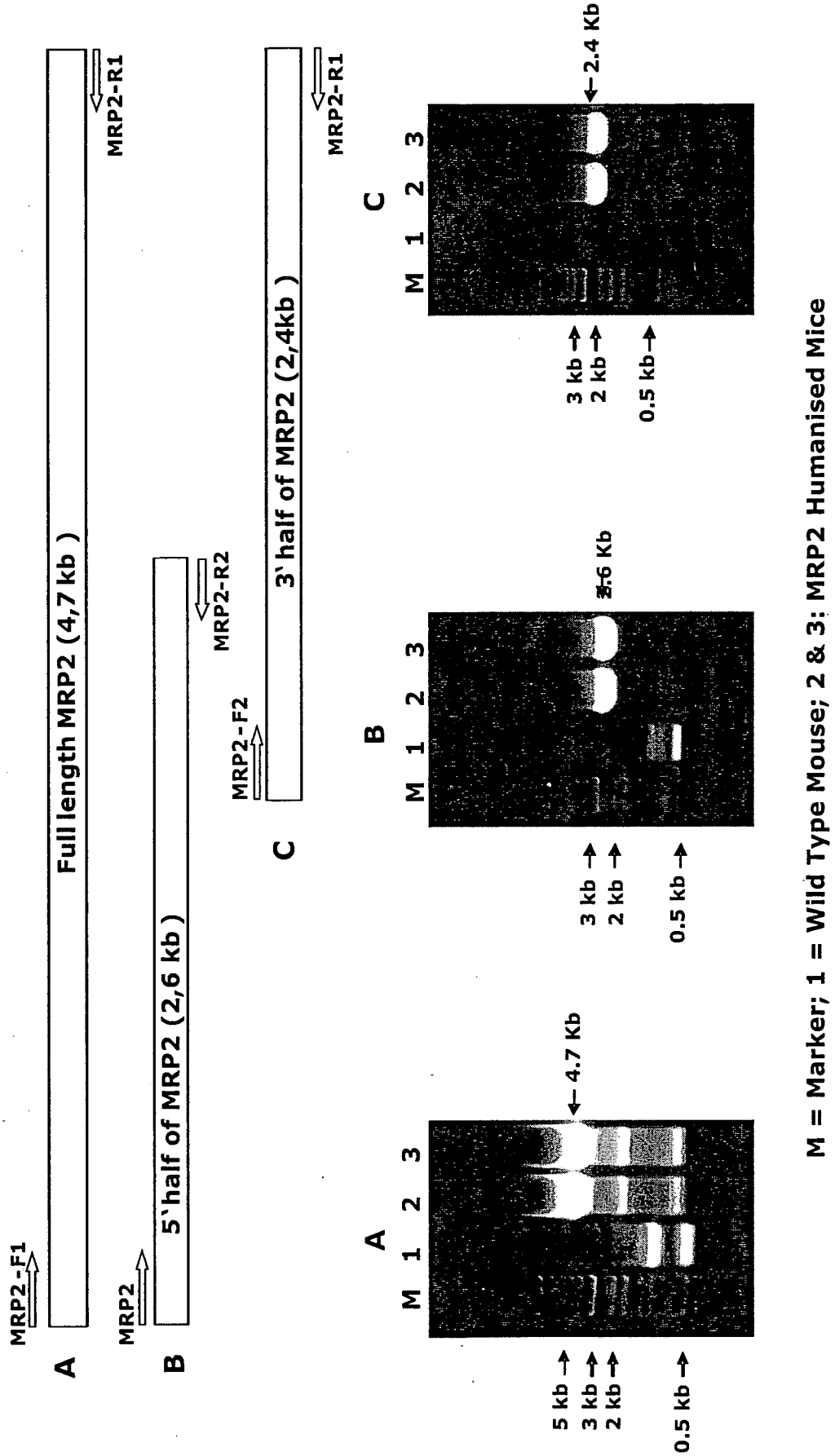
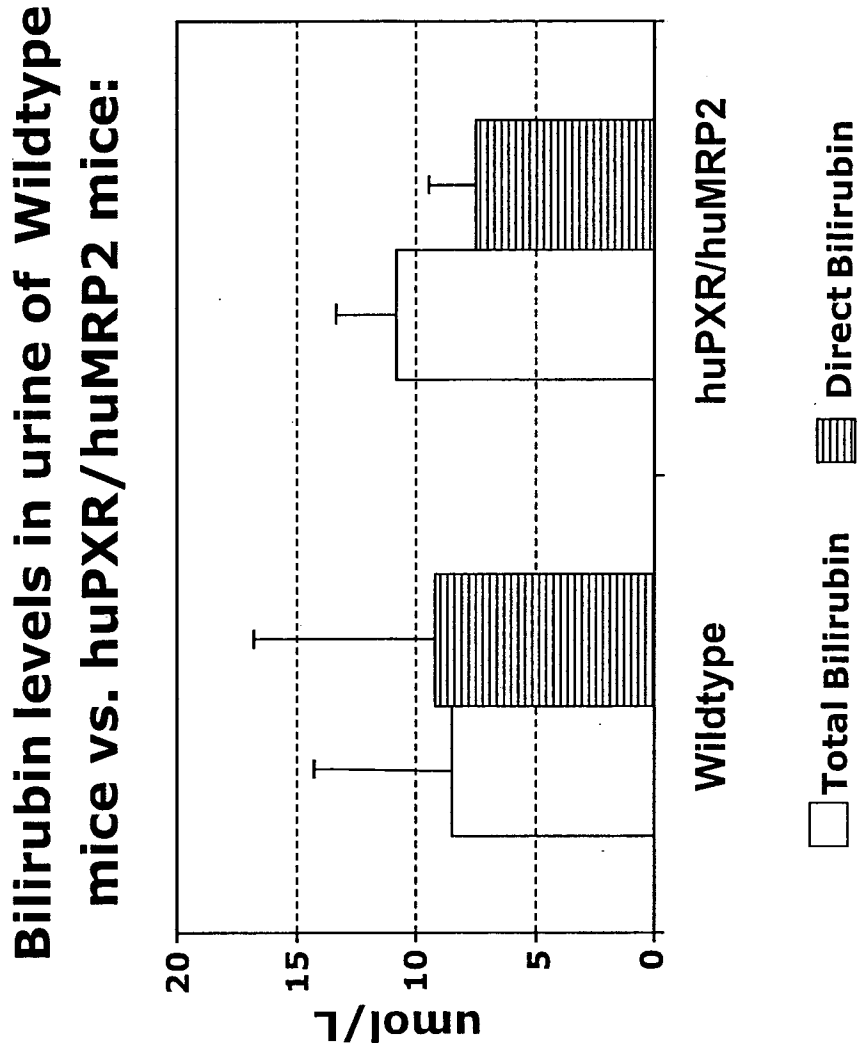

Cyp2c Cluster5' targeted
Cyp2c Cluster3' targeted
CYP2C9 expression cassette
Neo
1200kb mouse Cyp2cCluster
Hyg
Cross to Cre -deleter
CYP2C9 expression cassette
Cross to FLP -deleter
CYP2C9 humanised, Cyp2c wt
CYP2C9 humanised Cyp2c knockout
Complete Cyp2c knockout
sApA
pA
loxP
lox5171
FRT

FIG. 106



M = Marker; 1 = Wild Type Mouse; 2 & 3: MRP2 Humanised Mice

FIG. 107


Bilirubin levels in urine of Wildtype mice vs. huPXR/huMRP2 mice:
umol/L
20
15
10
5
0
Wildtype
huPXR/huMRP2
Total Bilirubin
Direct Bilirubin

CYP2B6 reporter

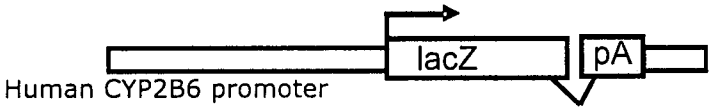


FIG. 108

untreated

80mg/kg Phenobarbital

CYP2D6 reporter

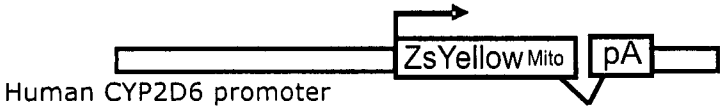
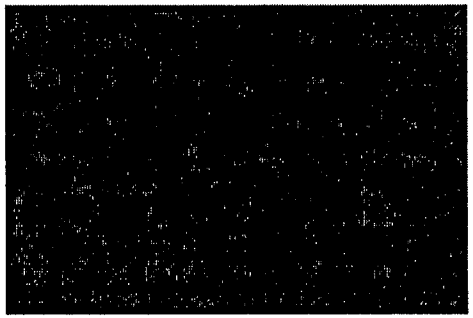
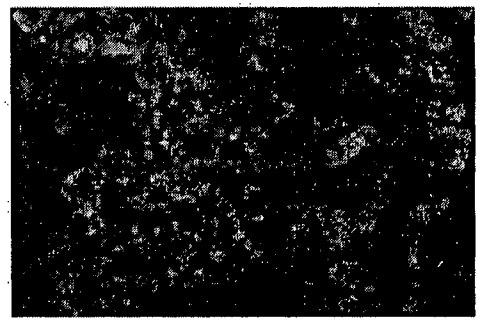


FIG. 109



WT control



CYP2D6 reporter

CYP3A4 reporter

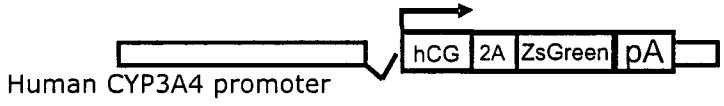
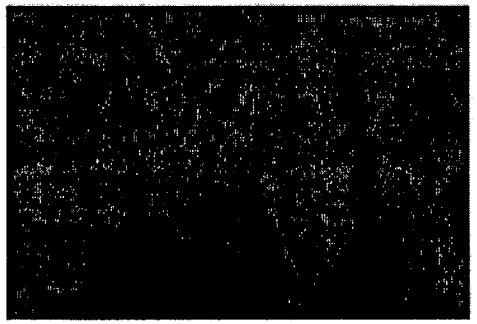
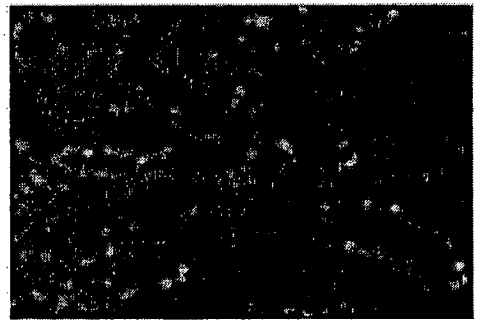


FIG. 110



WT control



CYP3A4 reporter

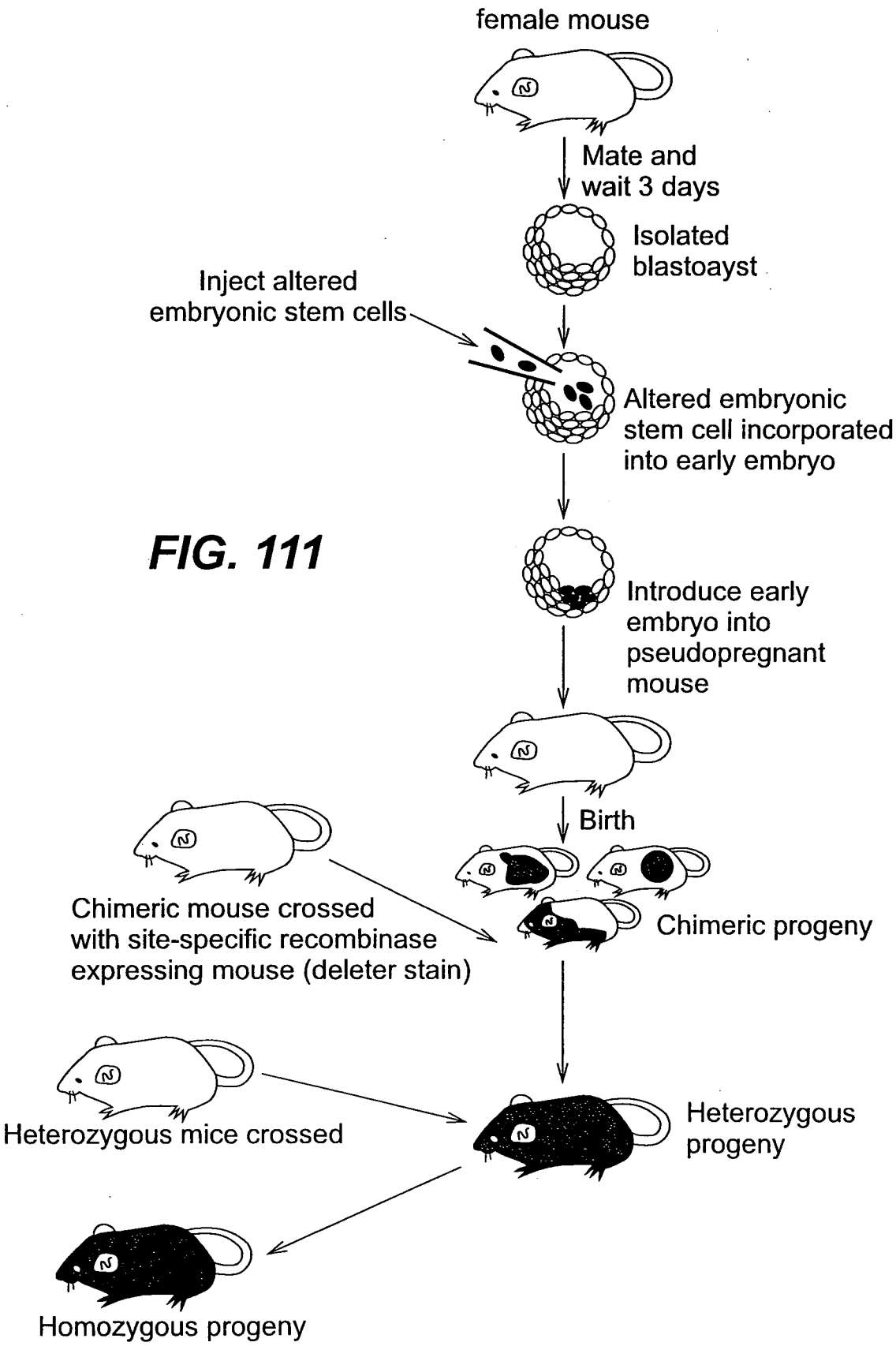

female mouse
Mate and wait 3 days
Isolated blastoayst
Inject altered embryonic stem cells
Altered embryonic stem cell incorporated into early embryo
Introduce early embryo into pseudopregnant mouse
Birth
Chimeric mouse crossed with site-specific recombinase expressing mouse (deleter stain)
Chimeric progeny
Heterozygous mice crossed
Heterozygous progeny
Homozygous progeny

FIG. 111

FIG. 112

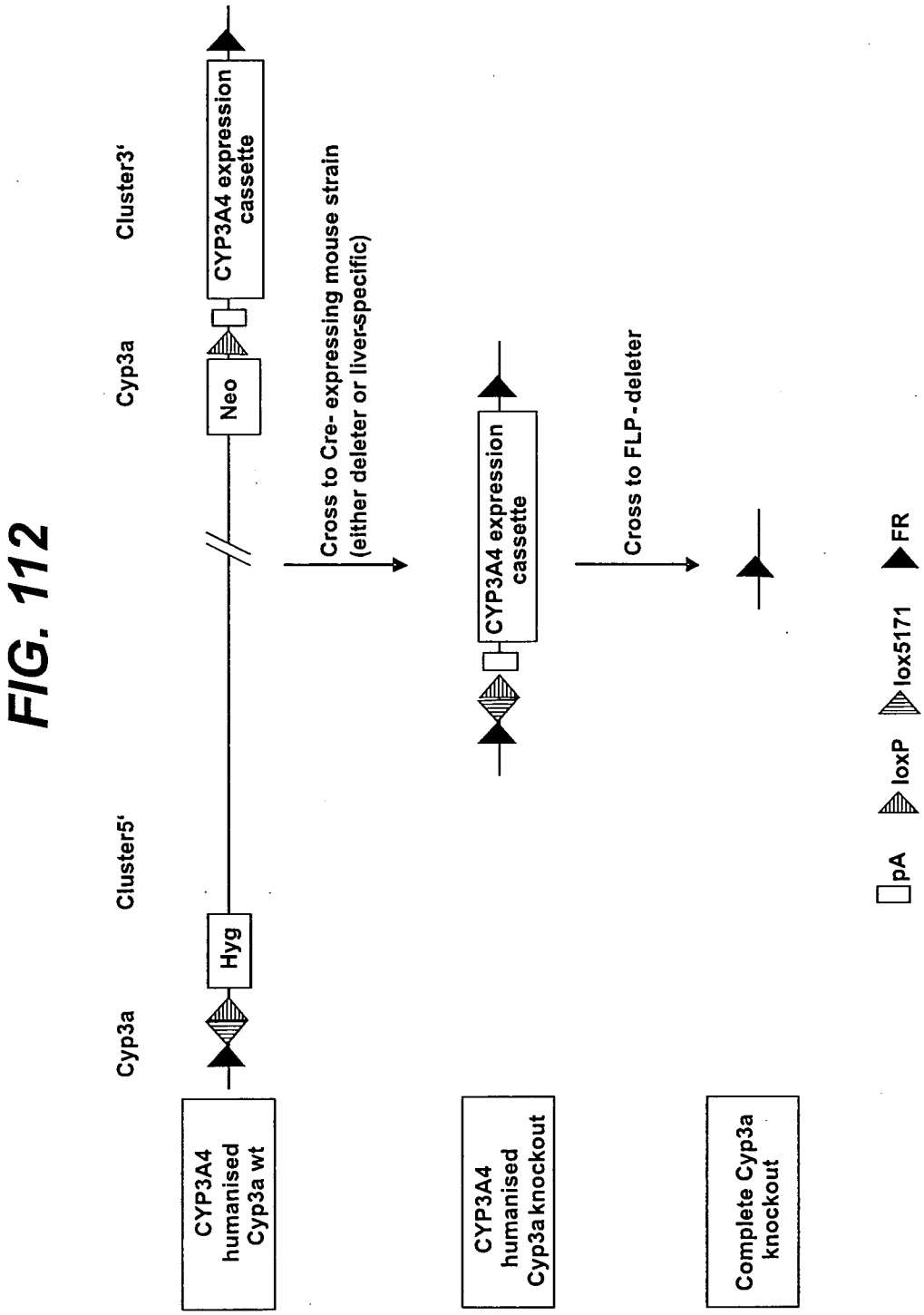

Cyp3a
Cluster5'
Hyg
Cyp3a
Neo
Cluster3'
CYP3A4 expression cassette
Cross to Cre- expressing mouse strain (either deleter or liver-specific)
CYP3A4 expression cassette
Cross to FLP-deleter
CYP3A4 humanised Cyp3a wt
CYP3A4 humanised Cyp3a knockout
Complete Cyp3a knockout
pA
loxP
lox5171
FR

FIG. 113

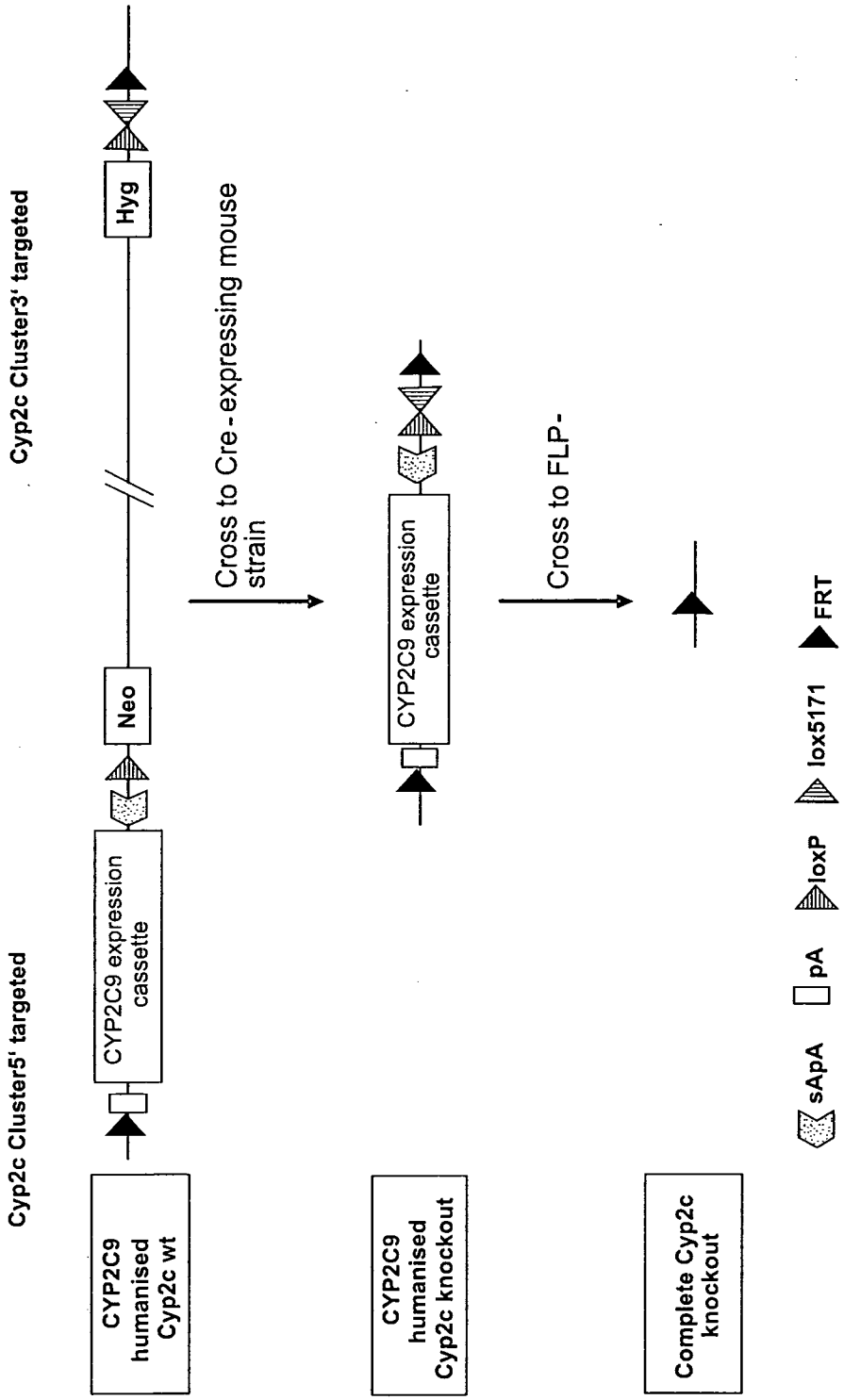
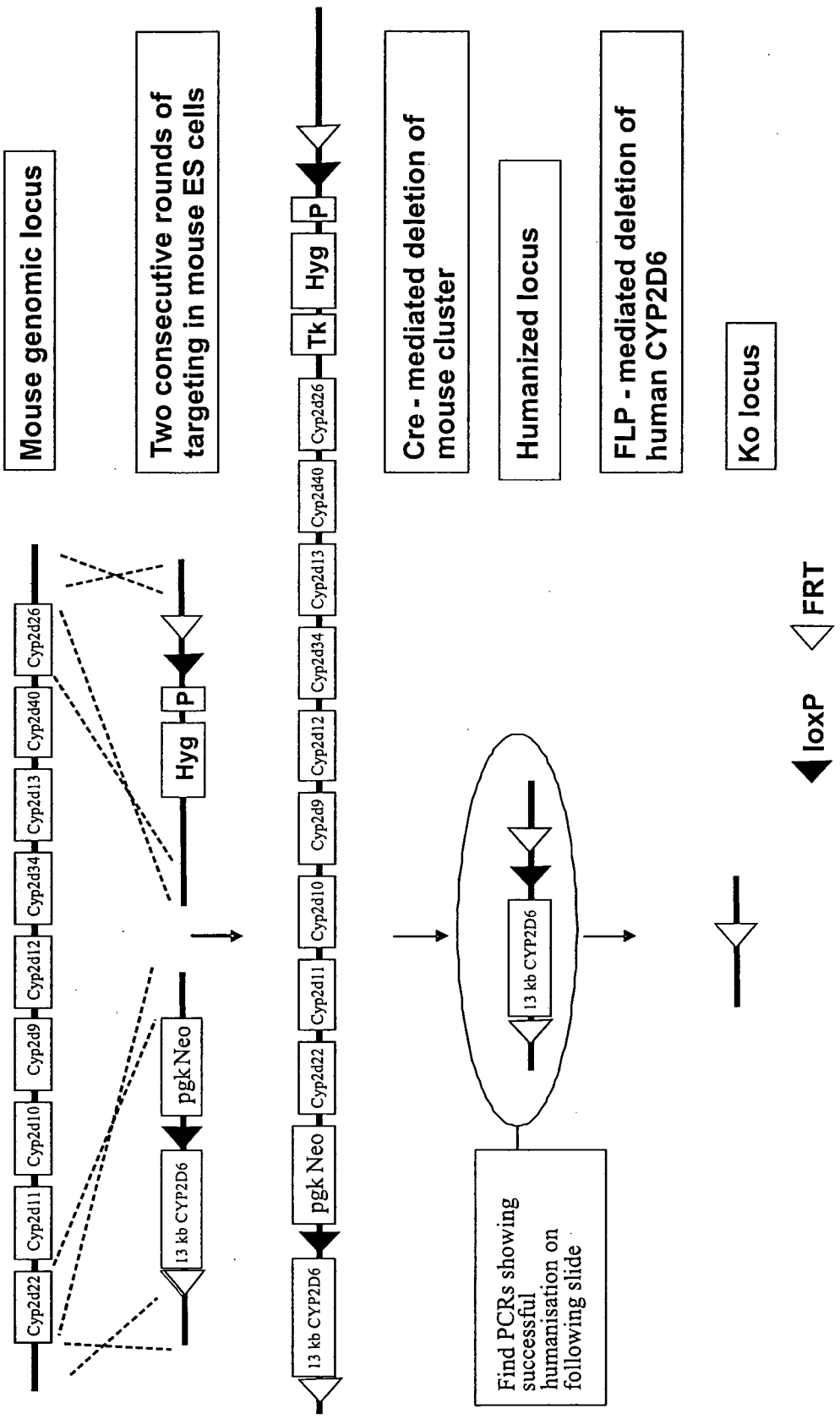
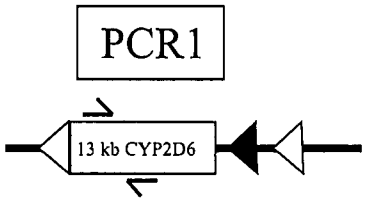
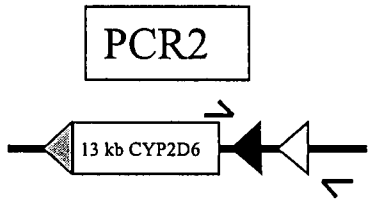

Cyp2c Cluster5' targeted
Cyp2c Cluster3' targeted
CYP2C9 expression cassette
Neo
Hyg
Cross to Cre-expressing mouse strain
CYP2C9 expression cassette
Cross to FLP-
CYP2C9 humanised Cyp2c wt
CYP2C9 humanised Cyp2c knockout
Complete Cyp2c knockout
sApA
pA
loxP
lox5171
FRT

FIG. 114


Mouse genomic locus
Two consecutive rounds of targeting in mouse ES cells
Cre - mediated deletion of mouse cluster
Humanized locus
FLP - mediated deletion of human CYP2D6
Ko locus
Cyp2d22
Cyp2d11
Cyp2d10
Cyp2d9
Cyp2d12
Cyp2d34
Cyp2d13
Cyp2d40
Cyp2d26
pgk Neo
13 kb CYP2D6
Hyg
P
Tk
Find PCRs showing successful humanisation on following slide
loxP
FRT

General primer position :





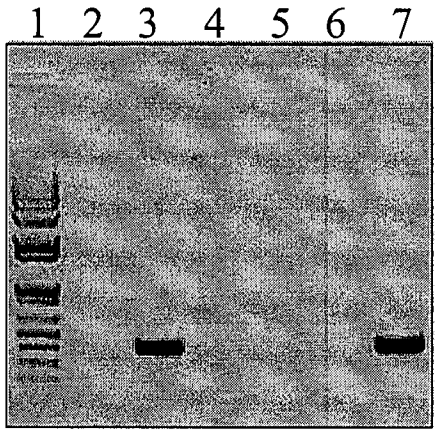
Primers : onC2D6tf1 (CCTCTCCCTCCATACCTC)
onC2D6tr1 (TTCTCTACCCGCCCCAGTG)

oC2Ddt_2 (TCAGCCCACCCCACATCAGG)
oC2Ddt_3 (GGCCAGAATGACCTAGAAC))

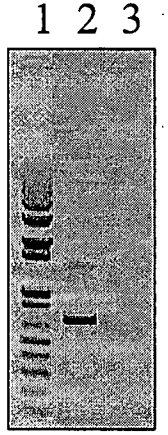
Expected fragment: 408bp

710 bp

PCR results :



1 Marker; 2 ID217878; 3 ID218641; 4 ID218642; 5 ID218936; 6 H_2O; 7 pos. control



1 Marker; 2 ID218641; 3 H_2O

FIG. 115

Conclusion: The mouse with the ID 218641 is heterozygously humanised for CYP2D6

FIG. 116

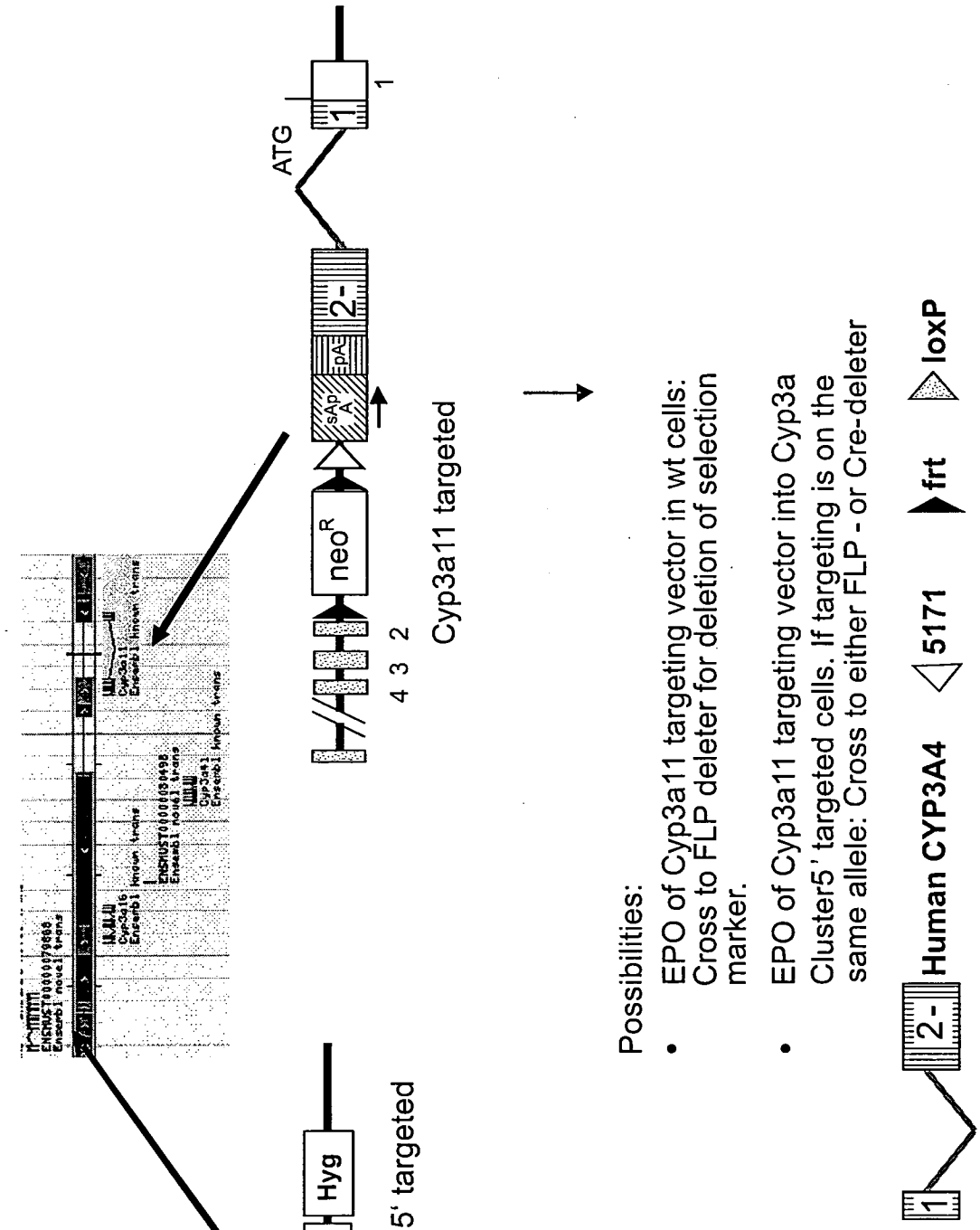
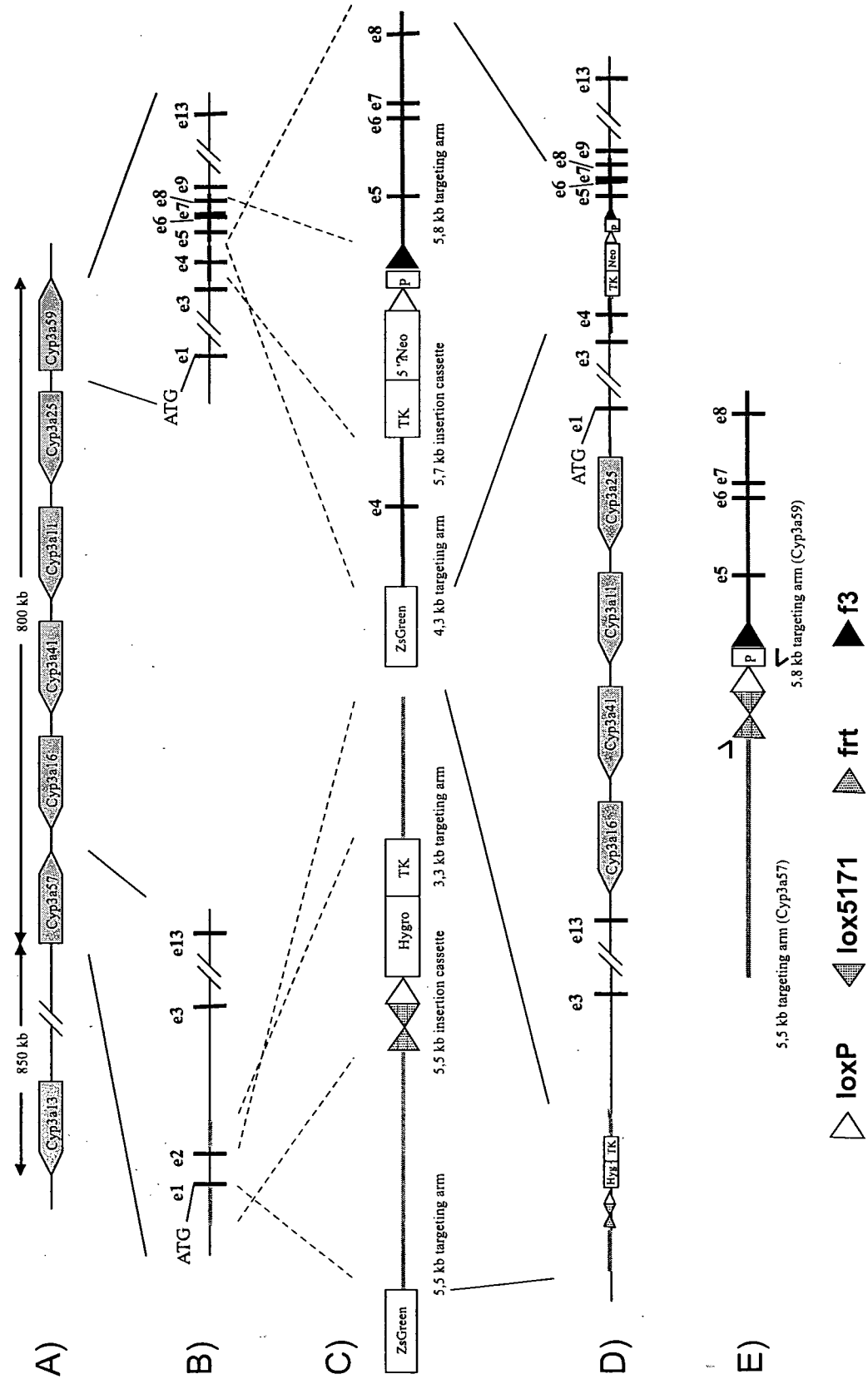

Cyp3a Cluster 5' targeted
P
Hyg
4 3 2
neo^R
sAp A
pA
2-
ATG
1
1
Cyp3a11 targeted
Possibilities:
EPO of Cyp3a11 targeting vector in wt cells: Cross to FLP deleter for deletion of selection marker.
EPO of Cyp3a11 targeting vector into Cyp3a Cluster5' targeted cells. If targeting is on the same allele: Cross to either FLP - or Cre-deleter
1
2-
Human CYP3A4
5171
frt
loxP

FIG. 117


A)
B)
C)
D)
E)
850 kb
800 kb
Cyp3a13
Cyp3a57
Cyp3a16
Cyp3a41
Cyp3a11
Cyp3a25
Cyp3a59
ATG
e1
e2
e3
e4
e5
e6
e7
e8
e9
e13
ZsGreen
Hygro
TK
5,5 kb targeting arm
5,5 kb insertion cassette
3,3 kb targeting arm
4,3 kb targeting arm
5,7 kb insertion cassette
5'Neo
P
5,8 kb targeting arm
Hyg TK
TK Neo
5,5 kb targeting arm (Cyp3a57)
5,8 kb targeting arm (Cyp3a59)
loxP
lox5171
frt
f3

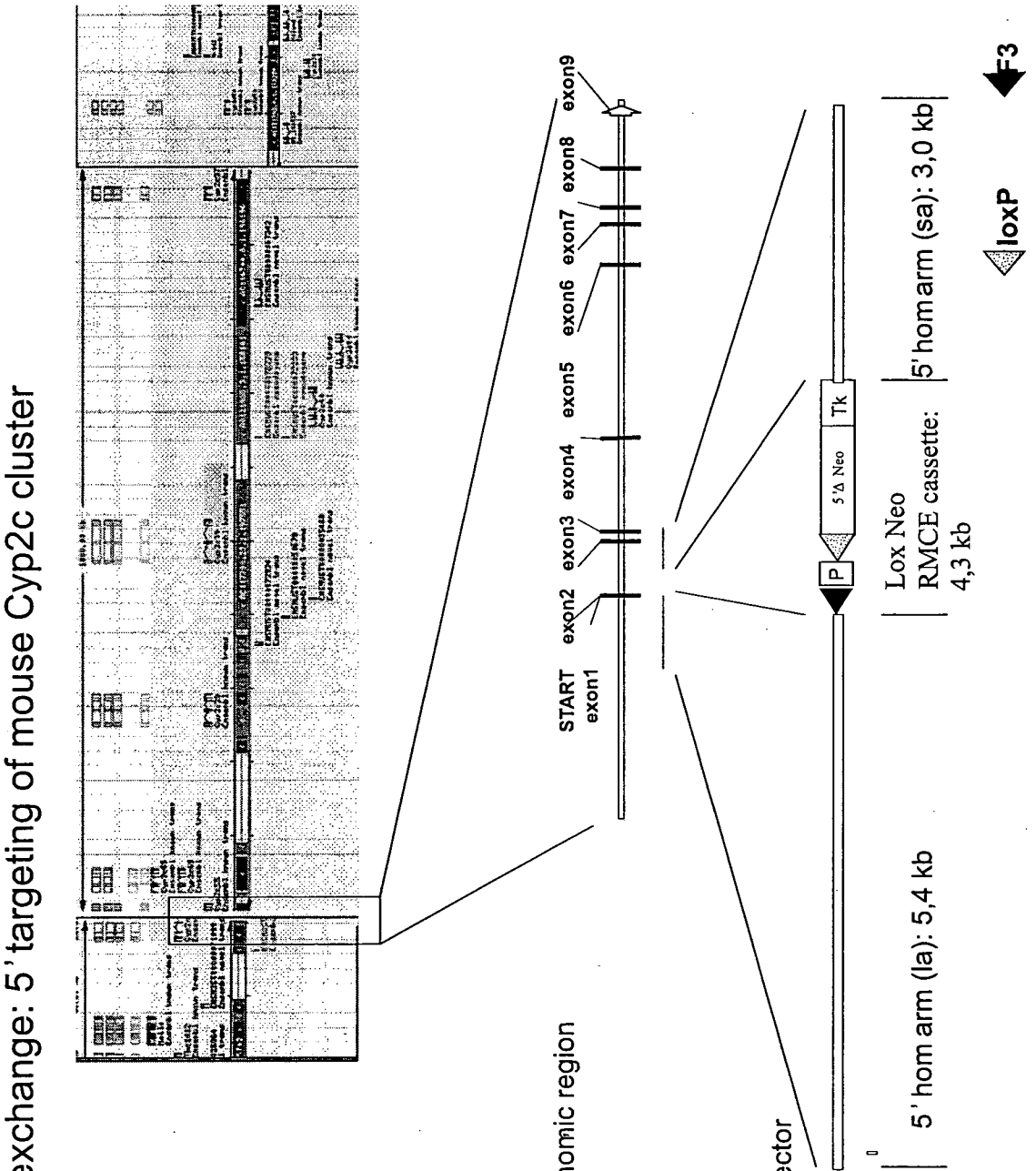

Cyp2c cluster exchange: 5' targeting of mouse Cyp2c cluster
Cyp2C cluster:
Overview genomic region
Cyp2C cluster 5 genomic region
= Cyp2c55 (52.8 kb)
START
exon1
exon2
exon3
exon4
exon5
exon6
exon7
exon8
exon9
Targeting vector
P
5'Δ Neo
Tk
Lox Neo
RMCE cassette:
4,3 kb
5' hom arm (la): 5,4 kb
5' hom arm (sa): 3,0 kb
F3
loxP

FIG. 118A

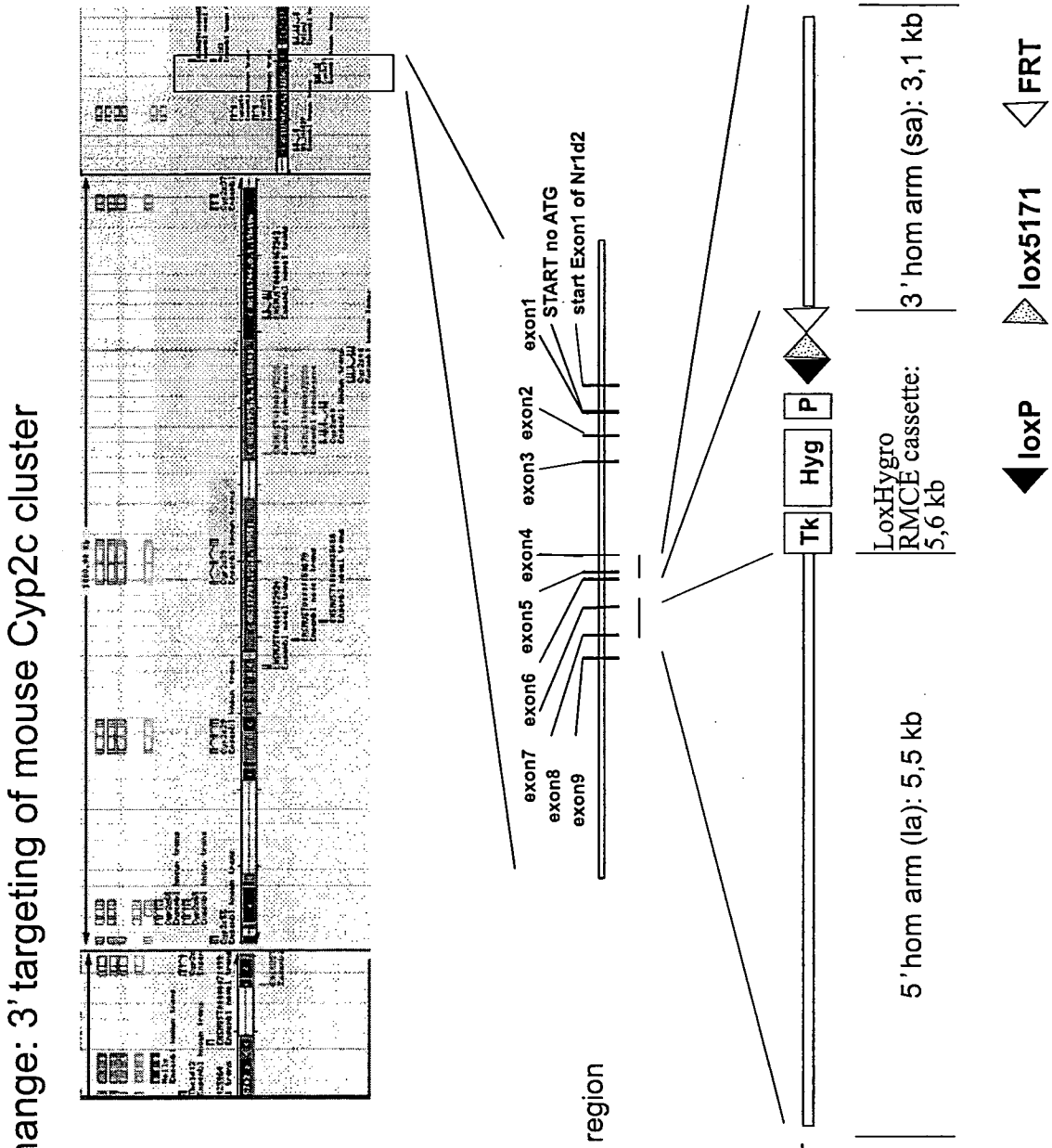
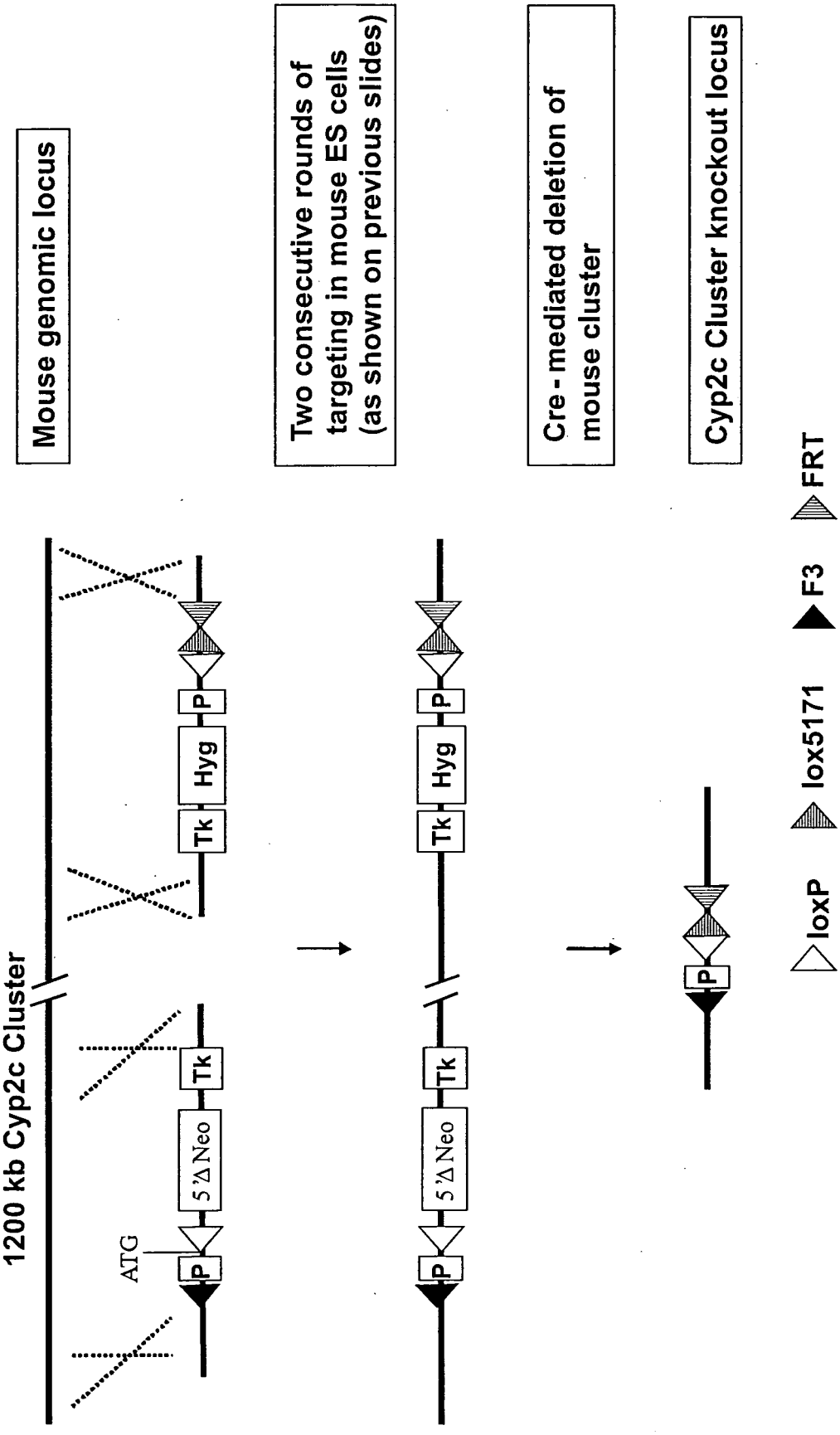

Cyp2c cluster exchange: 3' targeting of mouse Cyp2c cluster
Cyp2C cluster:
Overview genomic
region
Cyp2C cluster 3 genomic region
= Cyp2c70 (87 kb)
exon1
START no ATG
start Exon1 of Nr1d2
exon2
exon3
exon4
exon5
exon6
exon7
exon8
exon9
Targeting vector
Tk
Hyg
P
LoxHygro
RMCE cassette:
5,6 kb
5' hom arm (la): 5,5 kb
3' hom arm (sa): 3,1 kb
loxP
lox5171
FRT

FIG. 118B

FIG. 118C Overview: Cyp2c Cluster knockout



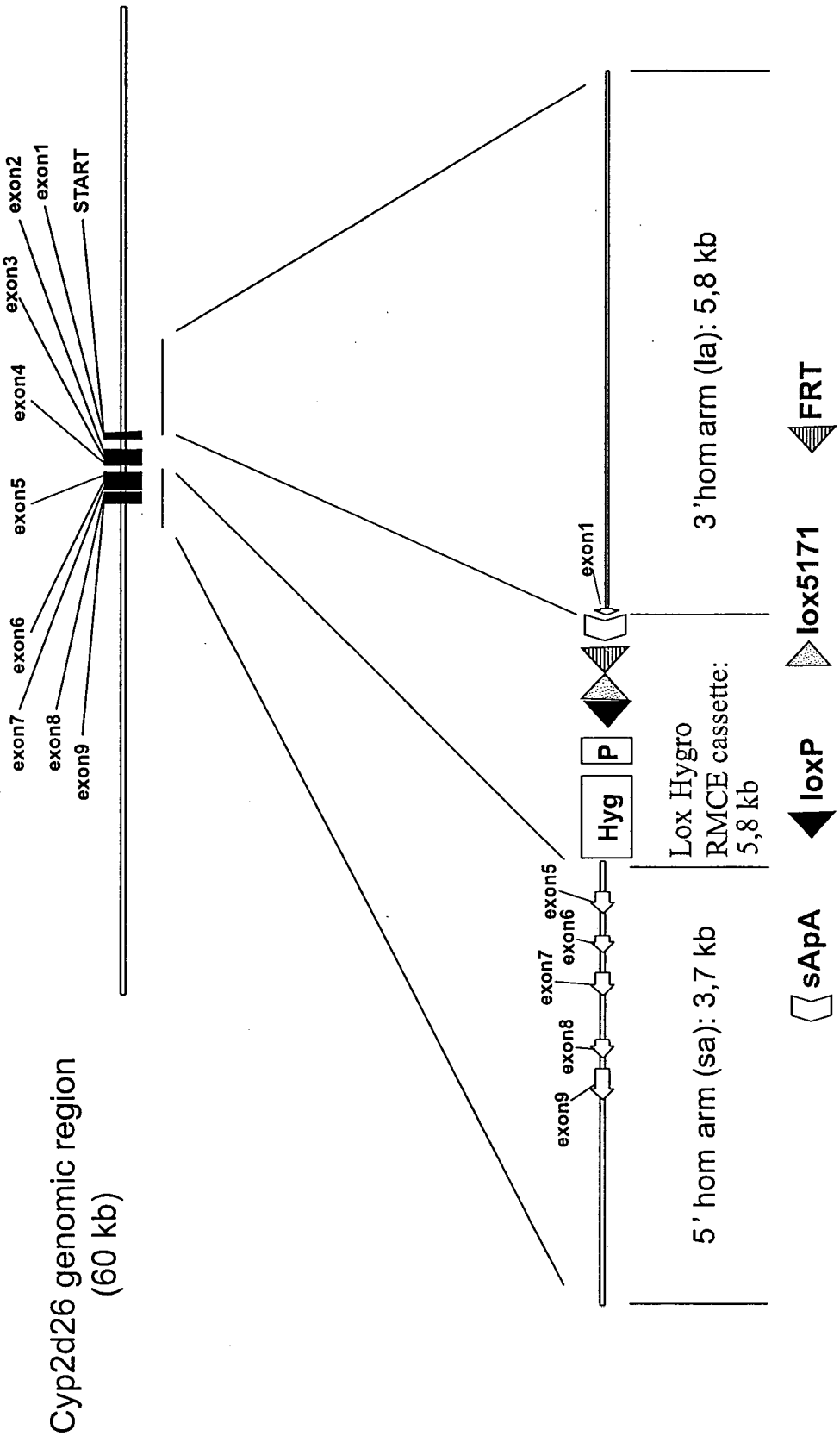


FIG. 119A Cyp2d Cluster exchange: '3' targeting of mouse Cyp2d26

FIG. 119B Cyp2d cluster exchange: 5'targeting of mouse Cyp2d cluster

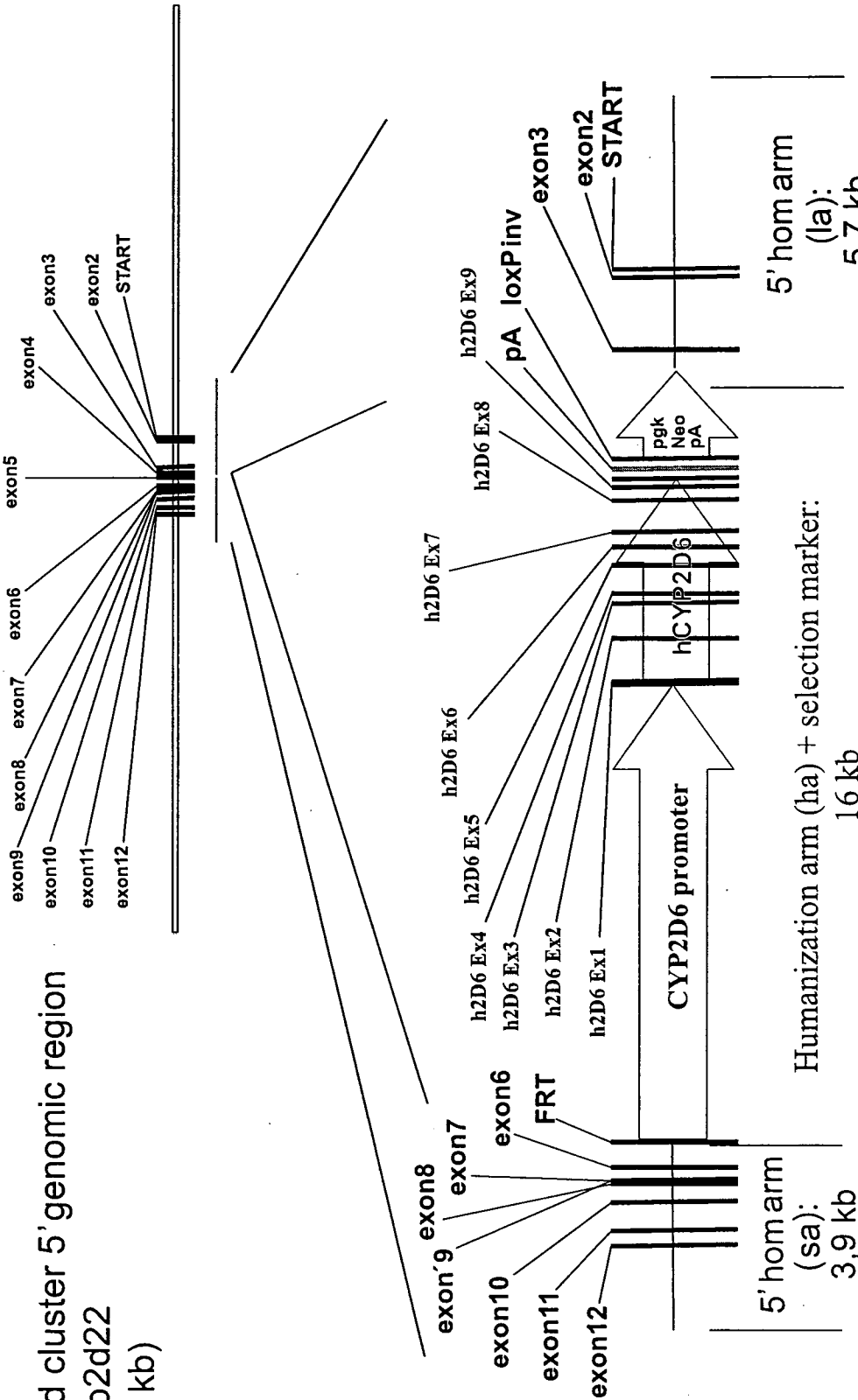
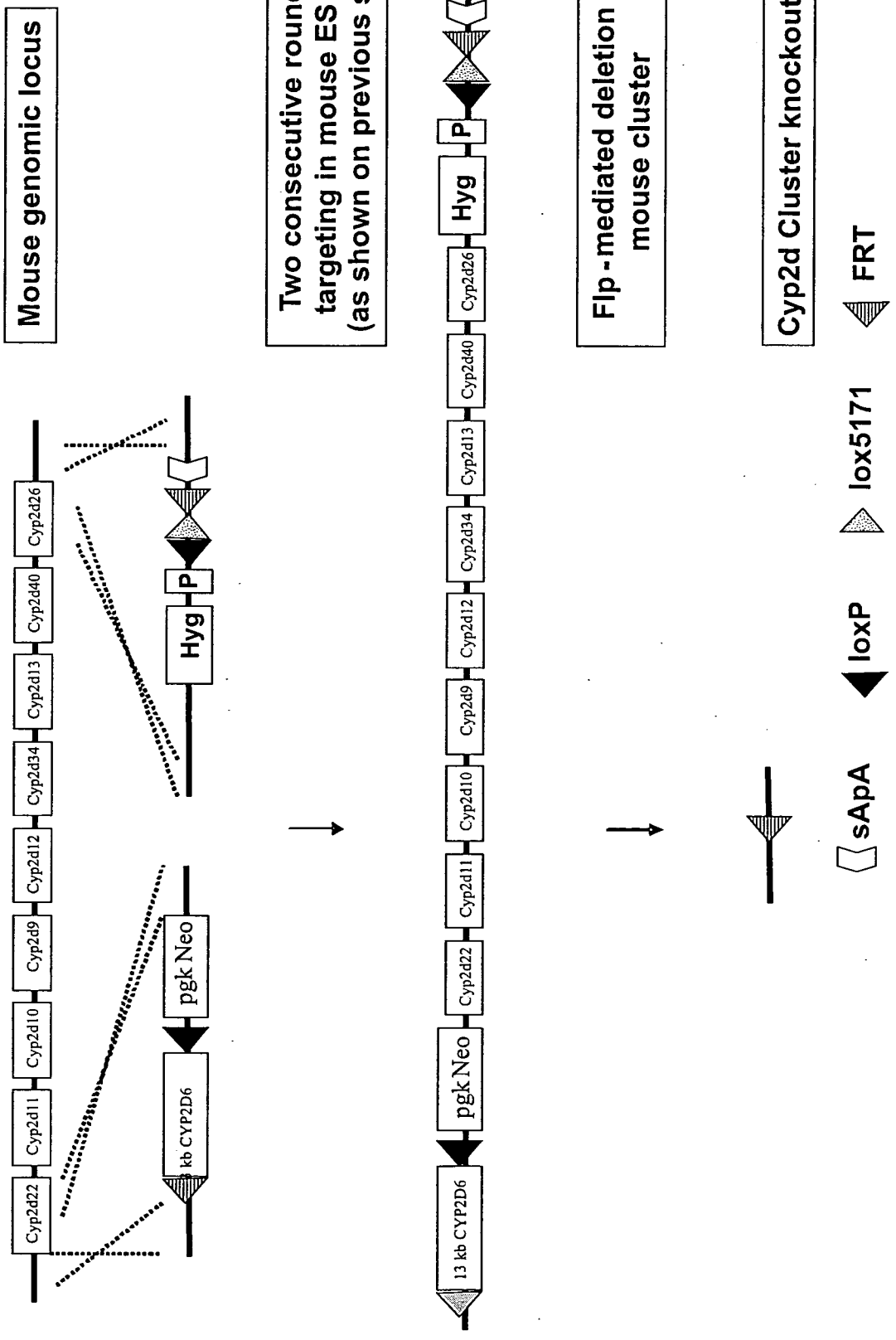


FIG. 119C Overview: Cyp2d Cluster knockout



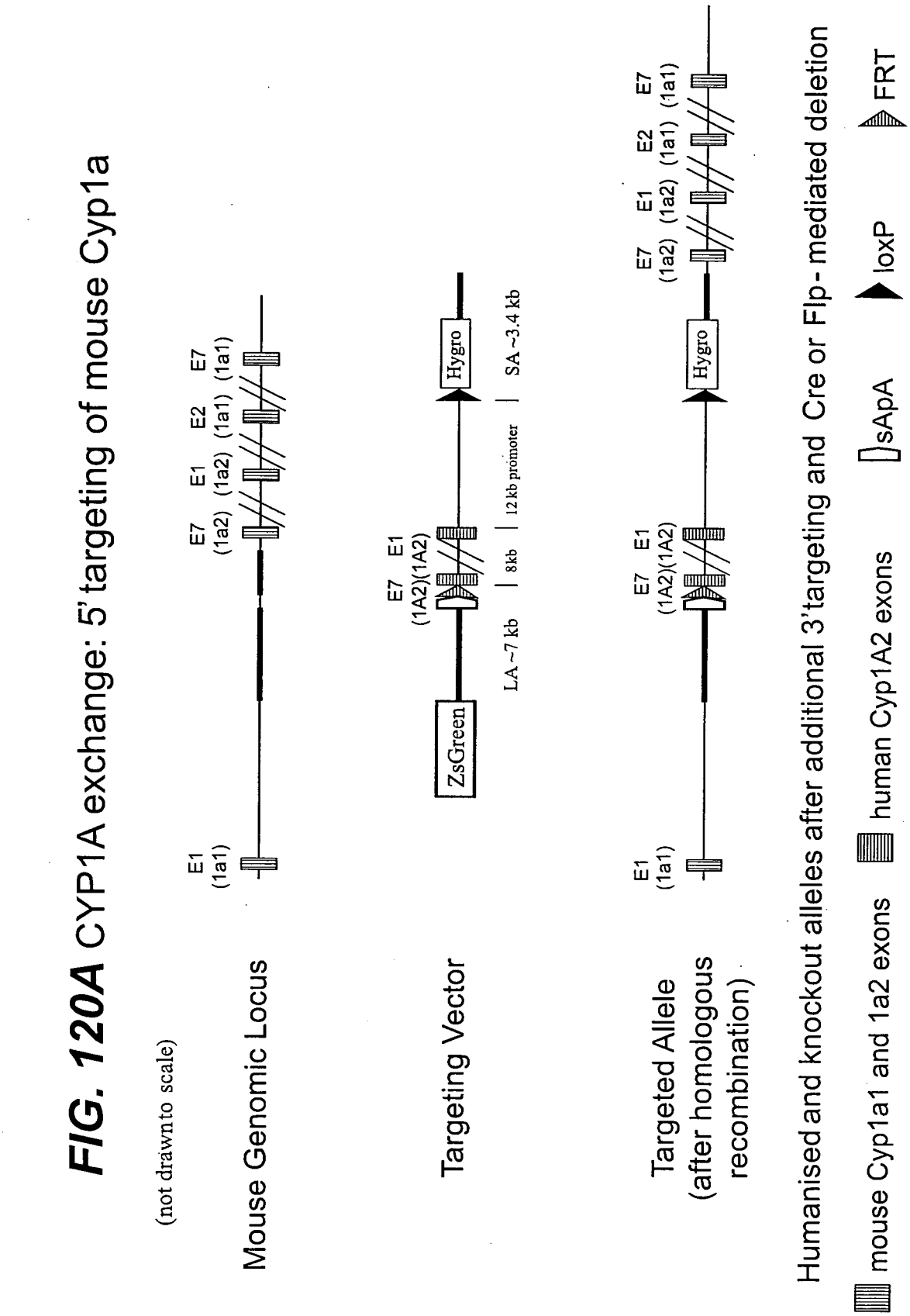
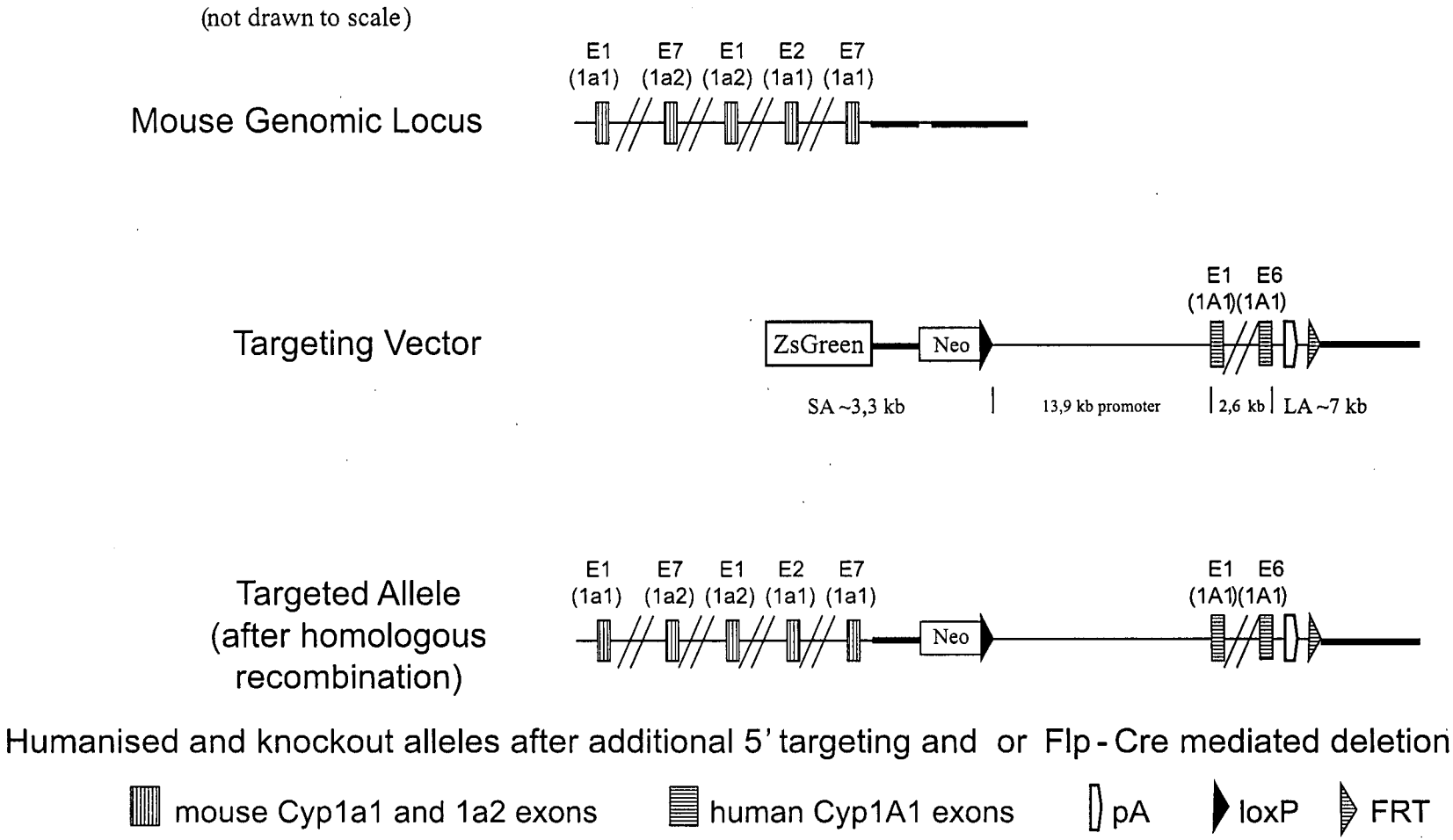


FIG. 120A CYP1A exchange: 5' targeting of mouse Cyp1a
(not drawn to scale)
Mouse Genomic Locus
E1 (1a1)
E7 (1a2)
E1 (1a2)
E2 (1a1)
E7 (1a1)
Targeting Vector
ZsGreen
E7 (1A2)(1A2)
E1 (1A2)(1A2)
Hygro
LA ~7 kb
8kb
12 kb promoter
SA ~3.4 kb
Targeted Allele (after homologous recombination)
E1 (1a1)
E7 (1A2)(1A2)
E1 (1A2)(1A2)
Hygro
E7 (1a2)
E1 (1a2)
E2 (1a1)
E7 (1a1)
Humanised and knockout alleles after additional 3'targeting and Cre or Flp- mediated deletion
mouse Cyp1a1 and 1a2 exons
human Cyp1A2 exons
sApA
loxP
FRT

FIG. 120B CYP1A exchange: 3' targeting of mouse Cyp1a



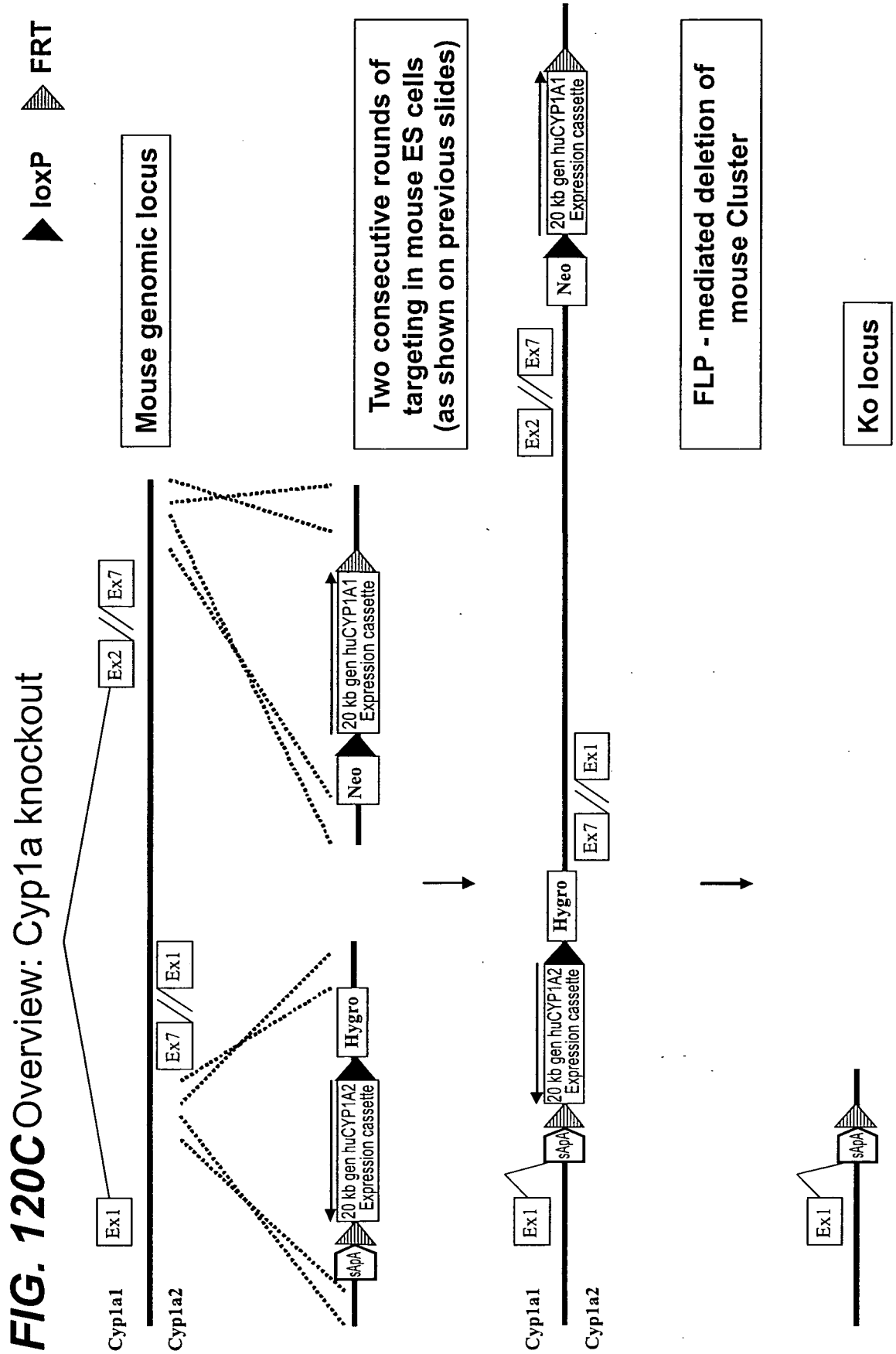


FIG. 120C Overview: Cyp1a knockout
loxP
FRT
Mouse genomic locus
Two consecutive rounds of targeting in mouse ES cells (as shown on previous slides)
FLP - mediated deletion of mouse Cluster
Ko locus
Cyp1a1
Cyp1a2
Ex1
Ex2
Ex7
Ex1
Ex7
Neo
20 kb gen huCYP1A1 Expression cassette
Hygro
20 kb gen huCYP1A2 Expression cassette
SApA

FIG. 121

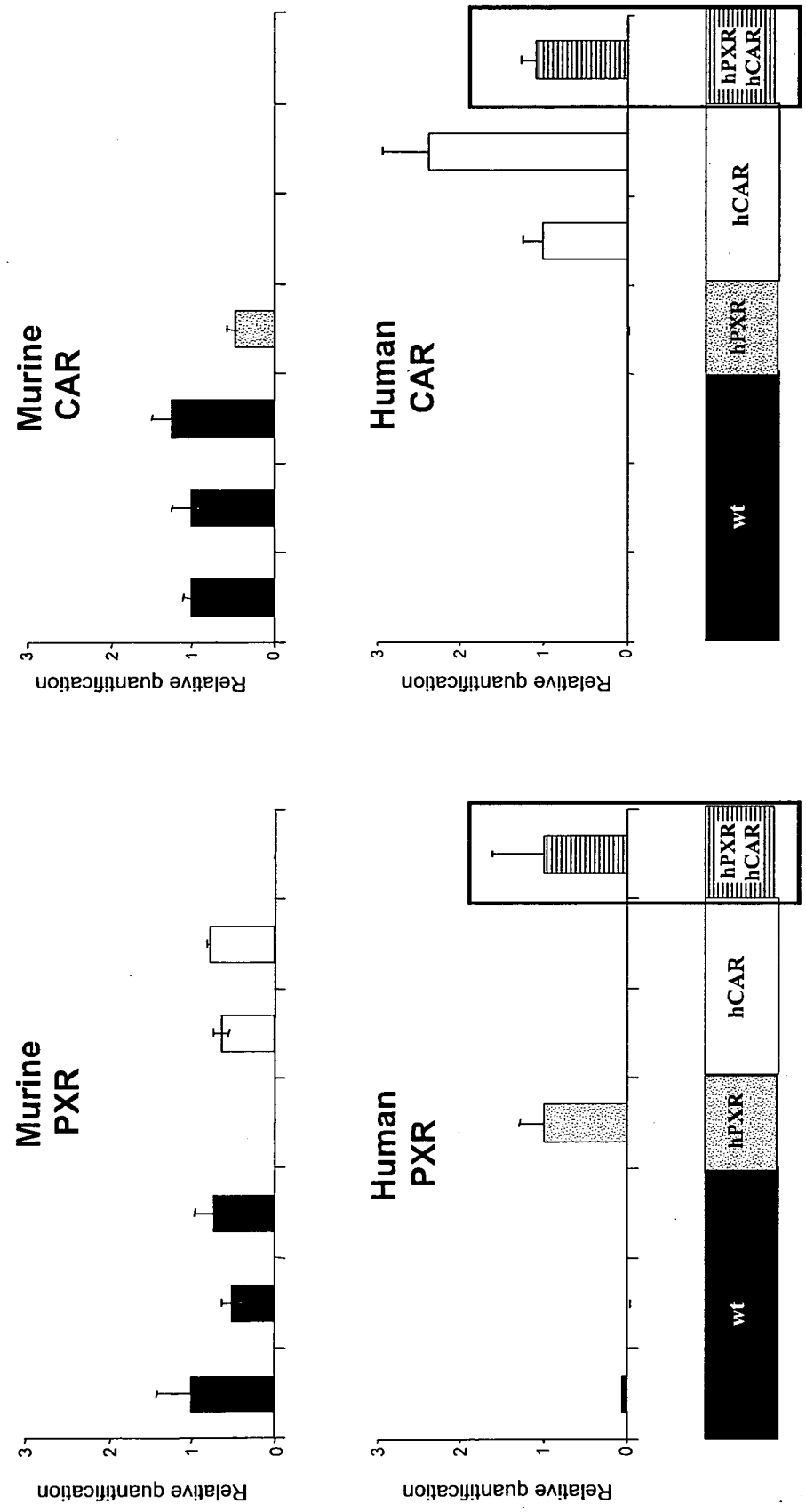

Murine CAR
Human CAR
Murine PXR
Human PXR
Relative quantification
0
1
2
3
wt
hPXR
hCAR
hPXR hCAR

FIG. 122

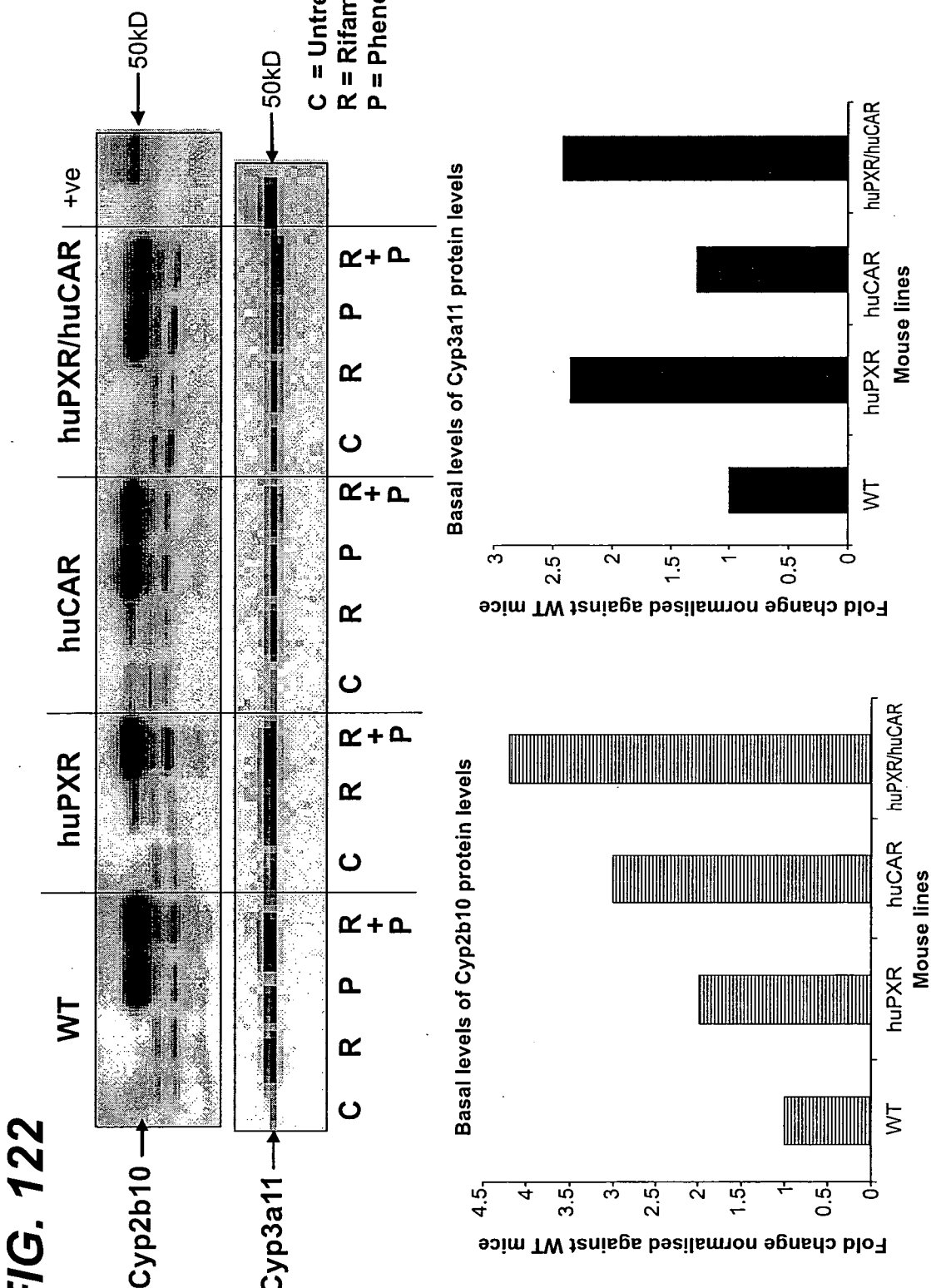

WT
huPXR
huCAR
huPXR/huCAR
+ve
50kD
50kD
C = Untreated
R = Rifampicin
P = Phenobarbital
C R P R + P C R P R + P C R P R + P C R P R + P
Cyp2b10
Cyp3a11
Basal levels of Cyp2b10 protein levels
Fold change normalised against WT mice
Mouse lines
WT
huPXR
huCAR
huPXR/huCAR
Basal levels of Cyp3a11 protein levels
Fold change normalised against WT mice
Mouse lines

FIG. 123

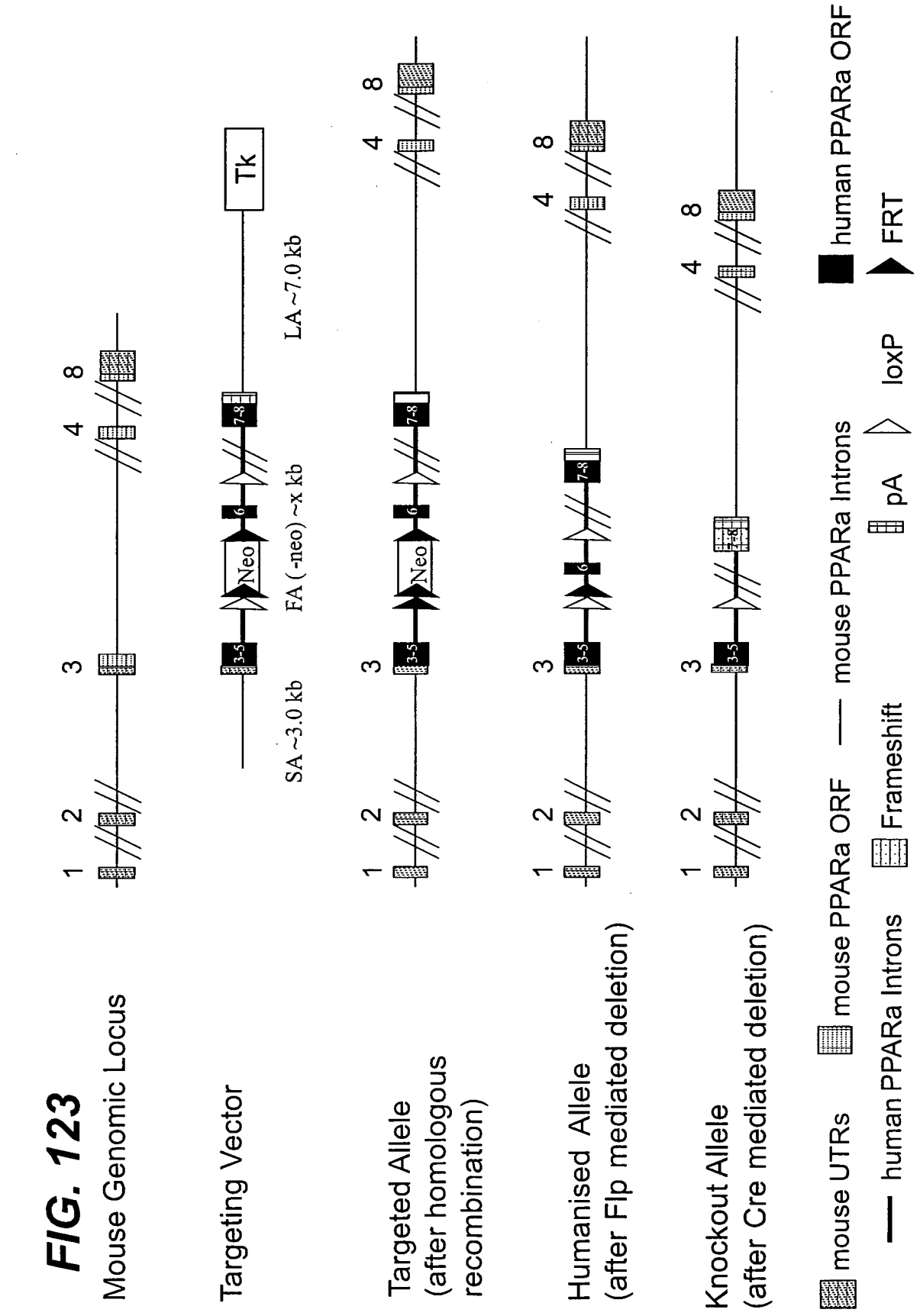

Mouse Genomic Locus
1 2 3 4 8
Targeting Vector
SA ~3.0 kb
3-5
Neo
6
7-8
FA (-neo) ~x kb
Tk
LA ~7.0 kb
Targeted Allele
(after homologous
recombination)
Humanised Allele
(after Flp mediated deletion)
Knockout Allele
(after Cre mediated deletion)
mouse UTRs
mouse PPARa ORF
mouse PPARa Introns
human PPARa ORF
human PPARa Introns
Frameshift
pA
loxP
FRT

FIG. 124

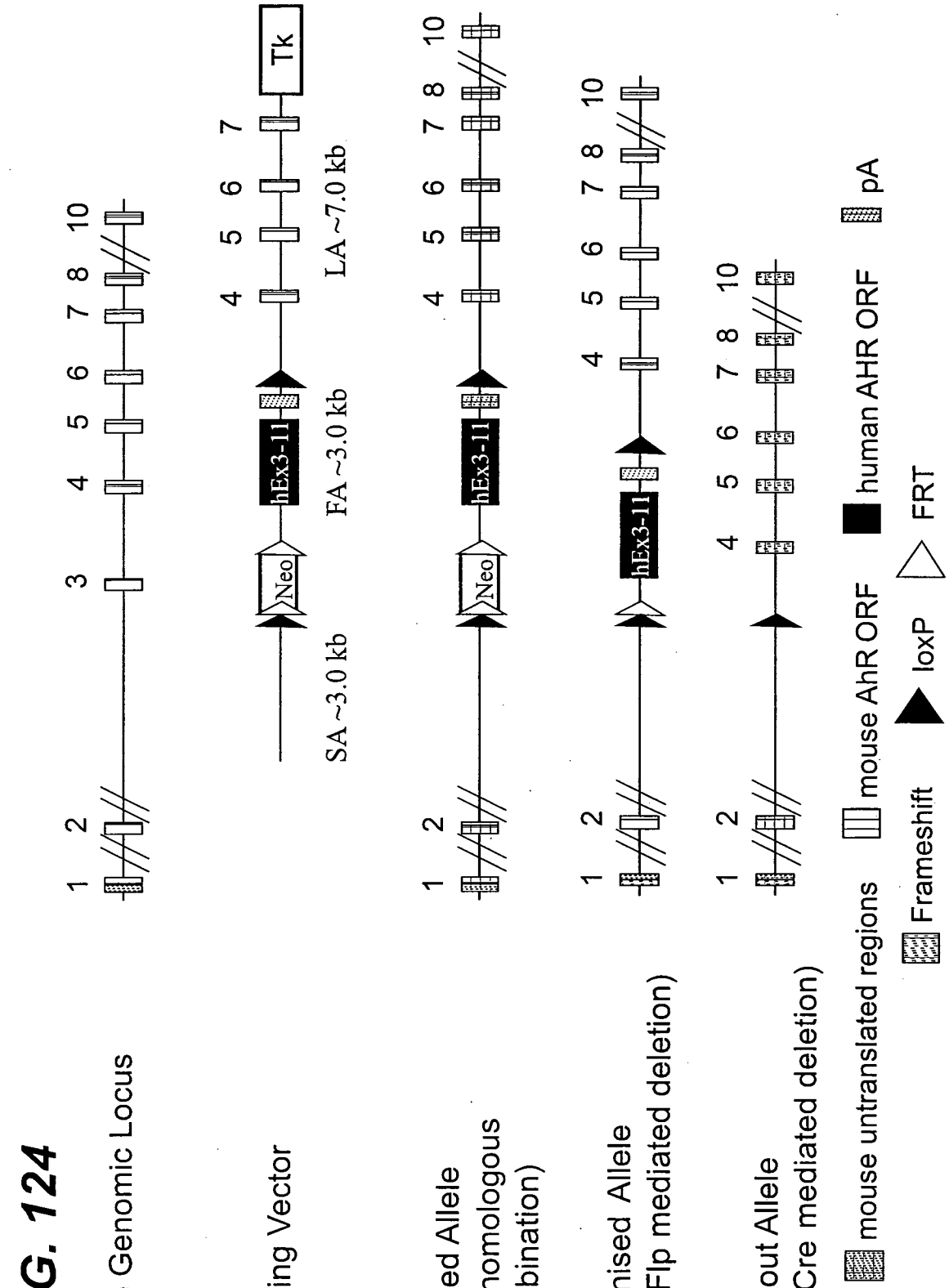

Mouse Genomic Locus
1 2 3 4 5 6 7 8 10
Targeting Vector
Neo
hEx3-11
Tk
SA ~3.0 kb
FA ~3.0 kb
LA ~7.0 kb
4 5 6 7
Targeted Allele
(after homologous recombination)
Neo
hEx3-11
4 5 6 7 8 10
Humanised Allele
(after Flp mediated deletion)
hEx3-11
4 5 6 7 8 10
Knockout Allele
(after Cre mediated deletion)
4 5 6 7 8 10
mouse untranslated regions
mouse AhR ORF
human AHR ORF
pA
Frameshift
loxP
FRT

FIG. 125

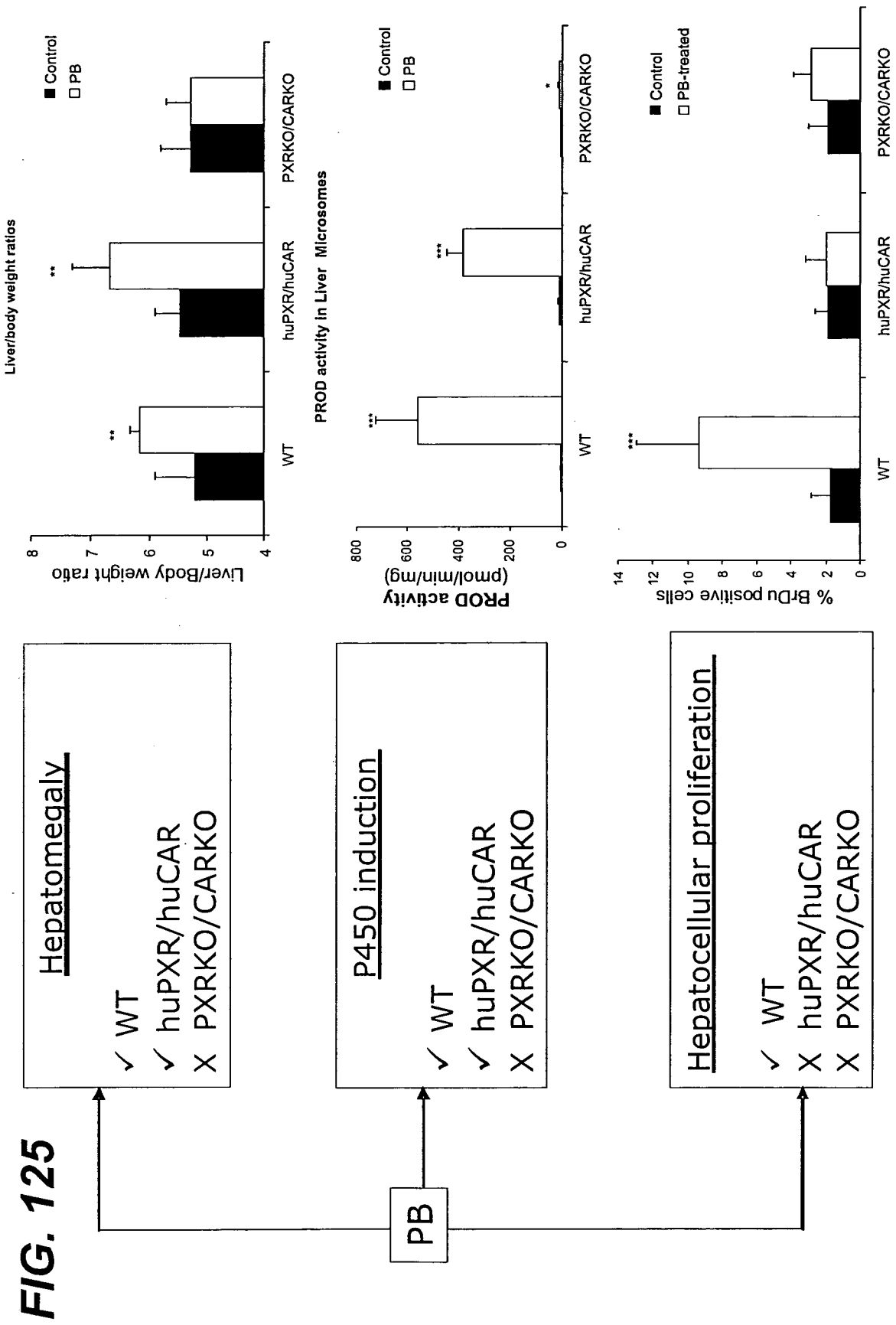

Hepatomegaly
✓ WT
✓ huPXR/huCAR
X PXRKO/CARKO
P450 induction
✓ WT
✓ huPXR/huCAR
X PXRKO/CARKO
Hepatocellular proliferation
✓ WT
X huPXR/huCAR
X PXRKO/CARKO
PB
Liver/body weight ratios
Liver/Body weight ratio
Control
PB
WT
huPXR/huCAR
PXRKO/CARKO
PROD activity in Liver Microsomes
PROD activity
(pmol/min/mg)
% BrDu positive cells
PB-treated

FIG. 126

Liver/body weight ratios

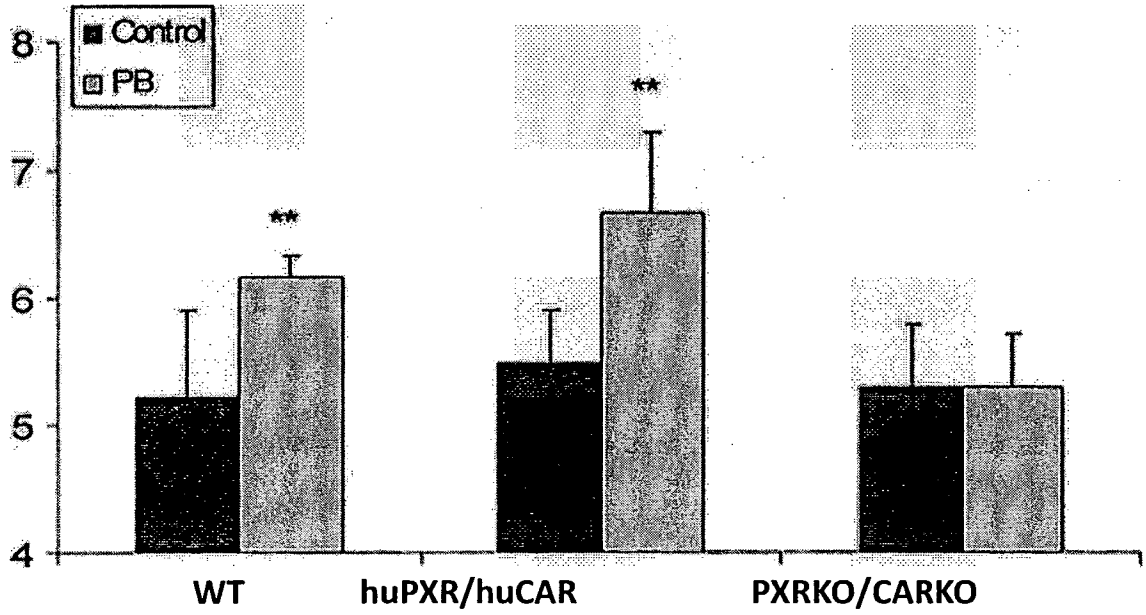


FIG. 127

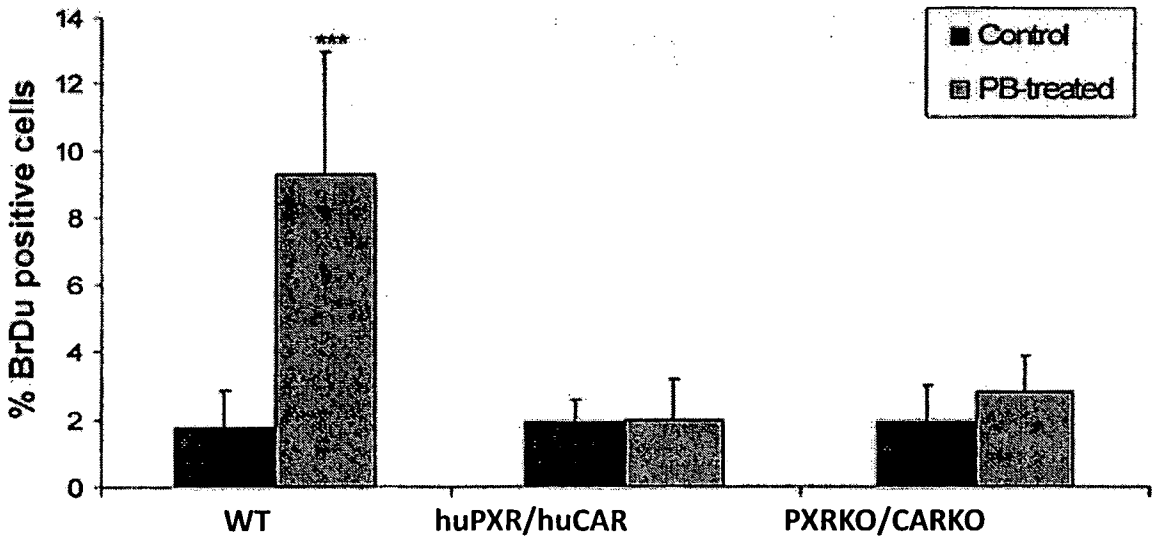


FIG. 128

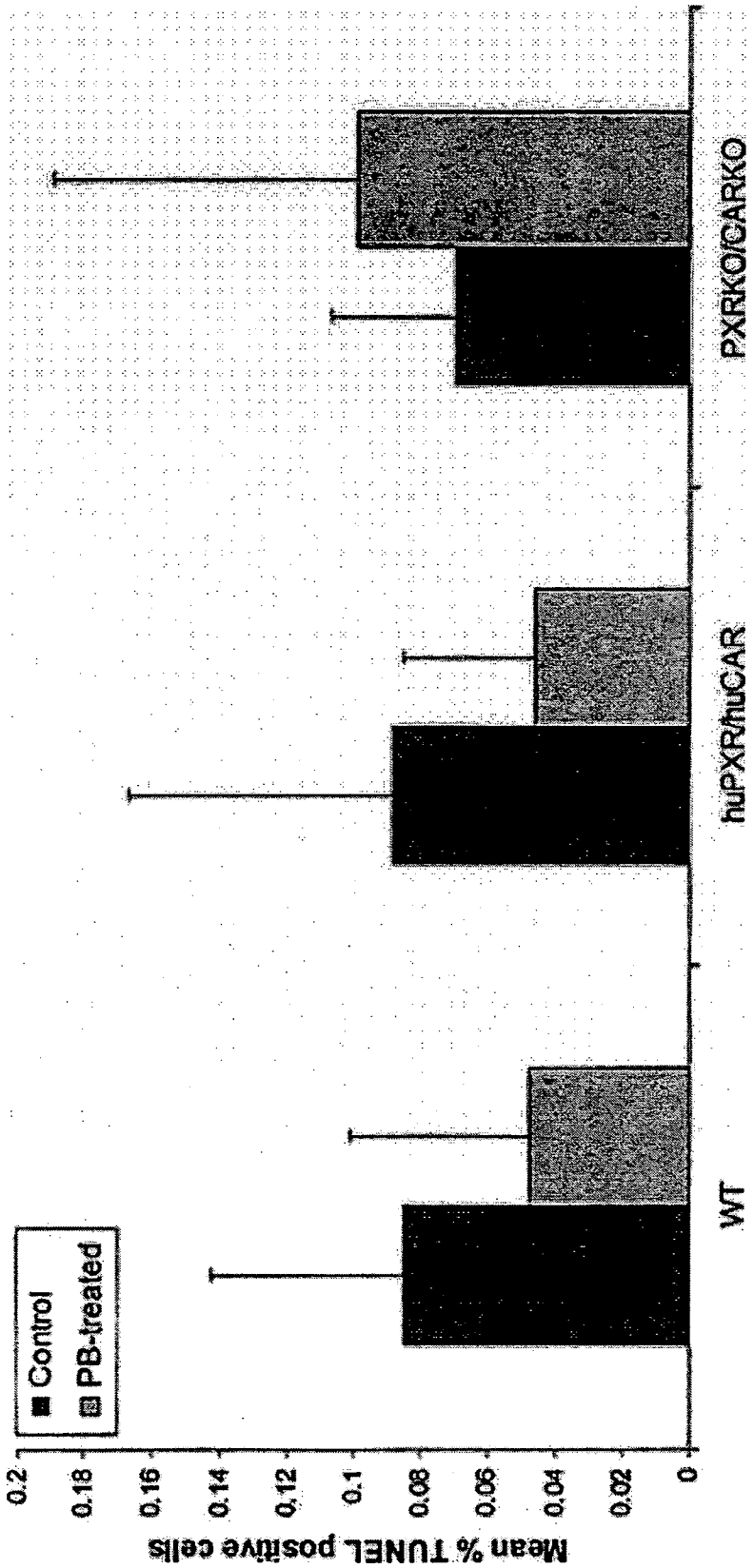

Mean % TUNEL positive cells
0.2
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
Control
PB-treated
WT
huPXR/huCAR
PXRKO/CARKO

FIG. 129

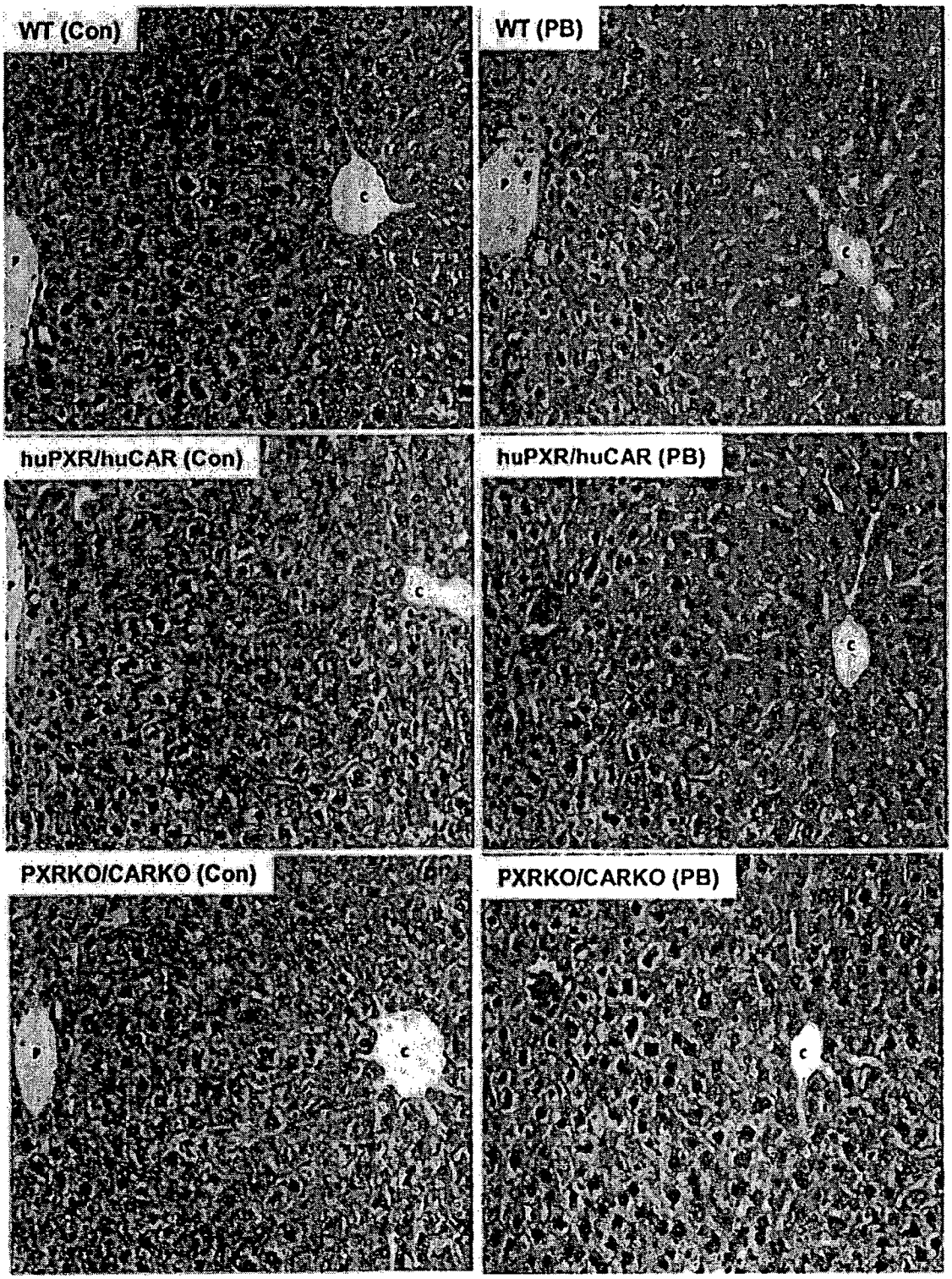

WT (Con)
WT (PB)
huPXR/huCAR (Con)
huPXR/huCAR (PB)
PXRKO/CARKO (Con)
PXRKO/CARKO (PB)

FIG. 130A

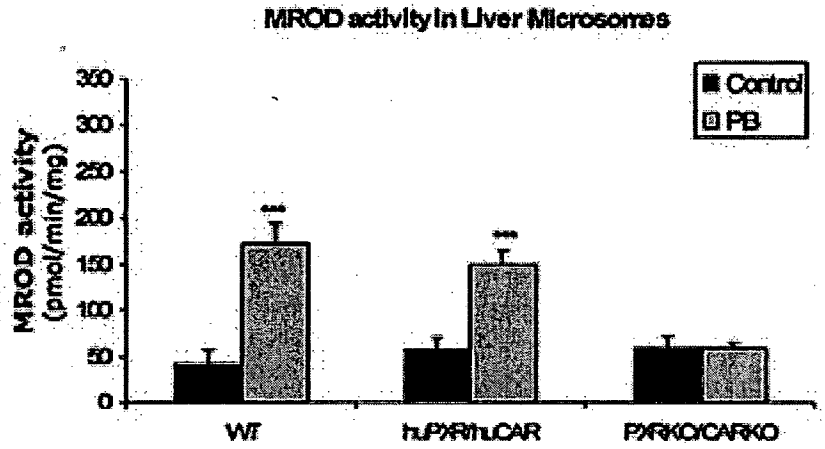


FIG. 130B

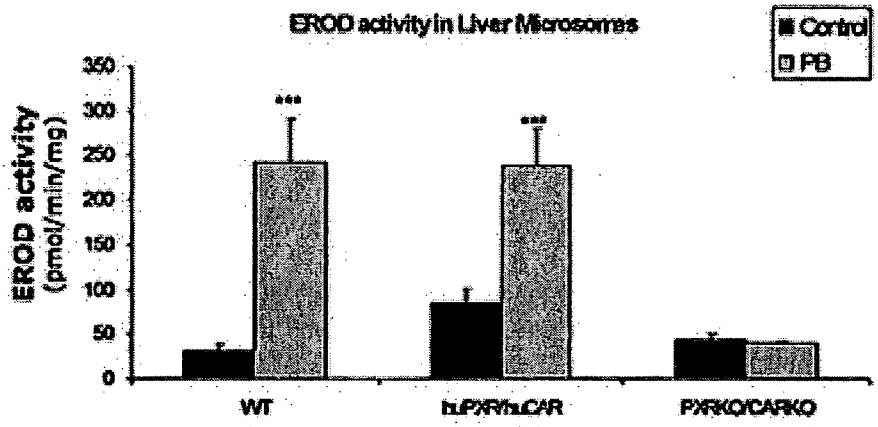


FIG. 130C

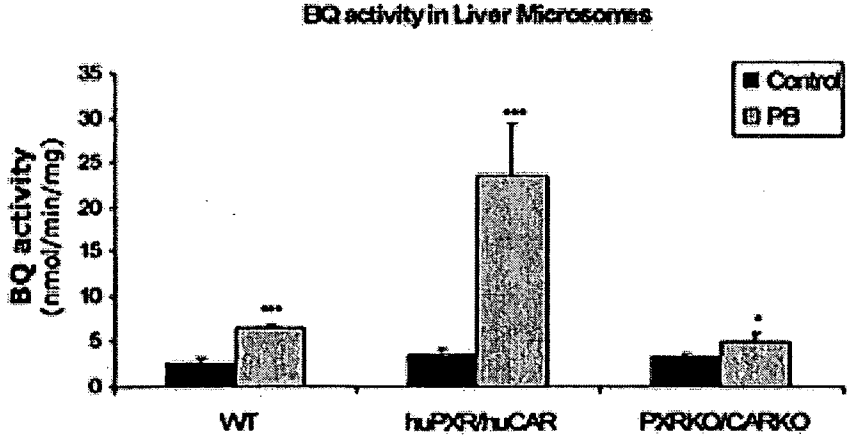


FIG. 130D

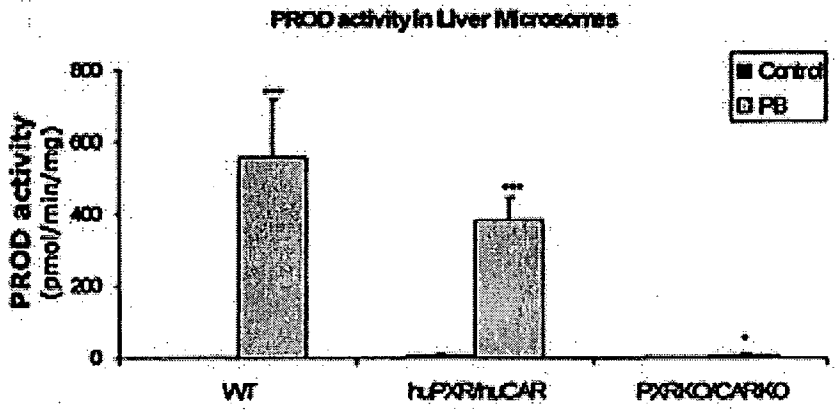
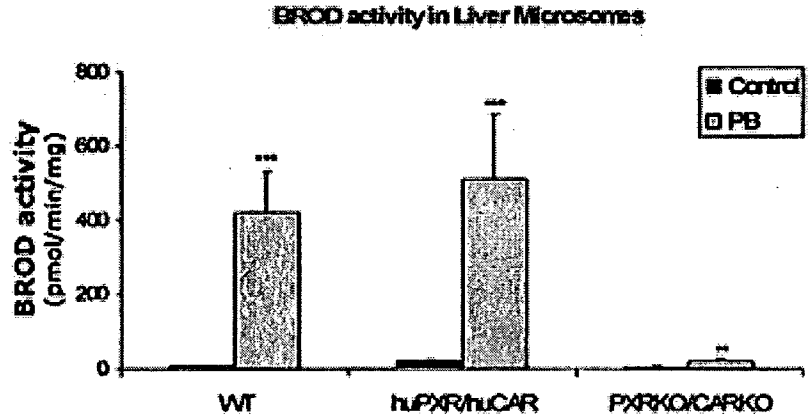


FIG. 130E



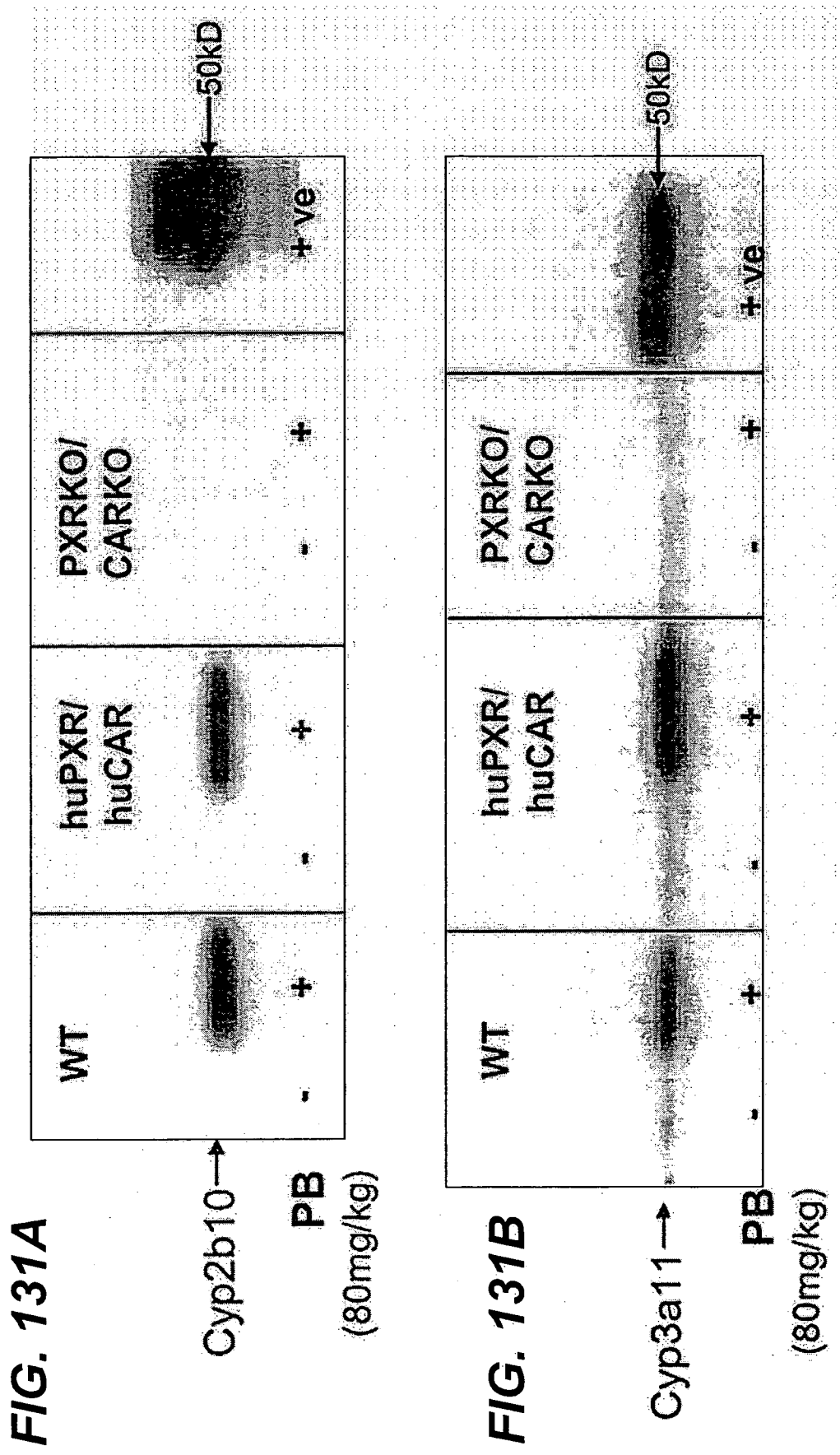


FIG. 131A

FIG. 131B

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2008/001897

A. CLASSIFICATION OF SUBJECT MATTER
INV. A01K67/027

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)
A01K

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

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2006/064197 A (ITI SCOTLAND LTD [GB]; WOLF CHARLES ROLAND [GB]; SCHEER NICO [DE]; FAU) 22 June 2006 (2006-06-22) the whole document	1

☐ 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 of particular relevance

"E" earlier document but 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 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	Date of mailing of the international search report
27 August 2008	03/09/2008

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Galli, Ivo

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB2008/001897

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

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

1. [X] Claims Nos.: 2-110
because they relate to subject matter not required to be searched by this Authority, namely:

--

2. [X] 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:

see FURTHER INFORMATION sheet PCT/ISA/210

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 No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

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

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

2. [ ] As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search reportcovers 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 and, where applicable, the payment of a protest fee.

[ ] The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box II.1

Claims Nos.: 2-110

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Continuation of Box II.2

Claims Nos.: -

The present application contains 110 claims, of which 43 are independent. There is no clear distinction between the independent claims because of overlapping scope. There are so many claims, and they are drafted in such a way that the claims as a whole are not in compliance with the provisions of clarity and conciseness of Article 6 PCT.

Moreover, the claims relate to an extremely large number of possible constructs (see in particular the nearly endless "shopping list" of claim 110). Support and disclosure in the sense of Article 6 and 5 PCT is to be found however for only a very small proportion therof.

In sum, the ISA finds that the claims as a whole have been drafted deliberately to make it burdensome for a skilled person to establish the subject-matter for which protection is sought. Aside from the fact that this is contrary to the provisions of Art. 6 PCT, the nefast consequence of this approach is that the claims also erect a smoke screen in front of the skilled reader when assessing what should be the subject-matter to search. The failure to comply with the substantive provisions of the PCT is so severe that a search on the basis of the claims is impossible.

A search for the closest prior art has been carried out based on the disclosure in the description and in respect of claim 1 -- in so far as possible under the circumstances. However, the results of the search are not to be considered complete or exhaustive.

The applicant's attention is drawn to the fact that claims relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure. If the application proceeds into the regional phase before the EPO, the applicant is reminded that a search may be carried out during examination before the EPO (see EPO Guideline C-VI, 8.2),

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

should the problems which led to the Article 17(2)PCT declaration be overcome.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/GB2008/001897

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2006064197 A	22-06-2006	AU 2005315438 A1	22-06-2006
		CA 2590010 A1	22-06-2006
		EP 1827087 A2	05-09-2007
		JP 2008522605 T	03-07-2008
		US 2008148416 A1	19-06-2008