Transgenic Animal Models in Toxicology: Historical Perspectives and Future Outlook


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Transgenic animal models are powerful tools for developing a more detailed understanding on the roles of specific genes in biological pathways and systems. Applications of these models have been made within the field of toxicology, most notably for the screening of mutagenic and carcinogenic potential and for the characterization of toxic mechanisms of action. It has long been a goal of research toxicologists to use the data from these models to refine hazard identification and characterization to better inform human health risk assessments. This review provides an overview on the applications of transgenic animal models in the assessment of mutagenicity and carcinogenicity, their use as reporter systems, and as tools for understanding the roles of xenobiotic-metabolizing enzymes and biological receptors in the etiology of chemical toxicity. Perspectives are also shared on the future outlook for these models in toxicology and risk assessment and how transgenic technologies are likely to be an integral tool for toxicity testing in the 21st century.

Key Words: transgenic models; knock-out models; humanized models; risk assessment.

In the last two decades, transgenic animal models have played a vital role in improving our understanding of gene regulation and function in biological systems and in human diseases. Perhaps one of the earliest and most impactful examples in biological research was when the regulatory region of the mouse gene for metallothionein-I was fused to the structural gene coding for human growth hormone (GH) and introduced into mice. The resultant transgenic mice exhibited altered growth characteristics and served as a valuable resource for analysis of gene inheritance and expression as well as the consequences of excess GH production on various physiological processes (Palmiter et al., 1983). This example is just one of many in the field that have highlighted the value of transgenic models in the study of biology. In the field of toxicology, transgenic animal models have primarily been used to screen for toxicity (mutagenicity/carcinogenicity) and to elucidate mechanisms of toxicity. Although transgenic animals have been widely accepted for the study of biological processes and diseases, their purposeful application in the assessment of chemical risk is less widely observed. It is hoped that with the recent drafting of an Organisation for Economic Co-operation and Development (OECD) guideline for transgenic rodent mutation assay (Lambert, 2008), the international adoption of transgenic mouse models in combination with the traditional 2-year cancer bioassay in rats (EC, 2006; Wells and Williams, 2009), and other advancements, there will be a more active use of transgenic animal data among risk assessors. An increased understanding and a consensus acceptance of biological mechanisms of toxicity and their relevance to humans should lead to increased use and application of data from transgenic models to human health risk assessments. This will be further fueled through the continued technological advancements in this area that will...
increase the ease of model generation, decrease the cost, and broaden biological pathway representation and model species availability. Furthermore, recent perspectives on the need to modernize our approaches to toxicity testing and risk assessment, through the application of new technologies to streamline and increase the human relevance of the process, are certain to leverage the powerful insights that can be provided by transgenic models.

For purposes of this review, a transgenic animal model is defined as a species in which specific DNA sequences have either been deleted or introduced. Transgenic animals can be constructed via retroviral infection of pre- or pro-implantation embryos (Wight and Wagner, 1994), DNA injection of pronuclear stage embryos, or microinjection of genetically modified embryonic stem cells (ESCs) into blastocysts (Capecchi, 1989; Hanahan, 1989; Hanson and Sedivy, 1995; Jaenisch, 1988). The first transgenic mice were generated through the microinjection of Simian virus 40 to explanted mouse blastocysts (Jaenisch and Mintz, 1974) and early embryonic exposure to retroviruses (Jaenisch, 1976). Subsequent to this, other techniques to generate transgenic animals have evolved.

A well-known technique to create transgenic animals is via pronuclear DNA microinjection of fertilized one-cell embryos with plasmids containing fused proteins. This method allows for foreign DNA to be introduced into the embryo after fertilization. A fine needle is used to microinject the DNA into a pronuclear stage embryo and integration occurs randomly as tandem arranged copies. The pronuclear stage embryos are transferred into the uterus of pseudopregnant recipient animals. Only if transgenic cells contribute to the germ line, animals will be transgenic in the next generation (Fig. 1A). Another method uses electroporation of DNA into pluripotent ESC derived from the inner cell mass of blastocysts. This approach has been particularly useful for mice because stable rat ESC have only recently become available (Kawamata and Ochiya, 2010). The development of knock-in, knockout, and conditional mutant transgenic mice is possible because of homologous recombination through gene targeting in ESC. The modified ESC are microinjected into blastocyst-stage embryos. The resulting chimeric animals will only transmit the recombinant genotype to the next generation if the ESC contributed to germ cells (Fig. 1B) (Capecchi, 1989; Hanson and Sedivy, 1995). For both methods, the stable integration and heritable germ line transmission of the genetic alteration can only be determined after multiple generations of breeding and selection of the most stable transgenic line.

**FIG. 1.** Classical methods to produce transgenic mice: (a) standard transgenic approach; (b) Gene-targeted transgenic approach. (Adapted from: (Manson and Tuzi, 2001.)
Initially, it was quite common to use DNA vectors for microinjection that simply contained the genomic sequence of interest coupled with the endogenous promoter. In addition, these systems can be set up as inducible models that include both monogenic and bitransgenic approaches. Monotransgenic approaches are those in which the transgene is controlled by a tissue-specific promoter (e.g., albumin) or an inducible promoter (e.g., metallothionein). Bitransgenic approaches offer better monitoring and selectivity of transgene expression. In this approach, the expression is controlled by another promoter that drives the expression of a regulator protein in a non-tissue-specific fashion which can then be activated (e.g., via ligand such as the tetracycline-regulated system) to regulate the expression of the transgene (Peters, 2006).

Conditional expression of the transgene expression is also possible through the Cre-lox system: the Cre recombinase and the lox site–containing target transgene (Feil et al., 2009; Grippo et al., 2002). This system allows for the genetic manipulation of specific cells in order to control gene expression, delete DNA sequences, or modify chromosomes. The Cre recombinase is a site-specific integrase isolated from the P1 bacteriophage that catalyzes the recombination of DNA between specific sites in DNA, the loxP sites. The loxP sites contain Cre-specific–binding sites (Sauer and Henderson, 1988). Typically, these models systems are created by starting with two separate strains. First a strain expressing the Cre recombinase is created that expresses the enzyme under the control of a ubiquitously expressed promoter (such as beta-actin) or a tissue-specific promoter (such as albumin), thereby allowing for tissue or cell-specific genetic manipulations. In some cases, the Cre recombinase models are developed as inducible systems such that the researcher can control the life stage at which the recombinase will start to be expressed. The loxP strain is then developed, wherein the loxP sites flank the target gene of interest. The two strains (Cre and loxP) are then crossed to produce a strain that expresses the Cre recombinase in the presence of the loxP sites that allows for recombination events to occur. The outcome of a Cre-lox recombination is determined by the orientation and location of flanking loxP sites. If the loxP sites are oriented in opposite directions, Cre recombinase mediates the inversion of the floxed segment (Fig. 2A). If the loxP sites are located on different chromosomes (trans arrangement), Cre recombinase mediates a chromosomal translocation (Fig. 2B). If the loxP sites are oriented in the same direction on a chromosome segment (cis arrangement), Cre recombinase mediates a deletion of the floxed segment (Fig. 2C).

Genetically modified rats have been more difficult to generate because stable rat ESC lines with high developmental potential are not readily available. Generally rat ESC have a low rate of egg survival after microinjection and transgene integration (Charreau et al., 1996; Heideman, 1991). An alternative approach is to generate transgenic rats through the male germ line. This has been investigated by using lentiviral transduction of spermatogonial stem cells followed by transplantation into recipient rat testes (Ryu et al., 2007). Recently, a specialized culture medium was developed, sustaining the efficient derivation and maintenance of germ line-competent ESC from rat blastocysts (Di Lorenzo et al., 2008). These rat ESC retain the capacity to differentiate into cells from all three germ layers and have the ability to produce chimerism at a high rate as well as germ line transmission (Li et al., 2008). The generation of Oct4-Venus transgenic rats where the fluorescence reporter Venus is used as monitoring system has resulted in the establishment of pluripotent cell lines with successful germ line transmission (Kawamata and Ochiya, 2010). This may be a suitable model for future gene targeting and transgenic manipulation in rats.

Transgenic models have found considerable utility in both basic and applied toxicology research and a survey of all relevant models is beyond the scope of the review. What follows is an overview of the major applications of transgenic

**FIG. 2.** The outcome of a Cre-lox recombination is determined by the orientation and location of flanking loxP sites. (A) If the loxP sites are oriented in opposite directions, Cre recombinase mediates the inversion of the floxed segment. (B) If the loxP sites are located on different chromosomes (trans arrangement), Cre recombinase mediates a chromosomal translocation. (C) If the loxP sites are oriented in the same direction on a chromosome segment (cis arrangement), Cre recombinase mediates a deletion of the floxed segment. (By J. Pendola in http://jaxmice.jax.org).
animal models in toxicology, with special emphasis on reviewing and highlighting advances in mutagenicity, cancer, reporter systems, metabolism/enzyme models, and nuclear receptors for the understanding of biological processes, disease, and the development of better methods for the identification and characterization of toxicants.

TRANSGENIC MUTATIONAL MODELS

Measuring gene mutation in vivo has long been a goal for genetic toxicology. A number of in vitro mutation assays have been developed that are used extensively for hazard identification, determining whether or not an agent has mutagenic potential. Mutations are important because of the potential effect they have on the phenotype of intact animals (e.g., cancer, diseases associated with germ cell mutations) and determining whether or not an agent is capable of inducing mutations in vivo requires using an animal model that can detect this end point in the tissues of interest. A number of in vivo mutation assays have been developed using endogenous reporter genes, for example, the Hprt and Pig-a assays (Aidoo et al., 1991; Dobrovolsky et al., 2010; Jones et al., 1985); however, they all measure mutation in only one or a few cell types; and with the possible exception of the Pig-a assay, are time consuming and challenging to perform.

The idea for using a transgene to measure in vivo mutation was first suggested by Malling et al. (1983). Transgenic in vivo mutation models use mice and rats in which every cell contains multiple copies of chromosomally integrated vectors carrying reporter genes for detecting mutation. Somatic and germ cell mutations arising in a rodent after treatment with a test article can be evaluated by recovering the vector as a bacteriophage or plasmid and analyzing the phenotype of the reporter gene in an appropriate bacterial host. These assays detect mutations induced in a transgene that is not expressed and hence genically neutral in the animal host. Thus, they can be used to quantitatively evaluate mutation in any tissue of the rodent from which microgram quantities of high–molecular weight DNA can be extracted. A further consequence of being neutral is that transgene mutations, unlike the end points measured in many other in vivo genetic toxicity assays, increase with repeated animal dosing (Heddle et al., 2003; Thybaud et al., 2003). This ability to accumulate mutations with repeated dosing is exploited in International Workshop on Genotoxicity Testing (IWGT) recommendations for conducting the assay (Thybaud et al., 2003). Although alterations to the basic protocol are permitted if scientifically justified, the basic experiment involves a 28-day repeat dose exposure of transgenic rodents to the test article, followed by tissue sampling for mutation after an additional 3 days. The OECD is in the beginning stages of developing a Test Guideline for the assay that relies heavily on the IWGT recommendations (OECD, 2010).

Mutational Models

MutaMouse. Goosen et al. were first to report the development of a transgenic mouse for mutation detection (Gossen et al., 1993). In their model (later referred to as MutaMouse), the lambda (λ) gt10 vector is the transgene, and the bacterial lacZ gene, encoding β-galactosidase, is the reporter of mutation. Mutation detection in this model is carried out by extracting high–molecular weight genomic DNA from the tissues of interest, packaging the λ shuttle vector in vitro into phage heads, and then testing for mutations following infection of an appropriate strain of Escherichia coli (E. coli C lacZ−).

MutaMouse, which is on a BALB/C × DBA2 murine background, carries 40 copies of the transgene in a head-to-tail manner at a single site on chromosome 3 (Blakey et al., 1995). Mice are commercially available from Covance Research Products (Denver, PA). The original assay involved scoring a small number of colorless mutant (LacZ−) plaques on a plate containing a large number of blue wild-type (WT, LacZ+) plaques, a very labor-intensive process. A simpler and faster system for the positive selection of mutant plaques was later developed using a new E. coli C (galElacZ) host and phenyl-β-d-galactopyranoside (P-Gal) medium (Vijg and Douglas, 1996). P-Gal medium is toxic to galE strains that express a functional lacZ gene; thus, only phages that contain a mutated lacZ will be able to form plaques. In this system, LacZ mutant frequency is calculated by dividing the number of plaques containing lacZ mutations on the selection plates by the total number of plaques estimated on nonselective titer plates.

Because the assay relies upon making infectious phage particles, it is generally limited to detecting point mutations and deletions and insertions of a few hundred base pairs within the 3100-bp LacZ gene. The multiple copies of the reporter gene per cell is somewhat counterbalanced by the low packaging efficiency of the recovery method (rate at which infectious phage are made from genomic DNA of the transgenic mouse).

LacZ plasmid mouse. The lacZ plasmid mouse contains approximately 20 copies of the pUR288 plasmid per haploid genome integrated into multiple chromosomes of the C57BL/6 mouse (Gossen et al., 1995; Vijg et al., 1997). Genomic DNA carrying pUR288 plasmid is digested with HindIII to release single copies of the linearized plasmid. Lac repressor (Gossen et al., 1993)–coated magnetic beads are used to isolate plasmid DNA from the digest, and the recovered DNA then is recircularized into individual plasmid molecules by T4 DNA ligase. These plasmids are electroporated into E. coli C (galElacZ−), and mutant frequency is determined using P-Gal–positive selection.

There are several advantages to the plasmid mouse system compared with the bacteriophage-based models, including (1) plasmids can be isolated from genomic DNA with higher efficiency and (2) deletions within the concatamer as well as extending from a lacZ target gene into 3′-flanking
chromosomal sequences can be recovered and characterized (Vijg et al., 1997). A disadvantage of the plasmid system is that a “star” activity associated with the HindIII restriction enzyme can contribute to the background mutant frequency (Dolle et al., 1999).

Big Blue Rodents. Kohler et al. developed the Big Blue mouse model using the λLIZα shuttle vector containing the 1080-bp bacterial lacI gene as the transgene and reporter gene (Kohler et al., 1990, 1991). The model is available commercially from Agilent (La Jolla, CA) on either a C57BL/6 (homozygous for the transgene) or B6C3F1 (heterozygous for the transgene) background. The transgene is present in approximately 40 copies per chromosome with integration occurring in a head-to-tail fashion at a single locus on chromosome 4 (Dycaico et al., 1994). Subsequently, a lacI transgenic rat was developed in a Fischer 344 background (Dycaico et al., 1994) containing 30 copies of the shuttle vector per diploid genome.

The original (Big Blue) assay involves extraction of high-molecular weight genomic DNA from the tissues of interest, in vitro packaging of the λLIZα into phage heads, and infection of E. coli SC5-8 cells. When the SC5-8 host is plated on 5-bromo-4-chloro-3-indoly1-β-d-galactopyranoside (X-Gal) medium, phage bearing WT lacI (encoding functional Lac repressor) will produce colorless plaques. However, mutations in lacI will produce a Lac repressor that is unable to bind to the lac operator; consequently, lacZ transcription will increase and β-galactosidase will cleave X-Gal, producing a blue plaque. The mutant frequency is the ratio of total number of blue plaques to total plaques (blue and colorless).

The blue mutant plaques in the Big Blue assay are much easier to distinguish from the colorless WT plaques than are the clear mutant on blue WT plaques generated in the original MutaMouse assay. However, the Big Blue assay has similar limitations as the MutaMouse assay in terms of the types of mutations recovered and in the relatively low packaging efficiency of the recovery system.

After the introduction of the Big Blue assay, a mutation detection system was described using another reporter of mutation that is part of the Big Blue λLIZα transgene, the 294-bp cII gene (Jakubczak et al., 1996). The cII gene encodes a repressor protein that controls the phage lysogenic/lytic cycle. The major advantage of the cII assay is that, unlike the lacI assay, it is a positive selection assay: phage with a WT cII gene cannot enter a lytic cycle in hfl E. coli hosts; therefore, only phages with a mutated cII gene form plaques. Besides the limitations noted above that are common to all bacteriophage-based transgene systems, the cII assay has a higher background mutant frequency than the lacI assay. This higher background does not appear to impact the assay’s sensitivity relative to the original Big Blue assay, however, and the cII assay has become more popular than the original because it is not as resource intensive and the cII reporter gene is somewhat easier to sequence than the lacI gene. Although initially described for

the Big Blue assay, cII selection also can be used in MutaMouse (λgt10lacZ) (but not gpt delta mouse or a rat, which also contains a λ transgene, see below).

Gpt delta rodents. As noted above, one of the limitations of the MutaMouse and Big Blue assays is their inability to detect large deletions. The gpt delta mouse was developed to detect both point mutations and large deletions (up to 10 kb) using the same transgene (Nohmi et al., 1996). The λEG10 phage vector in this system was designed for performing two different positive selection assays: the gpt assay using the E. coli gpt gene and the Spi− sensitive to P2 interference assay using the red/gam genes of λ. In the gpt assay, recovered bacteriophages (expressed in the indicator bacteria as a multi-copy plasmid) are cultured on plates containing the toxic purine analog 6-thioguanine; the assay detects mainly point mutations, such as base substitutions and frameshifts, in the 460 bp gpt gene. In Spi− selection, only mutant λ phages that are deficient in the functions of both the gam and redBA genes can grow well in P2 lysogens. The Spi− phenotype is most usually generated by a deletion that is sufficiently large to inactivate both genes.

The model was constructed in the C57BL/6J mouse, with about 80 copies of the transgene per diploid genome maintained in a head-to-tail fashion at a single site on chromosome 17 (Masumura et al., 1999). More recently, gpt delta rats were developed in both Sprague-Dawley and F344 backgrounds (Hayashi et al., 2003). Gpt delta rats have approximately 10 copies of the λEG10 vector integrated into chromosome 4.

Applications of Transgenic Mutation Models

The most common use of transgenic mutation assays has been for mechanistic studies, especially for exploring the role of mutation in biological responses to toxic agents. For instance, transgenic animals have been used to explore relationships among DNA adduct formation, gene mutation in target tissues, and cancer (Chen et al., 2006). Also, Big Blue, MutaMouse, and gpt delta mice have been used to establish mouse models deficient in DNA repair proteins, including p53, Atm, Parp-1, and Ogg1. These models have helped to elucidate the mechanisms suppressing genome instability mediated by endogenous and exogenous environmental stressors (Klungland et al., 1999; Minowa et al., 2000; Shibata et al., 2005; Wijnhoven and van Steeg, 2003; Yatagai et al., 2002).

Lambert et al. found that transgenic rodent mutation assays have very high sensitivity and positive predictivity for carcinogens (Lambert et al., 2005;Table 1), suggesting that the assays may be useful for inclusion in genotoxicity screening batteries. Note that the relatively low negative predictivity for all the assays listed in Table 1 is undoubtedly influenced by the low number of noncarcinogens that have been tested in transgenic rodent assays. It was recognized early in their history that transgenic mutation assays were unlikely to be adopted for this purpose, principally because of cost and their reliance on genetically modified animals but were more
likely to be used as follow-up tests to answer questions raised by the initial screening (Gorelick and Mirsalis, 1996). This may be particularly important when the in vivo site of contact should be evaluated or germ cell mutation is a particular concern. The draft International Conference on Harmonization (ICH, 2008). To our knowledge, transgenic rodent assays have had increasing roles in other aspects of risk assessment. The U.S. Environmental Protection Agency (EPA) test guidelines for genotoxicity safety testing of human pharmaceuticals is consistent with this assessment, on one hand indicating that pharmaceuticals are consistent with this assessment, on one hand citing practicality issues but on the other hand noting that toxicologists and risk assessors have for decades relied on genetic toxicity screening or follow-up, the hazard identification phase of risk assessment, data from transgenic rodent assays have had increasing roles in other aspects of risk assessment. The U.S. Environmental Protection Agency (EPA) has been developing guidelines for carcinogen risk assessment that rely heavily on identifying the key events in tumor formation, including mutation, in order to establish the mode of action (MOA) for the carcinogen (U.S.EPA, 2005, 2007). Agents with a mutagenic (or genotoxic) MOA are evaluated differently, and in many cases more stringently, than those that have nonmutagenic MOAs. In the process of establishing an MOA, in vivo data are given particular weight. Although guidelines for establishing a mutagenic MOA have not been finalized, draft versions emphasize the value of in vivo mutation data and data that can be tied to the tumor target (U.S.EPA, 2007). Transgenic rodent mutation data have been important in establishing a cancer MOA for several agents, for example, cyclophosphamide (McCarroll et al., 2008), acrylamide (U.S.EPA, 2010a), and hexavalent chromium (U.S.EPA, 2010b). A general framework for using transgenic rodent mutational data to establish cancer MOAs has also been proposed (Moore et al., 2008).

Another potentially important application of transgenic rodent models in risk assessment is for dose-response assessment and risk characterization. The contamination of an AIDS drug, Viracept, with the known mutagen and carcinogen, ethylmethane sulfonate (EMS), prompted an assessment to determine whether or not patients taking the contaminated drug were at risk for cancer (reviewed in Pozniak et al., 2009). Central to this analysis were data on the in vivo mutagenicity of EMS using MutaMouse. The analysis indicated a threshold for the mutagenicity of EMS at a concentration in excess of the highest human exposure. Based on these data, the European regulatory authorities concluded that the contaminated drug posed little risk to exposed humans.

Transgenic rodent assays have the unique ability to detect and characterize in vivo mutation in different tissues and organs using relatively easy procedures. Thus, they will undoubtedly continue to play an important role in mechanistic studies of mutation and carcinogenesis. Although the transgenic models have certain advantages, like choice of target tissue evaluation, flexible mode of administration, and the prospect of an OECD Test Guideline, these models do have disadvantages, including the resources required to perform the assay, their reliance on unique (transgenic) animals, limitations in the types of mutations they can detect, high background mutant frequency in most somatic tissues, and the necessity for repeat-dose protocols to assure sensitivity. The plasmid-based lacZ and gpt delta mutation models provide additional capability to detect large deletions; however, as stated above, these assays will probably have little utility for primary safety assessments. Where transgenic rodent assays may be uniquely valuable for risk assessment is in cancer MOA analysis, where the induction of gene mutations in a cancer target tissue may be important for establishing a mutagenic MOA, and in dose-response analysis for mutagenic carcinogens. With regard to the later, the Viracept contamination case, in particular, may point the way for how the risks associated with drug contamination, and possibly drug impurities, can be addressed in the future.

### Table 1

<table>
<thead>
<tr>
<th>Short-term in vivo assays →</th>
<th>In vivo CA</th>
<th>In vivo MN</th>
<th>In vivo UDS</th>
<th>In vivo TGR</th>
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<tr>
<td>No. of chemicals tested</td>
<td>39</td>
<td>69</td>
<td>37</td>
<td>105</td>
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<tr>
<td>No. of carcinogens</td>
<td>38</td>
<td>67</td>
<td>32</td>
<td>92</td>
</tr>
<tr>
<td>No. of noncarcinogens</td>
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<td>2</td>
<td>5</td>
<td>13</td>
</tr>
<tr>
<td>Total no. of chemicals tested positive</td>
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<td>44</td>
<td>24</td>
<td>76</td>
</tr>
<tr>
<td>No. of carcinogens tested positive</td>
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<td>43</td>
<td>22</td>
<td>72</td>
</tr>
<tr>
<td>No. of carcinogens tested negative</td>
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<td>23</td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td>No. of noncarcinogens tested negative</td>
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<td>1</td>
<td>3</td>
<td>9</td>
</tr>
<tr>
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<tr>
<td>Negative predictivity (%)</td>
<td>8</td>
<td>4</td>
<td>23</td>
<td>31</td>
</tr>
</tbody>
</table>

*Note.* CA, chromosomal aberration; MN, micronucleus assay; UDS, unscheduled DNA synthesis; sensitivity (%), percentage of carcinogens tested positive in the short-term assays; specificity (%), percentage of noncarcinogens tested negative in the short-term assays; positive predictivity (%), percentage of chemicals that were positive in the short-term assays that were carcinogenic; negative predictivity (%), percentage of chemicals that were negative in the short-term assay that were not carcinogenic or probability that a negative short-term assay was correct.
bioassays for predicting human risk of exposure to chemical and physical agents. However, these assays are less than perfect. First, these assays take a long time to complete (up to three years) and use a lot of animals (up to 70/sex/dose level). Second, they are very resource intensive, costing up to 1 million U.S. dollars per species per chemical. Third, although the sensitivity of these assays in predicting human carcinogens is good, their specificity is generally considered to be very poor leading to many false-positive responses with little relevance to human risk assessment. Last, but not least, these assays generally do not provide information on the potential MOA leading to tumorigenesis which in turn leads to extensive/expense follow-up mechanistic studies.

Ideally, bioassays for identifying carcinogens should be capable of identifying all human carcinogens (100% sensitivity) and yield no irrelevant or false-positive findings (100% specificity). In addition, it is also desirable that there is concordance between the tumor sites observed in animals in these bioassays with those expected in humans. These assays should provide insight into the MOA and key events leading to tumor formation so as to enable dose-response characterization and human relevance analysis. Finally, given that in the real world, resources are limited and public health decisions have to be made in a timely manner, these tests should ideally be of low cost with a rapid turn around time.

For the reasons outlined above, this section will focus only on the use of transgenic mice in cancer assessment. Activated oncogenes were introduced into mice in the early 1980s. These animals were found to develop spontaneous tumors more rapidly than WT mice and appeared more susceptible to chemically induced tumors (Leder et al., 1990). These models would have remained merely interesting research tools if not for a change in the ICH S1B Guideline for Testing for Carcinogenicity of Pharmaceuticals that paved the way for short or medium-term in vivo rodent test systems including transgenic models (ICH, 1997). This guidance states that “Possibilities should focus on the use of in vivo models providing insight into carcinogenic endpoints. These may include models of initiation-promotion in rodents, or models of carcinogenesis using transgenic or neonatal rodents.”

A hallmark of cancer cells is the presence of multiple somatic mutations in genes such as in the tumor suppressor p53 (Hussain and Harris, 2000; Hussain et al., 2000), the proto-oncogenes ras (Lowy and Willumsen, 1993; Lowy et al., 1993), c-myc (Borresen, 1992; Riou, 1988), or in DNA repair genes. The observation that several human cancers have a high frequency of mutated genes has led to the development of transgenic or knockout mice to complement the traditional 2-year cancer bioassay.

The Tg. AC mouse strain, which is on an FVB background, has 40 tandem copies and at least one invert repeat of the v-Ha-ras oncogene with activating mutations in codons 12 and 59 (Leder et al., 1990). The transgene is not normally expressed in adult tissues (Hansen and Tennant, 1994). The skin of these animals behaves as if it is “genetically initiated” (i.e., exposure to promoters results in development of papillomas without the need for prior initiation with a mutagenic chemical). Fundamentally, this transgenic model is an alternative to the traditional initiation-promotion assay for dermally applied materials.

The hemizygous p53+/− knockout model was developed by Donehower et al. on a C57Bl/6 background (Donehower et al., 1992). These mice have a low spontaneous tumor incidence during the first 36 weeks of life. After 80 weeks, however, a large percentage of the animals (approximately 50%) develop lymphomas, osteosarcomas, and hemangiosarcomas. Earlier work has indicated that these mice develop tumors in approximately 6 months or less following exposure to genotoxic test materials but not to nongenotoxic carcinogens (Gulezian et al., 2000; Spalding et al., 2000; Tennant et al., 1996). It was initially assumed that the loss of activity of the functional p53 locus was a prerequisite for tumor development, and because of this, genotoxic agents such as benzene would induce a tumorigenic response in this test system (Boley et al., 2000). However, subsequent studies demonstrated this not to be the case with the mutagen p-cresidine (Venkatachalam et al., 2001).

The TgrasH2 strain containing a human c-Ha-ras gene with its own promoter was created by Saitoh et al. on a C57Bl/6 background (Saitoh et al., 1990). Mice used for the short-term carcinogenicity assay are obtained by crossing the parental strain with WT BALB/c to generate hemizygous CB6F1. The c-Ha-ras transgene, which is expressed in normal and tumor tissues, has a mutation in its intrinsic sequence but no mutations in its coding sequence. These mice have low background tumor incidence until the age of 8 months; however, up to 50% of the animals develop tumors (hemangiosarcomas, lung adenocarcinomas, skin papillomas, Harderian gland adenocarcinomas, and lymphomas) by 18 months of age.

The Xpa−/− knockout mouse is deficient in nucleotide excision repair. The combination of this genotype with p53+/− resulted in the Xpaip53+/−/− model, offering the advantages of the two models in one strain (van Kreijl et al., 2001; van Steeg et al., 2001). The Xpa knockout strain, maintained on C57Bl/6 background, is a murine homologue of the human genetic disease xeroderma pigmentosum, and accordingly, is highly susceptible to UV-induced skin tumors. This strain has a low-background tumor incidence similar to the WT during the first 9 months of age.

In 1997, an international multi-stakeholder (industry, academia, and the government) collaborative effort under the auspices of the International Life Sciences Institute’s Health and Environmental Sciences Institute was undertaken to validate the utility of the four alternate carcinogenicity models, Tg-AC, p53+/−, Tg-rasH2, and the Xpa−/− models (Robinson and MacDonald, 2001). More than 50 laboratories from the United States, Europe, and Japan participated in this effort at a cost of approximately U.S. $35 million. A total of 21 substances belonging to various carcinogen categories (based...
on conventional 2-year bioassays) were evaluated in one or more of the above models using standard protocols and sacrificing the animals at the end of 6 to 12 months. Overall, these assays proved to be useful as screening assays for the identification potential human carcinogens. In addition to using fewer animals and being quicker and less expensive than conventional 2-year bioassays, these assays did not seem to be overly sensitive in terms of identifying false positives nor did they have 100% specificity for identifying human carcinogens (Cohen, 2001). These assays were also unable to distinguish between genotoxic and nongenotoxic carcinogens.

**Regulatory Experience with Transgenic/Knockout Mouse Cancer Models**

As of January 2011, the United States Food and Drug Administration-Center for Drug Evaluation and Research (U.S. FDA-CDER) has received 211 protocols for review in alternative carcinogenicity models including the p53+/−, Tg-AC, TgRasH2, and XPA/P53+/− models (Table 2). Of these protocols, only 73 completed studies were submitted for agency review.

Considerations for assay selection are described below. The FDA-CDER’s Executive Carcinogen Assessment Committee gives concurrence for assay selection for a particular drug product before the study commences. Over the past few years, the number of TgRasH2 studies has increased, and the number of p53+/− and Tg-AC studies has decreased.

- **p53+/−**: Previously, this assay was acceptable for assessing carcinogenic potential if the drug was clearly or equivocally genotoxic. Now this assay is generally only acceptable only if the drug is clearly genotoxic.
- **TgRasH2**: This assay may be acceptable for assessing carcinogenic potential of both genotoxic and nongenotoxic drugs.
- **Tg-AC**: This assay is only for dermally applied products and is not currently recommended.

The incidence of positive studies using these models was as follows:

- **p53+/−**: 2/32 studies were positive for carcinogenicity. Phenolphthalein by oral administration and a vehicle used for the administration of povidone by subcutaneous route (and not povidone itself) gave positive results; there were no data with WT parental animals for the povidone vehicle. Another two drugs were positive but were also positive in WT animals, so the effect is not related to p53.
  - TgRasH2: 3/20 studies were positive for carcinogenicity. One negative study was inadequate because it did not have coverage of a major genotoxic metabolite. In one study, there was incomplete evaluation of low-dose groups although the high dose exceeded the MTD. One study had positive results in the 4-week dose-range–finding study.
  - Tg-AC: 8/16 studies were positive. One study was negative only at the application site. One study was not interpretable because of study conduct, but the drug may have been positive.
  - XPA/p53+/−: The one study conducted was inadequate, and it was noted that the drug was not genotoxic.

Clearly, some sponsors have adopted routine use of alternative, shorter term assays. However, transgenic studies have remained at approximately 25% of the mouse protocols reviewed by CDER’s Executive Carcinogen Assessment Committee in the last 8 years. Although transgenic studies offer some advantages in terms of time and resources, most drug sponsors still prefer 2-year bioassays.

Progress is being made with regards to more formalized adoption of these approaches in new regulations. The ICH Expert Working Group on Safety has adopted the use of transgenic mouse models in combination with the traditional 2-year cancer bioassay in rats for the evaluation of the carcinogenic potential of chemicals (ICH, 2007). Under the European REACH policy, these models are recommended as good alternatives to the 2-year bioassay (EC, 2006; Wells and Williams, 2009). Therefore, on paper, both genotoxicity and carcinogenicity tests with transgenic animals are accepted by regulatory bodies. Unfortunately, as illustrated above, these assays are still not commonly used.

The value of these models can also be observed with the development of transgenic animals mimicking human disorders with a high incidence of cancer that have been essential for the identification of important genes and pathways that result in genomic instability and early onset of carcinogenesis. For instance, hereditary nonpolyposis colon cancer (HNPPC) results from a heritable mutation in the HNPPC gene or in mismatch repair genes MutL Homologue 1 (MLH1), MutS Homologue (MSH) 2, and MSH6 (Abdel-Rahman et al., 2006; Watson and Lynch, 2001). Familial adenomatous polyposis arises from a heritable mutation in the tumor suppressor adenomatous polyposis coli or from a mutation in the DNA repair enzyme MUTYH (Calvert and Frucht, 2002; Vogelstein and Kinzler, 1993). Xeroderma pigmentosum arises from a defect in nucleotide excision repair in any of the 7 XP gene complementary groups, XPA to XPG (van Kreijl et al., 2001; van Steeg et al., 2001), or from a deficiency in DNA

### TABLE 2

Summary of Transgenic Cancer Models That Have Been Applied in Drug Development Based on Protocols and Reports Received by CDER/FDA (as of January 2011)

<table>
<thead>
<tr>
<th>Alternative Model</th>
<th>No. of Protocols Received</th>
<th>No. of Reports Received</th>
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<tbody>
<tr>
<td>p53+/−</td>
<td>81</td>
<td>32</td>
</tr>
<tr>
<td>TgRasH2</td>
<td>74</td>
<td>20</td>
</tr>
<tr>
<td>Tg-AC</td>
<td>44</td>
<td>18</td>
</tr>
<tr>
<td>XPA/P53+/−</td>
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polymerase eta (polh). The latter is a variant form of XPV, which is responsible for translesion synthesis at a relatively high accuracy (Biertumpfel et al., 2010; Cruet-Hennequart et al., 2010). Ataxia Telangiectasia develops from a mutation in the AT-mutated (ATM) gene encoding a Ser/Thr protein kinase crucial in G1-S and G2-M cell cycle checkpoints (Hurley and Bunz, 2007; Lavin, 2008; Slijepcevic, 2006). Other cancer transgenic models have been developed, such as the oncomouse that carries an activated v-Ha-ras oncogene under the control of the mouse mammary tumor virus promoter (Sinn et al., 1987) and a mouse breast cancer model in which a targeted human p53 mutation is conditionally expressed in the mammary gland (Wijnhoven et al., 2005). It is beyond any doubt that these transgenic models have been fundamental to the understanding of the mechanisms by which tumors arise in different organs and to the elucidation of the role that DNA repair genes, oncogenes, tumor suppressor genes, and progression factors play in carcinogenesis.

TRANSGENIC REPORTER MICE

Among the new transgenic animal models and tools available, reporter mice hold the promise to have a significant impact on the future design of experimental procedures as they provide the possibility to study the effects of a single or repeated exposure to a given compound or mixture directly on their targets in the context of an entire organism. In addition, the applicability of molecular imaging to these models enables the study of toxic effects in living animals, extending the assessment to the time dimension with the use of a small amount of animals. The definition of reporter mouse refers to an animal genetically engineered to express a protein easily detectable and measurable (such as luciferase, green fluorescent protein, thymidine kinase) in response to a specific stimulus. The use of bioluminescent reporters facilitates their detection by noninvasive imaging technologies that spare the life of the animals and can be repeated in time thus providing a complete spatiotemporal view of the effect of the toxic agent.

To obtain a reporter model system suitable for toxicological analysis, care must be taken: 1) to ensure that the expression of the reporter gene is ubiquitous and is regulated in all tissues, 2) the half-life of the reporter protein is short in order to facilitate the analysis of the exact kinetics of the toxicant interactions within any given tissue, and ideally, 3) the sensitivity of the reporter system must be set on physiological stimuli to be sufficient to measure amounts of toxic compounds that interfere with the physiology of the animal. A pioneering example of a reporter model is the estrogen-responsive element (ERE)-Luc, a transgenic mouse where the firefly luciferase reporter gene is driven by an ERE promoter. In this model, the generalized, hormone-regulated expression of the luciferase reporter was obtained by flanking the transgene with insulator sequences preventing position effects from the chromatin surrounding the transgene integration site (Ciana et al., 2003). The extensive and appropriate validation of the model has shown that the synthesis of luciferase mRNA and protein is strictly associated with liganded as well as unliganded estrogen receptor (ER) activity and is specific for ER as indicated by pharmacological and dose-dependent studies. The sensitivity of the reporter system is sufficient to detect subtle changes of circulating estrogen during the estrous cycle (Ciana et al., 2003; Maggi et al., 2004). Because of these characteristics, the ERE-Luc mouse was proposed as a novel system for the study of the effects of endocrine disrupters, and a series of studies were undertaken within the European Programmes CASCADE (http://www.cascadenet.org.html) and EDERA (http://www.edera-project.eu/) with the specific aim to generate protocols for toxicological application of the model. In the ERE-Luc mouse, luciferase measurements can be done in vivo counting the amount of photons generated by the substrate (luciferine) oxidation by bioluminescence imaging (BLI) using an appropriate charge-coupled device (CCD) camera apparatus or ex vivo by the appropriate enzymatic assay carried out in tissue extracts. BLI provides the unique opportunity to evaluate, in a single animal, the state of ER activity in time. Figure 3 (upper panel) shows that after a single administration of the natural hormone, 17β-estradiol (E2), luciferase content was increased with a maximum accumulation at 6 h, but by 17 h after the treatment, the effect of the hormone vanished, as expected, because of its rapid catabolism. The semi-quantitative analysis of ER activity in specific body areas was obtained by the analysis of photon emission in specific areas of the whole-body image (Fig. 3, lower panel). Using the ERE-Luc model, researchers were able to demonstrate the estrogenicity of compounds that were proposed as potential endocrine disrupters on the basis of studies carried out in cell-based reporter systems such as cadmium (Cd). Figure 4 shows the effects of long-term administration of CdCl2 to ERE-Luc mice. The treatment increased ER activity transiently in only one body area, the abdomen, and in none of the other body areas could we observe an effect of Cd treatment. In addition, when one compared the extent of ER activation by Cd and the natural hormone, estradiol, it is clearly demonstrated that the ability of CdCl2 to activate ER was far below the activity of the endogenous hormone thus demonstrating that it is unlikely that the toxic effects reported for Cd are ascribed to the effects of this metal on the ER.

Using this type of analysis, the activity of different compounds can be easily measured and compared with a significant advantage over other methods as in vivo imaging gives the possibility to assess the ability of a given estrogenic compound to modulate ER activity in time. However, from a practical standpoint, a typical long-term toxicological experiment carried out on experimental groups of 5–10 mice undergoing BLI on a daily basis is experimentally very challenging because of the number of data generated and the time necessary for the manual
definition of the region of interest (ROI). For instance, in a 21-day study, the analysis of ER activity in 10 experimental groups would (1) require one scientist working full time for an year, (2) generate 2100 single pictures, and (3) generate 16,800 data points on photon emission from the ROI. To speed up data analysis, specific algorithms may be designed (Rando et al., 2009a) for automatic segmentation and data storage.

In the past years, the ERE-Luc reporter system has been successfully applied to the study of the effects of well-known endocrine disrupters as indicated by several studies (Bondesson et al., 2009; Di Lorenzo et al., 2002, 2008; Montani et al., 2008; Penza et al., 2004; Rando et al., 2009a,b, 2010a,b), and high-throughput protocols were set up to provide a framework for the application of these novel model systems to toxicological studies of environmental and alimentary endocrine disrupters. Thus, the model may now be proposed for use in prevalidation studies for routine toxicological analysis encompassing the study of endocrine disrupters in the environment and in the food chain and to evaluate the efficacy and undesired effects of drugs for hormone replacement therapy.

The ERE-Luc model system has clearly demonstrated the tremendous potential of the application of reporter animals in toxicology which may be briefly summarized by the following points:

1. The action of the toxic compound is measured directly on its molecular target and is not inferred from measurements of its plasma or tissue content.
2. The measurement of reporter accumulation provides a complete view of the organs where the compound is active and the extent of its activity thus facilitating a comprehensive assessment.
3. In vivo imaging facilitates the study of the effects of exposure in time thus allowing the ability to measure cumulative effects because of the exposure to a mixture of toxic compounds or to prolonged exposure to low doses.
the use of live animals allows the researcher to closely mimic the extent of exposure to toxic compounds present in the environment or in the food chain, and the use of live molecular imaging reduces the number of animals to be used for temporal toxicological studies.

Having established that these models are applicable to systematic studies, it is felt that the advantages of the use of reporter mice could change the way researchers design whole-animal toxicological examinations, and the field should therefore focus future efforts in this area toward developing the types of reporter mice that are needed to advance toxicological research. The question to be asked is whether the ERE-Luc model system can be adapted to toxicological analyses other than those addressing the study of compounds active through intracellular receptors. From a technical point of view, reporter systems can be designed for any molecular event occurring within living organisms. However, before conceiving novel reporter models, researchers should better define the strategies to be applied for the use of reporter systems in the toxicological analysis.

From a practical perspective, the models most needed are those reporting on more general effects of toxic compounds such as proliferation, apoptosis, oxidative stress, and inflammation. These reporter mice would be extremely useful as a first screening test aimed at identifying unexpected events caused by the compound on the known as well as unknown target of action. This initial screen would highlight potential side effects of the chemical entity (e.g., in drug toxicology) and major effects of the toxicant under study (e.g., in alimentary or environmental toxicology). Once the compounds with the desired/undesired effects have been identified, a second level of screening would follow using more specific reporter systems where the effect of the toxic or therapeutic agent could be analyzed on its specific target to evaluate its potency and efficacy in space and time. In this case, the study would reveal the agonist/antagonist effects of the compound in study, its ability to interfere with the physiological system, and its potential for a desensitization of the target in repeated exposures and for the activation of signaling pathways outside of the organ of interest. The availability of a variety of novel

FIG. 4. Effects on luciferase activity of long-term exposure to cadmium or 17β-estradiol; 2-month-old ovariectomized female mice were treated daily by gavage with E2 (5 μg/kg/day or CdCl2 1 mg/kg/day) for 21 days and luciferase activity was measured weekly using the segmentation method. The activity of the natural hormone E2 is clearly visible in all organs, whereas CdCl2, at a daily dosage considered to be toxic, was unable to induce a significant or persistent activation of ER, thus demonstrating in an in vivo model that at this dosage the metal does not display the alleged estrogenic action.
reporter systems based on fluorescence and the constant improvement of the instrumentation for the measurement of bioluminescence will enable the generation of novel model systems where an appropriate combination of reporters could allow for the ability to measure at the same time, in the same animal, more general effects of a given toxic compound together with the potency and efficacy of its action on a given target.

Another major challenge will be provided by the study of the effects of toxic compounds in time and on a given organ. Experience with Selective Estrogen Receptor Modulators (SERMs) has demonstrated the immense variability of the in vivo action of estrogenic molecules: the long-term treatments performed induced a state of activation of ER which was dependent on the tissue evaluated, the dosage utilized, and the time of treatment, challenging the ability to establish the parameters necessary to evaluate the extent of beneficial/negative effects associated with each estrogenic compound. Novel algorithms to fully take into account the spatiotemporal effects of each chemical need to be generated. An example showing the feasibility of this approach is provided by an extensive study of SERMs recently published by Rando et al., where a systematic study of spatiotemporal effects was proposed as a measure of drug efficacy and the classification of pharmacologically active compounds and a mathematical model able to describe drug action on the ER led to the generation of families of compounds based on the ability of the drug to substitute for the natural hormone (Rando et al., 2010b).

Finally, the real limitation to the generation of novel reporter mice is the difficulties in creating a model where the reporter is completely deprived of any influence from the host genome. Because of that all models generated need an extensive validation to verify their applicability to pharmacotoxicological studies. Thus, more research in the field should be encouraged and aimed at simplifying the creation of these useful model systems.

**XENOBIOTIC METABOLISM MODELS**

*Xenobiotic Metabolizing Enzyme-Null Mice*

The cellular constitution of xenobiotic enzymes is vital for determining whether a chemical will be metabolized to induce a toxic response. In order to determine the role of metabolizing enzymes in mammalian development, physiological homeostasis, and xenobiotic metabolism, several mouse lines lacking xenobiotic-metabolizing enzymes, such as the P450s Cyp1a1, Cyp1a2, Cyp2e1, microsomal epoxide hydrolase, glutathione-S-transferase, or NADPH-quinone oxidoreductase, have been developed (Table 3). In general, these models are viable and show no developmental abnormalities which generally suggests a redundancy in xenobiotic-metabolizing enzymes and that their roles are primarily involved in metabolism of foreign compounds. However, the Cyp4a10 and Cyp4a14 P450s metabolize fatty acids and have a role in control of blood

<table>
<thead>
<tr>
<th>Table 3: A Listing of Xenobiotic Metabolism and Receptor Gene Knockout and Humanized Mouse Lines</th>
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<td><strong>Citation</strong></td>
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<td>P450-humanized mice</td>
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<td>Conjugation enzymes humanized</td>
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Note: AhR, aryl hydrocarbon receptor; CAR, constitutive androstane receptor; FXR, farnesoid X receptor; GST, glutathione S transferase; mEH, microsomal epoxide hydrolase; NQO1, NAD(P)H:quinone oxidoreductase; PPAR, peroxisome proliferator-activated receptor; PXR, pregnene X receptor; SULT, sulfotransferase; UGT, UDP-glucuronosyl transferase.
pressure because of their arachidonate monooxygenases activities as revealed with the knockout mice (Holla et al., 2001; Nakagawa et al., 2006). The various P450-null mouse lines have firmly established that these enzymes influence the metabolism, toxicity, and carcinogenicity of xenobiotics in vivo. The use of xenobiotic enzyme-null transgenic mice has provided great insights in toxicology by enabling the identification of the major metabolic enzymes involved in toxicant biotransformation in vivo. In addition, new metabolic pathways important for toxicant biotransformation have also been elucidated through the use of these models.

Mice lacking expression of P450s known to be involved in metabolism of carcinogens and toxins show marked resistance to cancer and toxicity upon chemical treatment. For example, Cyp1b1-null mice are resistant to 7,12-dimethylbenz[a]anthracene-induced ovarian cancers (Buters et al., 2003). Mice lacking expression of Cyp2e1 are resistant to acetaminophen (APAP)-induced hepatotoxicity (Lee et al., 1996; Zaher et al., 1998) and benzene-induced myelotoxicity (Valentine et al., 1996). These protective effects are compatible with the known role of Cyp2e1 in the conversion of APAP to the electrophilic derivative N-acetyl-p-benzo-quinone-imine and production of the reactive benzene epoxide, respectively. In contrast, Cyp1a1-null mice are more susceptible to benzo(a)pyrene (BaP)-induced toxicities indicating inducible Cyp1a1 functions in the detoxification and protection against oral BaP (Uno et al., 2004). This result was not predictable because Cyp1a1 can also metabolically activate BaP as revealed in vitro. Thus, in the gut, after oral administration, Cyp1a1 primarily functions to inactivate this procarcinogen through ring hydroxylation.

There are numerous other examples of how the in vivo roles of P450s in the metabolism, toxicity, and carcinogenicity of xenobiotics has been determined through the use of gene knockout mice (Gonzalez, 2002; Jiang et al., 2010).

Similar insights have been revealed with knockout models for phase 2 conjugating enzymes. Mice lacking expression of GSTP1/2, that are involved in inactivation of electrophilic metabolites and control of oxidative stress, have a lower incidence of 7,12-dimethylbenz[a]anthracene and 12-O-tetradecanoylphorbol-13-acetate-induced skin papillomas when compared with WT mice (Henderson et al., 1998). Mice lacking expression of microsomal epoxide hydrolase (mEH) and soluble epoxide hydrolase (sEH) and NQO1 are viable but exhibit altered chemical sensitivities due in large part to their function in hydrolyzing epoxides (Miyata et al., 1999; Recio et al., 2005; Sinal et al., 2000). The sEH-null mice have spontaneous low blood pressure, thus indicating that this enzyme may be potential drug target for controlling hypertension (Sinal et al., 2000).

Generation of Humanized Mice

There are many ways to make humanized mice. First, complementary DNAs to human proteins can be introduced into expression vectors and driven by exogenous promoters. These promoters can be tissue specific, such as the albumin promoter expressed in the hepatocytes or the villin promoter expressed in intestinal epithelial cells, or regulatable such as the tet-OFF and tet-ON systems, in which expression of the cDNA can be modulated by doxycycline. Transgenic mice expressing these vectors are then bred to mice that have the corresponding gene knockout. The humanized mouse can also be made by directly injecting the human transgene into pronuclear stage embryos from mice that are null for the mouse gene. The human gene can also be knocked-in to the corresponding mouse gene locus using a standard homologous recombination in ES cells strategy. Finally, genomic clones, usually bacterial artificial chromosomes (BACs) or P1 phage artificial chromosomes (PAC), can be used to generate transgenic mice that then are bred to the knockout mice. Transgenic lines made with BAC and PAC clones are of special value because the human genes are expressed under their own promoters and thus are regulated in mice similarly to their regulation in humans. Expression levels of the proteins are within the range of what is found in human tissues. In addition, the humanized mouse lines are stable over many generations with same expression levels of the corresponding proteins maintained. BAC and PAC clones that have been completely sequenced are commercially available. A listing of published humanized mouse lines are listed in Table 3.

Xenobiotic Metabolism-Humanized Mouse Models

Cytochrome P450s are some of the most important enzymes involved in chemical toxicity because they have the ability to either activate prototoxants and procarcinogens or inactivate potentially harmful compounds. The role of P450s in toxicology and carcinogenesis has been confirmed in vivo by the use of P450 knockout mice. However, a major limitation of animal models in drug development and human risk assessment is the extrapolation from animal to humans, particularly when there are marked species differences in xenobiotic-metabolizing enzymes. To this end, transgenic models harboring human genes that encode for metabolic enzymes have been developed to produce more predictive models to study xenobiotic metabolism. These transgenic models have catalytic activities that are comparable to human tissues and may reflect more accurately human xenobiotic responses. These humanized transgenic models may be crucial in the evaluation and prediction of toxicological responses, particularly in the drug development and carcinogen metabolism.

**CYP1A1/CYP1A2-humanized mice.** The CYPIA family of P450s are involved in metabolic activation of many chemical carcinogens including the polycyclic aromatic hydrocarbons (PAHs), heterocyclic amines, and heterocyclic arylamine “food mutagens.” These genes are regulated by the aryl hydrocarbon receptor (AhR) that is responsive to PAHs and dioxins such as...
2,3,7,8-tetrachloro-p-dioxin (TCDD), CYP1A1/CYP1A2-humanized mice were developed by crossing the Cyp1a1-null, Cyp1a2-null, or Cyp1a1/Cy1a2-null mice with mice containing a BAC that encompasses the human CYP1A1 and CYP1A2 genes (Dragin et al., 2007; Jiang et al., 2005). These knockout and humanized mouse models are responsive to AhR agonists and have been successfully used for studying the role of CYP1A1/1A2 in the pharmacokinetics of a number of drugs including caffeine and theophylline (Derkenne et al., 2005) and in the metabolism of PAHs (Uno et al., 2006) and food mutagens such as 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) (Cheung et al., 2005a). In the latter case, PhIP was found to be preferentially metabolized by N₂-hydroxylation by CYP1A2-humanized mice, whereas in WT mice, 4'-hydroxylation was the predominant pathway. Because the N₂-hydroxylation pathway for PhIP metabolism was reported to be the main pathway for PhIP metabolism in humans, these results suggest that CYP1A2-humanized mice may be more appropriate models for determining human health risks to PhIP and other heterocyclic amines instead of WT mice.

**CYP2E1-humanized mice.** CYP2E1 is an enzyme of tremendous toxicological importance because it metabolizes a vast array of low–molecular weight compounds to which humans are exposed, such as industrial solvents (i.e., benzene, carbon tetrachloride) and carcinogens (i.e., azoxymethane, dimethylnitosamine) (Guengerich and Shimada, 1998). A Cyp2e1-null mouse model was generated to evaluate CYP2E1-mediated biotransformation in vivo (Lee et al., 1996; Gonzalez, 2007). These Cyp2e1-null mice were considerably less sensitive to APAP-induced hepatotoxicity than WT mice, thus establishing an important role for Cyp2e1 in the activation of APAP to a hepatotoxic quinone metabolite. A BAC genomic clone containing the complete human CYP2E1 gene was introduced into the Cyp2e1-null mouse to create a CYP2E1-humanized mouse model. CYP2E1-humanized mice differed in their sensitivity to APAP as compared with WT mice (Cheung et al., 2005b). The CYP2E1-humanized mice should be of predictive value for assessing the pharmacological and toxicological effects of potential CYP2E1 substrates and thereby more accurately inform human risk assessment.

**CYP3A4-humanized mice.** CYP3A4 is the most abundant P450 in human liver and intestine and is involved in the metabolism of more than 50% of all clinically used drugs. It is the major enzyme induced by the pregnane X receptor (PXR). This broad substrate specificity and importance in metabolism of drugs implies the involvement of CYP3A4 in clinically relevant drug-drug interactions. Although mice have several Cyp3a P450s, in contrast to humans, they are expressed at low levels in both the liver and gut in the absence of PXR activation. To produce a mouse model to study CYP3A4 metabolism and its contribution to drug interactions, a humanized CYP3A4 transgenic mouse line was generated using a BAC clone containing the complete human CYP3A4 gene (Granvil et al., 2003). CYP3A4 was expressed at low levels in the liver and subjected to gender-specific hormonal control and regulation by mouse PXR activators (Cheung et al., 2006; Yu et al., 2005). Most importantly, this mouse line had high constitutive expression of CYP3A4 in the intestine and was used to determine the dominant role of the gut in drug metabolism and clearance using the probe substrate midazolam. Ketoconazole administration markedly decreased metabolism and clearance of midazolam demonstrating the value of this model in studying drug-drug interactions (Granvil et al., 2003). Nevertheless, the mouse Cyp3a enzymes are expressed in this line, especially after administration of PXR activators, which potentially confounds the interpretations of results in establishing a role for CYP3A4 in drug metabolism and toxicity. To this end, a Cyp3a-null mouse lacking all functional mouse Cyp3a were produced and used as recipients of human CYP3A4 cDNA-derived vectors expressing the enzyme in liver and intestine (Scheer et al., 2008). These mice were used to determine the role of intestinal human CYP3A4 in metabolism of orally administered triazolam and inhibition by ketoconazole (van Waterschoot et al., 2009). It is anticipated that the CYP3A4-humanized mouse models will be of great value for understanding CYP3A4-mediated drug metabolism and drug-drug interactions.

**TRANSGENIC RECEPTOR MODELS**

A selection of ligand-activated receptor transcription factors that respond to xenobiotics include the AhR, constitutive androstane receptor (CAR, NR1I3), farnesoid X receptor (FXR, NR1H4), PXR (NR1I2), and peroxisome proliferator–activated receptor (PPAR) α (NR1C1). The latter receptor is part of the PPAR family trio, including PPARβ (NR1C2) and PPARγ (NR1C3), which are critical for metabolic control of fatty acid and glucose homeostasis. CAR, FXR, PXR, and PPARα all belong to the metabolic sensor class of nuclear receptors that alter metabolism of xenobiotics and endogenous chemicals such as fatty acids, cholesterol, and bile acids (Gonzalez and Shah, 2008; Modica et al., 2009). They contain the ligand-binding, DNA-binding, transcription activator, and coactivator/corepressor-binding domains found in the nuclear receptor superfamily. Typical of all type 2 nuclear receptors, CAR, FXR, PXR, and PPARα require the retinoid X receptor as an obligate heterodimerization partner for activation of gene expression. The heterodimers can bind to specific direct repeat (DR) elements located upstream of target genes and, through interaction with coactivators and other components of the transcriptional machinery, activate target gene expression. Unlike the relatively specific binding of the type 1 steroid hormone receptors (i.e., ER, progesterone receptor, glucocorticoid receptor), CAR, FXR, PXR, and PPARα are capable of being activated by structurally diverse ligands including...
AhR Knockout and Humanized Mice

The AhR represents one of the most highly studied receptors in the field of chemical toxicology and is involved in mediating many of the toxic effects of various PAHs and halogenated aromatic hydrocarbons (HAHs), most notably the prototypical ligand TCDD (Rowlands and Gustafsson, 1997). Ligand activation of the AhR results in the induction of various phase I metabolizing enzymes including Cyp1a1 and Cyp1a2 and various phase II conjugating enzymes as well as a large number of additional responses that are thought to be critical in mediating chemical toxicity in animals (Rowlands and Gustafsson, 1997). Research with knockout mice for the AhR has indicated resistance to the toxicity of PAHs and HAHs demonstrating a central role for the AhR in their modes of action. These models have also identified a physiological role for the receptor in developmental biology (e.g., vascular development), endocrine function, cell growth and apoptosis, reproduction, and immunity (Luecke et al., 2010; McMillan and Bradfield, 2007; Nguyen and Bradfield, 2008; Stockinger, 2009; Veldhoen and Duarte, 2010).

A number of intra- and interspecies differences have been identified in the responses to the prototypical ligand TCDD which have been attributed to differences in receptor sequence and subsequent transactivation of target genes (Rowlands and Gustafsson, 1997). These differences also appear to translate to differences in susceptibility to the toxicity of ligands that activate this receptor. Large differences in AhR ligand-binding affinity have been observed in different mouse strains, most notably being the DBA and C57BL6 mouse strains. Molecular biology studies have revealed that the lower affinity in the DBA mouse AhR is due largely to a sequence alteration in the ligand-binding domain that results in an alanine-to-valine substitution at amino acid residue 375 (abbreviated Ala → Val375) (Ema et al., 1994). A second mutation located in the carboxy terminal transactivation domain results in a leucine-to-proline substitution at position 471 (Leu → Pro471) and also reduces ligand binding but with less impact than the ligand-binding domain mutation (Chang et al., 1993). The human receptor displays an approximate 10-fold lower affinity for TCDD than the C57BL6 mouse, which is largely due to a valine for alanine substitution at amino acid residue 381, which is equivalent to the mouse Ala → Val375 (Ema et al., 1994). The amino acid sequence of the transactivation domain of the AhR is not consistent across species, suggesting that recruitment of coactivator and thus transcription activation could significantly differ between species (Ramadoss and Perdew, 2004). In particular, comparison of the mouse and human AhR revealed only a 58% homology of amino acid sequence. Developing a better understanding of the human-relevant sensitivity and biological effects mediated by environmental contaminants that bind and activate the AhR has important implications for human risk assessment.

Two separate humanized AhR (hAhR) mouse models (hAhR knock-in mice) have been reported which were created on the C57BL6 strain background in order to study the species differences in AhR ligand-induced responses between humans and mice (Flaveny and Perdew, 2009; Moriguchi et al., 2003). The first hAhR mouse line was created by knocking-in a human AhR cDNA into the C57BL6 mouse Ahr locus by homologous recombination, thereby disrupting the mouse Ahr gene. The cDNA was recombined so that hAhR is expressed under the control of the endogenous mouse Ahr promoter. In the Moriguchi et al. (2003) hAhR cDNA knock-in mice, TCDD responsiveness was much lower as compared with either native C57BL6 mice or DBA/2 strain mice, with approximately 15- and 5-fold lower levels of CYP1A1 induction, respectively, at a TCDD dose of 100 μg/kg (Moriguchi et al., 2003). TCDD-induced teratogenic responses were significantly reduced in the hAhR knock-in mice, with the incidence of cleft palate and hydronephrosis in the pups completely abrogated or significantly reduced, respectively, compared with WT C57BL6 mice.

The second model generated a mouse line that in effect replaces mouse AhR (mAHR) expression with the hAhR specifically in hepatocytes (Flaveny and Perdew, 2009). This was accomplished by first making a transgenic mouse that expresses the hAhR under the Ttr promoter, which yields hepatocyte specific expression. This mouse line was then crossed with a conditional Ahr knockout mouse also expressing the Cre recombinase under the control of the albumin gene promoter, this mouse has the mAhR deleted from hepatocytes. From this cross, a double transgenic mouse on a conditional Ahr background was generated (strain name, B6.Cg-Ahrtm3.1Bra Tg (Alb-cre, Ttr-AhR)1Ghp)). It is important to point out that this line expresses the Ahd allele of the mAhR in tissues other than hepatocytes. This model has been used to examine differences in the specificity of ligand binding, comparing the mAhR and hAhR in mouse liver extracts (Flaveny et al., 2009). Results revealed significant quantitative differences in ligand affinity. As expected, some ligands appear to have higher affinity for the mAhR. In contrast, indirubin has much higher affinity for the hAhR and indirubin induces Cyp1a1 to a much greater extent in hAhR hepatocytes compared with cells derived from C57BL6/J mouse. The ability of the ligand-activated human versus mouse AhR to mediate gene expression changes in primary mouse hepatocytes was also examined (Flaveny et al., 2010). Both receptors were able to induce Cyp1a1 mRNA to a similar extent. DNA microarray studies utilizing primary mouse hepatocyte cultures derived from hAhR expressing mice and C57BL6/J mice revealed a dramatic qualitative difference in gene expression, with only ~18% of the induced genes were in common between each genotype. These results suggest that the hAhR regulates gene expression differentially compared with the mAhR, and TCDD-mediated studies have
recently been performed in these mouse models and have revealed that the hAhR can only modestly drive TCDD-mediated toxicity.

**PPARα Knockout and Humanized Mice**

PPARα is the target of lipid lowering fibrate drugs. Treatment of rats and mice with PPARα activators, such as clofibrate and Wy-14,643, results in a stereotypical pleiotropic response which is tissue specific but appears to most predominantly affect the liver. After short-term treatment with ligands, activation of PPARα results in hepatomegaly because of both hypertrophy and hyperplasia which is accompanied by a marked proliferation of peroxisomes and the smooth endoplasmic reticulum. Coincident with these responses is the increase in fatty acid catabolism as a result of the elevated expression of genes encoding proteins involved in lipid transport and fatty acid β-oxidation, which have made the receptor a key target for lipid and cholesterol lowering drugs. Targeted disruption of the mouse-PPARα gene demonstrated that mice lacking expression of the receptor do not respond to ligand exposure and lacked the associated downstream hepatic hypertrophic and hyperplastic response and induction of target genes involved in lipid metabolism and transport (Lee et al., 1995). Interestingly, rats and mice treated with PPARα activators develop liver cancers within 1 year, and the response has been shown to be mediated through a PPARα-mediated mechanism as PPARα-null mice are resistant to the hepatocarcinogenic response (Gonzalez and Shah, 2008; Peters et al., 1997). In contrast to rodents, humans are resistant to the carcinogenic effects of fibrate drugs. A pathway involving the microRNA let-7c and c-myc accounts in part for the hepatocarcinogenic effect of PPARα activators in rodents and the resistance of humans to liver toxicity and cancer when chronically administered fibrate drugs such as fenofibrate, chlolfibrate, and gemfibrozil. Activation of PPARα suppresses expression of let-7c resulting in stabilization of c-myc mRNA and increased c-myc protein (Shah et al., 2007). Induction of c-myc contributes to cell proliferation, and in the presence of increased reactive oxygen species, which likely causes genetic damage, hepatocytes are transformed (Gonzalez and Shah, 2008).

Mice humanized for PPARα were produced because of the great importance of this transcription factors as a drug target and for preclinical drug development and human risk assessment (Cheung and Gonzalez, 2008; Cheung et al., 2004; Yang et al., 2008). Two lines of PPARα-humanized mice were produced. One line was generated using a human PPARα cDNA under control of the tet-OFF system (Cheung et al., 2004) and another line made with a PAC containing the complete human gene (Yang et al., 2008). Treatment of these mice with the potent experimental PPARα ligand Wy-14,643 and the fibrate drug fenofibrate resulted in marked induction of hepatic genes involved in fatty acid β-oxidation and a lowering of serum triglycerides. However, these mice were resistant to PPARα activator–induced cell proliferation and hepatocarcinogenesis (Morimura et al., 2006; Yang et al., 2008). Although ligand treatment lowers let-7c expression in WT mice, let-7c is not suppressed in PPARα-humanized mice. This lack of suppression of let-7c by human PPARα likely accounts for the resistance of humans to the proliferative properties of PPARα activators. The PPARα-humanized mice will be of great value in preclinical analysis of PPARα pure agonists, PPARα/PPARγ dual agonists, and PPAR-pan agonists under development as drugs for treatment of metabolic disorders because these mice would not develop liver toxicities and hepatocarcinogenesis and thus can be monitored for a sufficient length of time to research possible extrahepatic toxicities and cancers.

**PXR Knockout and Humanized Mice**

Human PXR (NR1I2) has a flexible ligand-binding pocket capable of binding to structurally diverse compounds (Kliewer et al., 2002), drugs, dietary supplements, natural products, environmental pollutants, endogenous hormone metabolites, and bile acids (Ma et al., 2008b). PXR is generally regarded as a sensor activated by endogenous and exogenous chemicals and regulates a large number of enzymes and transporters involved in chemical metabolism and elimination, most notably CYP3A4 which is one of the most important enzymes involved in drug metabolism in humans (Cheng et al., 2010a). Because of its key role in the regulation of drug metabolism, there has been a strong interest in further understanding the biological role of his receptor because of its potential influence on clinical responses leading to drug-drug interactions that could result in decreased therapeutic efficacy or increased drug toxicity (Ma et al., 2008b). PXR-null mice have been generated and indicate that the receptor is not essential for development or physiological homeostasis as the mice do not display any phenotypic abnormalities or alterations is general biochemical parameters. As expected, PXR-null mice did not respond to known PXR-ligands. PXR-null mouse models have served as useful research tools to better understand the role of PXR in mediating drug toxicity or efficacy (Ma et al., 2008b).

Importantly, there exist species-specific responses to ligand activation by human and mouse PXR; rifampicin does not significantly activate mouse PXR but is a very potent activator of human PXR, whereas pregnenolone-16α-carbonitrile (PCN) only weakly activates human PXR but potently activates mouse PXR (Lehmann et al., 1998). Because PXR is the principle regulator of CYP3A4 involved in metabolism of many clinically used drugs, it is of great importance for drug clearance and drug-drug interactions. Thus, preclinical studies must determine whether a drug is a substrate for CYP3A4 and whether it activates PXR. Although human hepatocytes and other in vitro systems can be used to make this determination,
mice humanized for PXR and CYP3A4 offer in vivo models for preclinical drug studies.

PXR humanized were made using a BAC genomic clone introduced on the Pxr-null mouse background (Ma et al., 2007, 2008a,b). Another humanized line was produced using a human PXR-cDNA (Xie et al., 2000). Both Pxr-null mouse lines have no deleterious physiological or reproductive phenotypes thus allowing them to be used as recipients for the human transgenes (Staudinger et al., 2001; Xie et al., 2000). The use of genomic clones under control of their native promoters results in expression and regulation of the transgenes in the proper tissues. Indeed, the PXR-humanized mice accurately express functional PXR protein in tissues where the receptor is normally found in humans, notably the gut and liver (Ma et al., 2007). To produce a humanized mouse line containing the main PXR target gene CYP3A4, a BAC genomic clone containing the CYP3A4 and CYP3A7 genes was introduced onto the PXR-humanized mouse line to generate PXR/CYP3A4 double humanized mice (Ma et al., 2008a). Both lines of single and double humanized mice are stable with no loss of PXR or CYP3A4 activities after more than 5 years of breeding. These mice were used to determine the role of PXR in drug toxicity and efficacy. Treatment of PXR-humanized mice with the human-specific PXR ligand rifaximin resulted in induction of PXR target genes such as Cyp3a11 and in the PXR/CYP3A4 double humanized mouse line, the induction of CYP3A4. In contrast, no induction was found with the mouse PXR ligand PCN, thus validating the PXR-humanized mice and its derivatives.

The PXR/CYP3A4 double humanized mice were first employed to investigate the role of human PXR in the hepatotoxicity of the widely used over-the-counter analgesic APAP (Cheng et al., 2009). Induction of human CYP3A4 by the human PXR activator rifampicin resulted in increased production of the quinone metabolite N-acetyl-p-benzoquinone imine, associated elevated oxidative stress, and massive hepatotoxicity; mice lacking human PXR were resistant to APAP-induced toxicity. This study revealed the utility of PXR/CYP3A4 double humanized mice in preclinical toxicity and drug interaction studies and the data obtained suggest the possibility of drug-drug interactions involving APAP and other PXR activators that could lead to liver damage.

Rifaximin was identified as a gut-specific PXR agonist that exerts a therapeutic role in inflammatory bowel disease (IBD), as revealed with the PXR-humanized mouse, whereas no activity was found in WT or Pxr-null mice (Cheng et al., 2010b). A dextran sulfate sodium (DSS)–induced IBD model, that causes massive inflammation of the colon, was applied, and mice were treated with rifaximin. The symptoms of IBD were considerably lower in mice either pretreated with rifaximin before application of DSS or PXR-humanized mice treated after the onset of IBD, thus establishing the therapeutic utility of the drug. Rifaximin had no effect on DSS-induced IBD symptoms in WT and Pxr-null mice. Amelioration of the IBD symptoms in PXR-humanized mice was because of PXR-mediated inhibition of nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor (NF-κB) (Cheng et al., 2010b). Although the precise mechanism by which PXR inhibits NF-κB requires further experimentation, studies using the PXR-humanized mice revealed that PXR is responsible for the therapeutic effects to rifaximin.

**CAR Humanized Mice**

Human CAR (NR1I4) shares functions similar to that of PXR that involves induction of a range of xenobiotic-metabolizing enzymes. CAR was first shown to be involved in the induction of CYP2B gene expression but since this characterization, a wide range of genes have been shown to be regulated in a CAR-dependent manner (Ueda et al., 2002). CAR is activated by a range of chemicals, most notably phenobarbital (PB). As with PXR, CAR-null mice have been generated as important research tools for understanding the role of CAR in mediating drug toxicity and/or efficacy. As with PXR-null mice, CAR-null mice had no phenotypic or physiological abnormalities indicating the receptor is not essential for normal development or physiology (Scheer et al., 2008). CAR-null mice have been used to show that the receptor plays a critical role in APAP metabolism and toxicity, and pretreatment with CAR activators can significantly potentiate the toxicity of APAP in WT mice, whereas CAR-null mice were resistant to the toxicity (Zhang et al., 2002).

As with PXR, there exist species-specific responses to ligand activation by human and mouse CAR. For example, human CAR strongly activated the chemical 6-(4-chlorophenyl)imidazo-[2,1-b][1,3]thiazole-5-carbaldehydeO-(3,4-dichloro-benzyl)oxime (CITC/O), whereas murine CAR shows only weak activation. In contrast, the mouse CAR receptor is more sensitive to 4-bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) when compared with the human receptor. Mouse CAR is also inhibited by a limited set of steroids related to 3x-androstenol whereas chlorpromazine and 17α-ethynyl-3,17β-estradiol are potent activators of mouse CAR, whereas they do not activate human CAR (Jyrkkarinne et al., 2005).

The species-specific ligand sensitivities and the role of CAR in the induction of drug metabolizing enzymes indicate that receptor activation has the potential to give rise to species-specific differences in drug metabolism and toxicity. To help develop a better understanding of these potential effects and their implications for human risk assessment, humanized CAR mice were generated using knock-in strategies with mice that resulted in the expression human CAR under the control of the corresponding mouse promoter (Scheer et al., 2008). CAR knockout (CARKO) models were also generated by the same group to assess the specificity of the observed responses for this receptor. These models were used to further characterize the species-specific responses for mouse and human CAR within the context of the whole animal. Consistent with previous
data, TCPOBOP resulted in a very high induction of the CAR-responsive genes Cyp2b10 and Cyp3a11 in WT mice, but in mice with human CAR, only a minor induction was noted and only at high doses relative to that observed for WT mice. Administration of the human-specific ligand CITCO to WT mice resulted in only a slight induction of CAR-responsive genes and a very profound induction in the humanized CAR mice. These induction responses were also completely absent in the CARKO mice indicating the specificity for the regulation of these genes by CAR.

An example of the use of such a humanized model for CAR in an application to assess human hazard potential is presented in the case study below. Because of the overlapping roles that CAR and PXR play in drug metabolism and toxicity, their shared gene targets, and the fact that many chemicals can interact with both receptors, combined CAR/PXR double knockouts and double humanized mice were used in the assessment.

APPLICATION OF HUMANIZED MICE TO THE ASSESSMENT OF HUMAN HAZARD POTENTIAL.

PB—A CASE STUDY

Background

Animal cancer bioassays have long been employed in order to determine whether a variety of types of chemical might cause cancer or other health problems in humans. They are still very much the “gold standard” for the assessment of carcinogenic potential where human use or exposure is anticipated. It is, of course, necessary to extrapolate this data, both in terms of dose and species, in order to accurately predict and determine human risk. MOA studies have generated molecular and cellular data that have enabled an improved understanding of the mechanisms underlying the induction of neoplasia. This, in turn, has raised concerns regarding the suitability of the animal data to enable extrapolation to humans. The use of rodent liver tumor responses in human cancer risk assessment has historically been controversial and has been extensively debated within the scientific community (Holsapple et al., 2006), and a human relevance framework has been developed that describes a means of establishing a relationship between observed cellular perturbations and the development of cancer (Boobis et al., 2006; Cohen et al., 2003; Sonich-Mullin et al., 2001; Whysner et al., 1996). Knowledge of these key events and the identification of an MOA provide a more rational basis for human hazard and risk assessment.

There is thus a very real need for improved animal models that more closely resemble the human situation. Transgenic mouse models that harbor human genes have been developed, and they represent very powerful tools to help address interspecies extrapolations for the application of MOA data to human hazard and risk assessment. Liver carcinogenicity is a relatively common finding in long-term rodent carcinogenicity studies. Indeed, many marketed chemicals and drugs are known to be rodent nongenotoxic hepatocarcinogens. When liver carcinogenicity is observed in rodent studies, the challenge is to demonstrate whether the findings are relevant to humans in a mechanistic, rapid, and cost-effective manner.

PB is a barbiturate that has been widely used as an anticonvulsant. Several studies have demonstrated that chronic administration of PB results in altered hepatic foci and liver tumors (Jones et al., 2009; Thorpe and Walker, 1973; Whysner et al., 1996). It is the prototype of several nongenotoxic rodent hepatocarcinogens that elicit hepatomegaly that is characterized by both hepatocellular hypertrophy and hyperplasia. The hypertrophy is associated with smooth endoplasmic reticulum proliferation that, in turn, is associated with the induction of cytochrome P450 isoforms belonging to the CYP2B, CYP2C, and CYP3A families. The hyperplasia is characterized by increased replicative DNA synthesis and cell proliferation. An inhibition of apoptosis has also been reported (Foster, 2000; James and Roberts, 1996; Jones et al., 2009; Kolaja et al., 1996; Ross et al., 2010). Similar nongenotoxic hepatocarcinogens include other CYP2B inducers such as TCPOBOP, chlordane, DDT, dieldrin, and oxazepam (Dickins, 2004; Martignoni et al., 2006).

The key role of CAR in mouse liver growth and cytochrome P450 induction has been shown in studies with WT and CARKO mice (Huang et al., 2005; Scheer et al., 2008; Wei et al., 2000). These studies have demonstrated that the deletion of CAR results in the absence of increases in relative liver weight, replicative DNA synthesis, and cytochrome P450 induction after treatment with PB and TCPOBOP. The role of CAR in tumor promotion has also been determined. Wild-type and CARKO mice were initiated with a single ip 90 mg/kg dose of diethylnitrosamine, then given 0 or 500 ppm PB in drinking water. After 32 weeks, none of the CARKO mice had foci or liver tumors, whereas all the PB-treated WT mice had hepatic carcinomas and/or adenomas (Huang et al., 2005; Yamamoto et al., 2004). This highlights the key role that CAR plays in tumor promotion.

A number of epidemiological studies, examining the long-term effects of PB administration, have been performed, and the results of these studies have demonstrated that when humans have been administered PB for many years at doses resulting in plasma concentrations similar to those that are carcinogenic in rodents, there is no evidence of an increased risk of liver tumors (Olsen et al., 1989, 1995; Holsapple et al., 2006; Whysner et al., 1996). However, prolonged administration of PB in human studies has been shown to increase liver size, which is associated with hepatocellular hypertrophy (Aiges et al., 1980; Pirttiaho et al., 1978, 1982).

The available data suggest that, although the hypertrophic effects of PB are observed in human liver, data from epidemiological studies indicate an absence of evidence that PB increases the risk of liver tumor formation in humans. PB is known to induce CYP2B in cultured rat, mouse, and human
hepatocytes, but it only induces replicative DNA synthesis in cultured rat and mouse hepatocyte cultures and not in human hepatocytes (Hasmall and Roberts, 1999; James and Roberts, 1996; Jones et al., 2009; Parzefall et al., 1991; Plant et al., 1998).

In light of these data, researchers have used mice nulled for CAR and PXR and mice humanized for CAR and PXR to further examine species similarities and species differences in the functional properties of these nuclear hormone receptors. There is a considerable degree of similarity and cross-talk between these two nuclear receptors (Dickins, 2004; Lin, 2006), so it is perhaps not surprising that some compounds (e.g., PB, clotrimazole) are activators of both receptors (Moore et al., 2000, 2003). Therefore, the studies described here are aimed at understanding the potential liver cancer hazard of “PB-like” enzyme inducers to humans and have utilized mice that are nulled or humanized for both CAR and PXR (Ross et al., 2010).

**Application of PXRCO/CARKO and huPXR/huCAR Mice to Evaluate Species-Specific Hepatic Responses to PB**

The effects of PB were investigated in double “humanized” PXR and CAR (huPXRxhuCAR), double knockout PXR and CAR (PXRCO/CARKO), and WT (C57Bl/6) mice. Homozygous humanized and knockout mice for PXR and CAR were generated and characterized as described in Scheer et al. (2008). Wild-type C57BL/6 (WT) animals of the same genetic background and age purchased from Harlan (UK) were used for control experiments. Male, sexually mature huPXRxhuCAR mice, PXRCO/CARKO mice and WT mice were implanted with osmotic pumps (Alzet model 2001) containing bromo-deoxyuridine (15 mg/ml in phosphate-buffered saline, pH 7.4), 5 days before termination. For PB treatment, animals were then dosed daily by i.p. injection with either the vehicle (saline) or PB (80 mg/kg/day) for 4 days. Following treatment, all mice were sacrificed 24 h after the last dose.

Increased liver weights, hepatocellular hypertrophy, and induction of the CAR and PXR target genes CYP2b10 (pentoxyresorufin-O-depentylation) and Cyp3a11 (data not shown) were observed in both WT and huPXRxhuCAR mouse lines following treatment with PB (Fig. 5 and Ross et al., 2010). In the PXRCO/CARKO, mice none of these parameters were affected. In WT mice, PB treatment increased the hepatocellular labeling index (S-phase) by approximately sevenfold (Fig. 5). However, no change in S-phase was detected in either the huPXRxhuCAR or the PXRCO/CARKO mice following PB administration. PB caused alterations in a number of cell cycle genes consistent with the induction of cell proliferation in WT mice (data not shown). However, these gene expression changes did not occur in PXRCO/CARKO or huPXRxhuCAR mice. These data are summarized in Table 4 and clearly demonstrate the CAR/PXR dependence of these effects and the profound species differences. Similar data have been demonstrated using chlordane, another nongenotoxic hepatocarcinogen (Ross et al., 2010).

The data from these models provide additional strength and confirmation to the mechanistic understanding, derived from previous rodent and human hepatocyte studies, that...
TABLE 4
Summary of Responses to PB in WT, PXR/CAR Knockout Mice, PXR/CAR Humanized Mice, and Rodent and Human Hepatocytes

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mouse In Vivo</th>
<th>WT Knockout</th>
<th>PXR/CAR Knockout</th>
<th>Humanized</th>
<th>PXR/CAR Humanized</th>
<th>Mouse/ Rat</th>
<th>Human</th>
</tr>
</thead>
<tbody>
<tr>
<td>Induction of CYP2B</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>—</td>
<td>—</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Induction of CYP3A</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>—</td>
<td>—</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Liver weight increases</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Hypertrophy</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Hyperplasia—cell proliferation</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

human PXR/CAR can support the hypertrophic hepatic response but not the hyperplastic response to the mouse hepatocarcinogen PB (Table 4). Compound-specific studies using the combination of human hepatocytes in vitro (human cell and human CAR and PXR) and humanized mice in vivo (mouse cell and human CAR and PXR) greatly strengthen the assertion that human hepatocytes will not exhibit increased cell proliferation in response to CAR or PXR activation. If the receptor-mediated stimulation of cell proliferation is pivotal to the nongenotoxic hepatocarcinogenicity of these materials, then these “liver growth carcinogens” are likely to pose no hepatocarcinogenic hazard to humans.

OUTLOOK ON TRANSGENIC MODELS IN TOXICOLOGY AND RISK ASSESSMENT

Transgenic models have certainly had an important impact on the field of toxicology, and there are many factors that are likely to influence and expand the use of these models in both basic toxicity research and risk assessment. In the past, the mouse has been the predominant animal model for generation and subsequent research with transgenic models, owing largely to the success of ESC-based targeting technology (Capecchi, 2005). Such an approach has not been amenable to application in other species such as the rat; however, recent approaches have been described that will open the door for the creation of a more diverse set of transgenic models. For example, the microinjection of specifically designed zinc finger nucleases into embryos have been used to introduce nonhomologous end-joining deletions or insertions at a target gene site to generate knockout rats and mice (Carbery et al., 2010; Geurts et al., 2009). More recently, this approach has been extended in both species to introduce sequence-specific modifications through homologous recombination (Cui et al., 2011). The approach enables precise alteration to the genome to generate insertions, deletions, and even point mutations in a whole animal system. Such approaches will clearly pave the way for more expanded research using transgenic models that will include extension into species beyond the mouse and allow for alterations that may more accurately mimic the genetic make up of the human population. Conceivably, this could also be applied to understand and compare human susceptibilities based on known polymorphisms in the human population.

Although this review has focused solely on rodent transgenic models, another expanding and promising area of research surrounds the use of alternative model systems such as zebrafish and Caenorhabditis elegans, both as screening tools for toxicity assessment and in MOA-based research. In zebrafish, various approaches to manipulate the genome have been used to generate both knockout and knock-in models that have been applied extensively to characterize vertebrate development and have served as models for research on human diseases (Yang et al., 2009). Transgenic zebrafish have also been engineered to express fluorescent reporter genes in response to tissue damage, oxidative stress activation, or other indicators of toxicity that could be used in a toxicity screening approach or MOA research (Yang et al., 2009). Similar applications have been published for C. elegans including transgenic reporter systems for biomonitoring and model systems for researching neurodegenerative diseases (Candido and Jones, 1996; Wu and Luo, 2005).

There have been widespread discussions in recent years on the need to overhaul our approaches to toxicity testing and risk assessment with the application of new technologies that will help to streamline and increase the human relevance of the process. One of the key drivers for these discussions has been the U.S. National Research Council (NRC) report—Toxicity Testing in the 21st Century: A Vision and a Strategy (NRC, 2007). The United States Environmental Protection Agency’s MOA framework and Strategic Plan for Evaluating the Toxicity of Chemicals is also helping to facilitate change in the risk assessment process for environmental chemicals (U.S.EPA, 2009). According to EPA, the purpose of these plans is to serve as a blueprint for incorporating advances in molecular biology and computational sciences into toxicity testing and risk assessment practices across the EPA.

The NRC report and the EPA strategy both outline the promise that the new technologies could dramatically increase both the numbers of chemicals comprehensively evaluated while also broadening and improving the human relevance of the assessed toxicity endpoints. According to the NRC report, this would be accomplished in the not-so-distant future through the use of human cells and cell lines by evaluating cellular “toxicity pathways” using high-throughput testing approaches. Risk assessments would be based on the results of these assays and focus on avoiding perturbations in key cellular pathways as assessed through comprehensive dose-response analysis and extrapolation to human-relevant exposure conditions. Such
outcomes, if successfully achieved, would significantly improve and inform science-based health assessments on a broad range of chemicals. However, further reflection has indicated that such a change will require a strategic and systematic transition with a continued reliance on whole animal testing in the near-term (Andersen and Krewski, 2010; Hartung and Daston, 2009). Such a transition could certainly benefit from the strategic implementation of transgenic models as part of an integrated testing strategy. Transgenic models have the potential to serve as important tools in this process including, but not limited to, characterization of the MOA, decreasing uncertainty in cross-species extrapolations, and defining the shape of the dose-response and the existence of thresholds, all with the ultimate goal of establishing more relevant measures of human risk.

With the ever increasing ability to create transgenic models with greater ease and within a wider range of research species (both rodent and nonrodent), our ability to characterize the biological pathway perturbations that lead to toxicity will certainly increase. This in turn will foster better mechanistic understandings within the field of toxicology which will enable the subsequent development of more relevant and specific in vitro assays for predicting toxicity that will help to deliver on the vision of the NRC report, the EPA Strategic plan, and other international initiatives focused on streamlining the risk assessment process while improving human relevance and decreasing animal use. The application of these models to human risk assessment will require appropriate and consistent interpretation of the data, recognition of the limitations of these models, and acceptance of the resultant data and its relevance to humans. Such progress will not be achieved without concerted efforts and focused communication from all stakeholders (Andersen and Krewski, 2010), most notably between research toxicologists and regulatory toxicologists to ensure that these new technologies can be applied and accepted within a regulatory context. The field of toxicology and risk assessment has entered an exciting period wherein the biological tools to streamline and advance the human relevance of toxicology testing are available. What is now required are creative and consensus approaches for their successful application to risk assessment.

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