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Transgenic mice as alternatives in carcinogenicity testing: current status

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Abstract

The correct prediction of human risk after exposure to chemical and physical compounds has long been a major challenge. For this, the lifetime bioassay using rats and mice is routinely used. However, over the years this assay has clearly come to reveal several drawbacks; for example, large numbers of animals are used, and the assays are slow, insensitive and expensive. On top of this, there is considerable scientific doubt about the reliability of the assay, since too many false positive results (so-called rodent carcinogens) have been observed. Therefore, there is an urgent need for alternative, more valid, carcinogenicity assays. One specific approach in finding alternatives is the use of transgenic mice with modifications in genes associated with the development of cancer, thereby increasing their sensitivity to carcinogens. Several transgenic mouse models have now been generated and studied for their usefulness as replacements for the lifetime bioassay in carcinogenicity testing. Generally, the transgenic mouse models could reliably predict the carcinogenic potential of compounds, and importantly, the number of false positives was reduced significantly. For this, 3 to 4-fold fewer animals are used in about a three-time shorter test period. This also enormously reduces costs associated with performing these *in* vivo studies. However, the transgenic models were not capable of identifying all known human carcinogens when applied as single assays. Using a short-term transgenic mouse assay in combination with a rat lifetime bioassay, however, completely eliminated the occurrence of false negatives, and moreover, increased the overall accuracy of detecting carcinogens and non-carcinogens enormously (85%) compared to using only the lifetime bioassay (69%). These marked advantages of transgenic mouse models have resulted in the FDA in the USA and the European CPMP now allowing the use of transgenic mice in regulatory testing of pharmaceutical compounds as an alternative for a second lifetime bioassay, when a two-year bioassay with rats was carried out. Nonetheless, a more extensive evaluation of transgenic mice using newly developed models and more compounds with different modes of action needs to be carried out in the future.

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Samenvatting

Nieuwe chemische stoffen en geneesmiddelen worden continu ontwikkeld. Voordat deze agentia worden toegelaten, moeten ze getest worden op mogelijke schadelijke effecten voor de mens in een panel van toxiciteitstesten. De test die wordt voorgeschreven om stoffen te identificeren die kanker veroorzaken is de 'chronische bioassay'. In deze chronische bioassay worden ratten en muizen hun leven lang blootgesteld aan de te testen stof, waarna gekeken wordt of de dieren tumoren ontwikkelen. Inmiddels is echter duidelijk geworden dat aan deze test nogal wat nadelen kleven. Er worden grote hoeveelheden dieren gebruikt (>1000 per stof), de testen duren erg lang (3 tot 5 jaar) en zijn als een gevolg hiervan erg duur. Verder worden in de bioassay veel vals-positieve resultaten gevonden, vermoedelijk omdat langdurig extreem hoge doseringen worden gebruikt. Dergelijke stoffen worden ten onrechte geïdentificeerd als kankerverwekkend, met onnodige gevolgen voor toelating en risico evaluatie. Er is daarom al jaren dringend behoefte aan alternatieve testsystemen, die recent nog meer is toegenomen omdat door de invoering van het REACH systeem grote hoeveelheden toxiciteitstesten zullen moeten worden uitgevoerd. Een alternatieve test zal de kankerverwekkende eigenschap van een stof met minder dieren, in kortere tijd en met een hogere betrouwbaarheid moeten kunnen vaststellen, om het testen van naar schatting duizenden stoffen mogelijk te maken voor REACH.

Een potentieel bruikbaar alternatief zijn transgene muizen met modificaties in genen betrokken bij het ontstaan van kanker. Verschillende transgene muismodellen zijn inmiddels geëvalueerd als alternatief voor de chronische bioassay, en de eerste resultaten zijn bemoedigend. Transgene muismodellen bleken goed in staat te discrimineren tussen kankerverwekkende- en niet-kankerverwekkende stoffen, met een enorme reductie van het aantal vals-positieve stoffen. Bovendien bleek dit mogelijk met 3 tot 4 keer minder dieren in een 3 keer kortere tijdsduur. Nadeel van de transgene muismodellen is echter het incidenteel voorkomen van vals-negatieve resultaten. Een combinatie van een chronische bioassay met ratten met een kortdurende carcinogeniteits assay met transgene muizen elimineert deze vals-negatieve resultaten volledig, bovendien kunnen kankerverwekkende en niet-kankerverwekkende stoffen met een zeer hoge nauwkeurigheid (85% correct) geïdentificeerd worden. In vergelijking met de nauwkeurigheid (69%) van de huidige test strategie (twee chronische bioassays) is dit een aanzienlijke verbetering, en toepassing van deze combinatie lijkt daarom op dit moment de beste strategie om kankerverwekkende stoffen te identificeren.

De genoemde voordelen van transgene muismodellen hebben er inmiddels toe geleid dat de FDA en de EMEA/CPMP de combinatie van één chronische bioassay met ratten en een test met transgene muizen accepteren als reguliere testmethode voor geneesmiddelen. De tot nu toe gebruikte tweede chronische bioassay met muizen komt hierbij te vervallen. Echter, voor een bredere toepassing van transgene muismodellen in reguliere carcinogeniteitstesten zal een uitgebreidere evaluatie met meer stoffen en nieuw ontwikkelde transgene modellen moeten worden uitgevoerd, met de focus op modellen die geen vals-negatieve resultaten meer laten zien.

1 Introduction

In modern, western society, new chemical compounds and pharmaceuticals are constantly developed that need to be tested for their potential hazardous effect to the human population. Of these, especially compounds capable of inducing cancer are of major health concern to the population. Therefore, proper toxicological testing is required for all these chemicals before being applied.
On top of this, large amounts of existing chemicals need to be tested the next years owing to the REACH Consultation Document, describing the future chemicals legislative system for Europe. The REACH document requires that data must be available on intrinsic properties (for example toxicity, genotoxicity, carcinogenicity) of all substances manufactured in volumes greater than 1 ton per year. At the moment, only a small proportion of all chemicals (~ 3%) is completely analyzed and meets the requirements of REACH. Obviously, it is to be expected that large amounts of regulatory toxicity tests will be carried out the coming years under the newly proposed REACH system. Therefore, the search for alternative test systems that will practically enable the analysis of probably thousands of chemical compounds is of the highest importance. Especially alternative test systems that will require less time, animals and money, and that will furthermore improve the accuracy of the classical tests will be essential. As such, short-term carcinogenicity assays with transgenic mice appear an attractive candidate to substitute for the lifetime bioassay currently routinely used.
Over 30 years ago, the lifetime rodent bioassay was developed to test compounds for their carcinogenic potential, after it was found that both rats and mice developed tumors following exposure to a known human carcinogen (Goldberg 1973; Boorman et al. 1994; Rall 2000). The lifetime bioassay with both rats and mice is currently mandatory in carcinogenicity testing of new chemicals and pharmaceuticals (Goldberg 1973; Lave et al. 1988; Gold et al. 1998). However, as is probably the case for any assay applied in toxicology testing, the lifetime bioassay has numerous disadvantages (Huff 1999). Namely, the bioassay is an experimental ‘black box’, since no information becomes available on the mechanism of action of a compound. Next, bioassay animal results require extrapolations from animals to humans and from high dose to low dose, frequently over enormous dose range findings, thereby increasing the probability of miscalculations. The assay is furthermore associated with extremely high costs (about 1 million euro per chemical), enormous amounts of animals (approximately 1000 per compound for two species), and a very long experimental time period (3-5 years total including organizing, conducting, analyzing and interpreting the results). Recently, it has also become clear that some of the tumor responses in rats and mice are due to specific responses of these species that are not relevant for human risk. Examples of these are the development of nephropathy and tumors in the kidney of male rats (induced by for example D-limonene, unleaded gasoline) (MacClain 1994), and liver tumors in the mouse induced by a large class of chemical compounds (Bucher 2000). Finally, and perhaps most importantly, many of the compounds tested in the lifetime bioassay are carcinogenic only at doses at or near the “Maximal Tolerated Dose”, raising questions whether these compounds possess a true risk for the human population (Swenberg 1995; Foran 1997). Indeed, since for a large number of these chemicals no indication can be found for an increased cancer risk in humans, they are frequently referred to as so-called ‘rodent carcinogens’. Clearly,

considering all these shortcomings, problems arise when the results of these bioassays need to be used for regulatory purposes, i.e. risk assessment.
Therefore, there is an urgent and continuing need for alternative, better assay systems to predict hazardous effects in humans, with the ultimate goal to better protect humans from these potentially adverse effects. In these alternative assay systems the shortcomings of the lifetime bioassay as described in the previous section should preferable be eliminated, or at least limited significantly. In addition, a valid alternative carcinogenicity assay should successfully identify the known or suspected human carcinogens. Likewise, the new assay should identify compounds negative in the lifetime rodent bioassay as non-carcinogens, and ideally, such an alternative model should also test negatively for the previously mentioned false positive 'rodent carcinogens'.
One such alternative assay system could be a short-term carcinogenicity assay with transgenic mouse models (Ashby 2001). Transgenic mice, with modifications introduced in genes known to be important in the process of tumor development, could potentially provide advantages compared to the lifetime bioassay. Namely, the assays can be much shorter, since tumor development in general is faster in these mice, both spontaneously as well as after exposure to chemical compounds (Marx 2003). Also the number of animals used per dose group can be reduced, since the number of animals responding to the treatment will be increased compared to wild type mice. As a result of all these reductions, costs of the assay will be reduced enormously. It is also to be expected that transgenic animals might provide insight into the mechanism of action of a chemical compound, due to the specific defect introduced into the animal. Finally, since a broad panel of different transgenic models is available, all with modifications in different genes, the models or combinations of the models might provide more accurate information on the cancer risk in humans after exposure to a given agent (Marx 2003).
The goal of this report was to give an update on the usefulness of transgenic mice as alternatives in routine, regulatory carcinogenicity testing. First, we will describe the current procedure for classification of chemical compounds and pharmaceuticals in the EU, and the role of the lifetime bioassay for this. Next, the nature of four transgenic mouse models, most intensively analyzed with regards to their possible application as alternatives for carcinogenicity testing, will be briefly described. In section 4, the guidelines for a lifetime bioassay and a short-term carcinogenicity assay with transgenic mice will be compared. In sections 5 and 6, the performance and prediction of the transgenic mouse models will be presented and discussed, and compared with the lifetime bioassay. Finally, the overall advantages and disadvantages of the transgenic mouse models will be discussed, and recommendations for their use in the future will be presented.

2 Classification of chemicals and the use of lifetime bioassays

Around the world, different protocols and systems are followed to classify chemical compounds and pharmaceuticals. The IARC classification system is well known and IARC classifications are generally recognized and accepted (www.iarc.fr), and therefore, also in this report IARC classifications are provided. However, authorities follow different guidelines. To prevent discrepancies in classification of chemicals in the future, and to stimulate the use of a uniform system, the Globally Harmonised System (GHS) for classification of chemicals has been initiated a few years ago, with the ultimate goal to have a common classification system that is used worldwide (Pratt 2002). To accomplish this, also researchers at the RIVM are involved in drafting the guidelines of this GHS system.
According to IARC classifications, agents are assigned to either group 1 (known human carcinogens); group 2A or 2B (probable or possible human carcinogens) or group 3 (inadequate evidence of carcinogenicity). In the Netherlands, the EU classification system is currently used for classification of chemical compounds (EU document 2001). The European Union describes in their guideline 2001/59/EG the definitions of classification and labeling of dangerous compounds. Compounds that might cause cancer, are classified in three categories:

Category 1: compounds known to be carcinogenic to humans. Enough evidence is available to demonstrate a causal relation between exposure and the development of cancer.

Category 2: compounds treated as if carcinogenic to humans. Enough evidence exists for the assumption of a causal relationship between exposure and the development of cancer. This evidence is based on:
- suitable long-term animal bioassays,
- other relevant information

Category 3: Compounds raising concerns with regards to their potential carcinogenicity in humans, but adverse effects can not be determined properly given lack of information. Animal exposure studies might give indications, however, these are not valid enough for classification in category 2.

Classification in category 1 is based on epidemiological data, whereas classification in category 2 or 3 is based on animal exposure studies. To classify a compound in category 2, positive tumor response data must have been observed in two rodent species in a lifetime bioassay, or a positive response in one species in a bioassay supplemented with knowledge concerning genotoxicity, metabolic and/or biochemical assays, induction of benign tumors, or data from epidemiological studies pointing towards a relationship.
To discriminate between category 2 and 3, a number of criteria are important:
- are carcinogenic responses only observed at MTD dose,
- do tumors only develop in tissues with a high background incidence in the specific strain used (for example liver tumors in specific mouse strains)

- do tumors only develop at the site of application, that is irrelevant for human exposure
- are there results provided from *in vivo* and *in vitro* genotoxicity assays
- is there evidence for the existence of a secondary mode of action (for example chronic stimulation of cell proliferation)
- is the observed response a species specific mechanism of tumor development, not relevant for humans (kidney tumors rat for example)

To discriminate between category 3 and non-classifiable, several important issues are raised that exclude a potential hazard of the compound to humans:

- a compound will not be classified when the mechanism of tumor induction is clearly known, and can not be extrapolated to the human situation
- the only data available are obtained using specific, sensitive for liver tumorigenesis, mouse strains, without additional evidence
- special emphasis should be given to tumor development only at target sites known to be sensitive in that specific strain spontaneously.

Clearly, results obtained with long-term *in vivo* experiments with rodents play an important role in the process of classification of chemical compounds into the different categories in the EU. More specifically, usually a long-term rodent bioassay with two species is required to enable classification (Boorman et al. 1994). For this, rats and mice are the most frequently used ones, given their relatively short life span, the limited cost of their maintenance, their widespread use in pharmacological and toxicological studies, their susceptibility to tumor induction, and the availability of inbred or sufficiently characterized strains.
Lifetime bioassays are carried out following OECD guidelines (see for a detailed description the "OECD guidelines for testing of chemicals, section 4: Health effects"). For pharmaceuticals, additional guidance on the evaluation of carcinogenicity is provided in EMEA/CPMP guidelines (CPMP/SWP/2877/00; Note for guidance on carcinogenic potential).
Briefly: both sexes, post-weaning animals, 50 animals of each sex/dose group, at least three dose levels (necessary for risk assessment) with the highest dose inducing minimal toxicity (< 10% reduction in body weight) and proper control groups should be used. The main routes of administration applied are oral, dermal and inhalation, depending on the characteristics of the test compound. The duration of the study comprises the majority of the normal life span of the animals. For mice, this currently means termination of the study at 18-24 months, and for rats the bioassay is terminated at 24 months, but these time periods alter according to the life span of the specific strain applied. Finally, extensive histopathological examination needs to be carried out on all animals and all tissues.

3 Transgenic mouse models analyzed as alternatives in carcinogenicity testing

The rational for using transgenic mice in regulatory carcinogenicity testing is that modifying the mouse genome by either removing or adding specific genetic material might result in animals more sensitive to carcinogenic compounds (Tennant et al. 1995; 1999). As such, these animals would respond more strongly to the potential carcinogenic effect of unknown compounds, and would therefore be a more sensitive predictor of carcinogenic risk to humans. The increased sensitivity of mice can be created by:
I) removing genes whose gene products are necessary to prevent cells to become cancerous, for example tumor suppressor genes and DNA repair genes. Removing genes in mice is routinely carried out through a process called homologous recombination in Embryonic Stem (ES) cells followed by the generation of chimaeric animals (Robertson 1991; Bradley et al. 1992).
II) adding (multiple) copies of activated genes such as oncogenes to the genetic material of the mouse, thereby changing normal cells into initiated, pre-cancerous ones (Hogan et al. 1986). Adding copies of active genes to the mouse is done by pronuclear injection. Since the discovery of transgenesis in the early 1980s the technique of generating transgenic mice has evolved enormously, resulting in a broad panel of different transgenic mouse models in which genes somehow involved in the process of (inhibition of) tumor growth are either removed or added (Marx 2003). Recently, the analysis has started of the most promising ones with respect to their possible application in routine carcinogenicity testing; the $p53^{+/-}$, $Xpa^{-/-}$, Tg.AC and rasH2 model. Therefore, a brief introduction to these models will be given below.

3.1 Increased sensitivity by deleting tumor suppressor genes: $p53^{+/-}$ knockout model

3.1.1 The p53 protein

p53 is a tumor suppressor protein, primarily involved in preventing cells to become cancerous (Lane 1992; Levine 1997; Prives and Hall 1999). Its importance is clearly demonstrated by the observation that over 50% of human cancers harbor mutations in this gene (Hainaut and Hollstern 2000). Moreover, patients with a hereditary form of cancer have been identified carrying germ-line mutations in p53 (Malkin 1994). These so-called Li-Fraumeni patients develop a wide spectrum of tumors at an early age (~30 yrs). Clearly, mutations in p53 are associated with the development of tumors in humans.
In a normal, non-cancerous cell, p53 is present in the cell in a latent form. However, the protein acts upon the induction of DNA damage by chemical compounds or physical compounds such as UV-light and gamma radiation. As a result, p53 directs the cell either into a 'programmed cell death' pathway also called apoptosis, thereby removing cells with damage. Alternatively, p53 arrests the cell in its cell division cycle to allow for removal of the DNA damage by repair pathways before the damage will be fixed into mutations (Vousden and Lu 2002). Malfunctioning of p53 therefore results in sustained DNA damage in cells. As a consequence, the presence of DNA damage in cells may lead to mutations in critical genes, for example proto-

oncogenes and tumor suppressor genes, ultimately resulting in cancer (Kinzler and Vogelstein 1996), see also Figure 1 at page 12).

3.1.2 $p53^{+/-}$ mice

To further elucidate the normal function of p53 as well as its preventive role in the development of tumors, *p53* knockout mice that completely lack the gene product were generated and extensively studied by different groups (Donehower et al. 1992; Jacks et al. 1994). Homozygous *p53* knockout mice ($p53^{-/-}$) are viable but develop tumors at a very early age. Already at 6 months of age, most *p53* knockout mice die because of the development of mainly lymphomas and sarcomas. Heterozygous *p53* mice ($p53^{+/-}$) have a less severe phenotype and a low spontaneous tumor incidence up to 9 months of age, but are more prone to tumors when they get older, with a 50% survival of the animals at 18 months of age. The spontaneous tumors these animals develop comprise again sarcomas and to lesser extent lymphomas. In the first studies employed, $p53^{+/-}$ mice appeared highly sensitive to tumor induction by a number of different, in particular genotoxic compounds, for example gamma radiation and 7,12-dimethylbenz[a]anthracene (see for extensive review Attardi and Jacks 1999). Approximately 50% of the tumors these mice develop have lost their remaining wild type p53 allele, through a process called Loss of Heterozygosity (LOH). But, apparently also reduction of gene dosage has an impact on tumor growth (Venkatachalam et al. 1998). Given their diverse response to chemical and physical compounds $p53^{+/-}$ mice seemed an attractive candidate for routine testing. Based on the diverse functions of the p53 protein after the induction of DNA damage, it is to be expected that the $p53^{+/-}$ model will mainly respond to genotoxic compounds, but a response to non-genotoxic compounds cannot be excluded.

3.2 Increased sensitivity by deleting DNA repair genes: $Xpa^{-/-}$ knockout model

3.2.1 Nucleotide Excision Repair and the Xpa gene

Nucleotide Excision Repair (shortly NER) is one of at least 5 different DNA repair pathways used by the cell to remove DNA damage. Each repair pathway is involved in the removal of a specific subset of DNA damage, and as such, NER is involved in the removal of DNA damage introduced by UV-light and bulky adducts and cross links introduced in the DNA by a broad range of chemical compounds. Several genes have been shown to be involved in NER, and human patients exist with mutations in some of these genes. The majority of these so-called Xeroderma Pigmentosum (XP) patients is highly sensitive to UV-light, and develops skin cancer with high incidence at a very young age. So clearly, a dysfunctional NER pathway is associated with increased tumor development upon exposure to DNA damaging agents (Hoeijmakers 2001). Due to these skin cancers, frequently accompanied by metastases, in combination with neurological abnormalities some of these XP patients develop, XP patients die at young age (Cleaver and Kraemer 1995). One of the genes involved in NER is XPA (see also Figure 1), and Xeroderma Pigmentosum patients carrying germ line mutations in this XPA gene are amongst the most tumor prone XP patients. It is now well understood that the Xpa protein plays an important role in DNA damage verification (Hoeijmakers 2001).

3.2.2 Xpa$^{-/-}$ mice

Two groups have generated mice lacking the Xpa gene, both yielding mice with an interesting and highly comparable phenotype (De Vries et al. 1995; Nakane et al. 1995). Xpa$^{-/-}$ mice appear normal and are viable, and hardly develop tumors if not exposed to physical or chemical compounds. Only a very low incidence of liver adenocarcinomas is observed after the age of 15 months (De Vries et al. 1997). However, the first *in vivo* experiments using different exposure routes and a broad panel of genotoxic compounds, such as benzo[a]pyrene (oral gavage), UV-light (skin), DMBA (skin) and 2-AAF (in feed), show that Xpa$^{-/-}$ mice develop tumors more rapidly and with a higher incidence compared to wild type mice (De Vries and Van Steeg 1996; Van Steeg et al. 2000; Van Steeg et al. 2001). This increased tumor development is preceded by increased levels of primary DNA damage, followed by increased mutation frequencies (De Vries 1997; Van Steeg et al. 2000).

3.3 Increased sensitivity by adding Ha-ras (proto)-oncogenes: Tg.AC and rasH2 models

3.3.1 v-Ha-ras oncogene

The ras gene family comprises a group of (proto)-oncogenes, including Harvey (Ha)-, Kirsten (Ki)- and Neuroblastoma (N-) ras. In a normal cell, ras genes play important roles in controlling cell proliferation, cell differentiation and inhibition of cell death by apoptosis (Bos 1998). Therefore, defects in ras proto-oncogenes can lead to its activation into an oncogene, ultimately resulting in uncontrolled cell growth. Overexpression of or mutations in ras genes are frequently observed oncogenetic changes in specific human tumor types, for example pancreatic-, colon-, and lung-cancer (Bos 1989). Moreover, defects in ras genes are also frequently observed in spontaneous and chemically-induced tumors in rodents. (Maronpot et al. 1995). Evidently, modifications of ras genes are associated with human cancer (see Figure 1).

3.3.2 Tg.AC mice

Tg.AC mice are transgenic mice harboring multiple copies (approximately 20) of a mutant mouse *v-Ha-ras* gene under control of a ξ-globin promoter (Leder et al. 1990). The transgene is silent, until activated by wounding, UV irradiation or specific chemical exposure. The model can be used testing chemical compounds on the skin after topical application. In response to these treatments, Tg.AC mice develop squamous cell papillomas or carcinomas. Also oral administration results in tumor responses in the skin of these mice, in addition, tumors in the forestomach are observed (Tennant et al. 2001). For testing however, the dermal application is currently the most acceptable route. Interestingly, the Tg.AC model responds to topical application with tumor promoters such as phorbol esters. Therefore, the Tg.AC ras model can be viewed as a genetically initiated model, and as such, is expected to respond to both genotoxic and non-genotoxic agents. Spontaneously, less than 4% of the control animals develop tumors at the site of application when treated with the solvent, other tumors occur in a broader range of tissues with low incidence (Tennant et al. 2001). Major drawback of this model however is that the mechanism of response by the Tg.AC model to chemical carcinogens is not well understood.

3.3.3 rasH2 mice

rasH2 mice carry multiple (3) copies of a normal (non-mutated) human H-ras gene under control of its own (human) promoter (Saitoh et al. 1990). Low expression levels of the transgene are observed in almost all mouse tissues, but after exposing the mice to chemical carcinogens, the expression of H-ras is increased (approximately 2-fold). The idea is that increased expression of wild type ras cooperates with other, additional, changes induced by the carcinogenic compound, eventually resulting in a predisposition to develop tumors. Since these additional changes can be both genetic as well as epigenetic, the rasH2 model is expected to respond to both genotoxic as well as non-genotoxic agents. Spontaneously, the life span of the transgenic mice is reduced compared to wild type mice (Tamaoki 2001). Also, RasH2 transgenic mice start developing tumors spontaneously at the age of 6 months, including lung adenomas/adenocarcinomas, forestomach papillomas, Harderian glandadenomas, splenic hemangiomas/hemangiosarcomas and lymphomas.

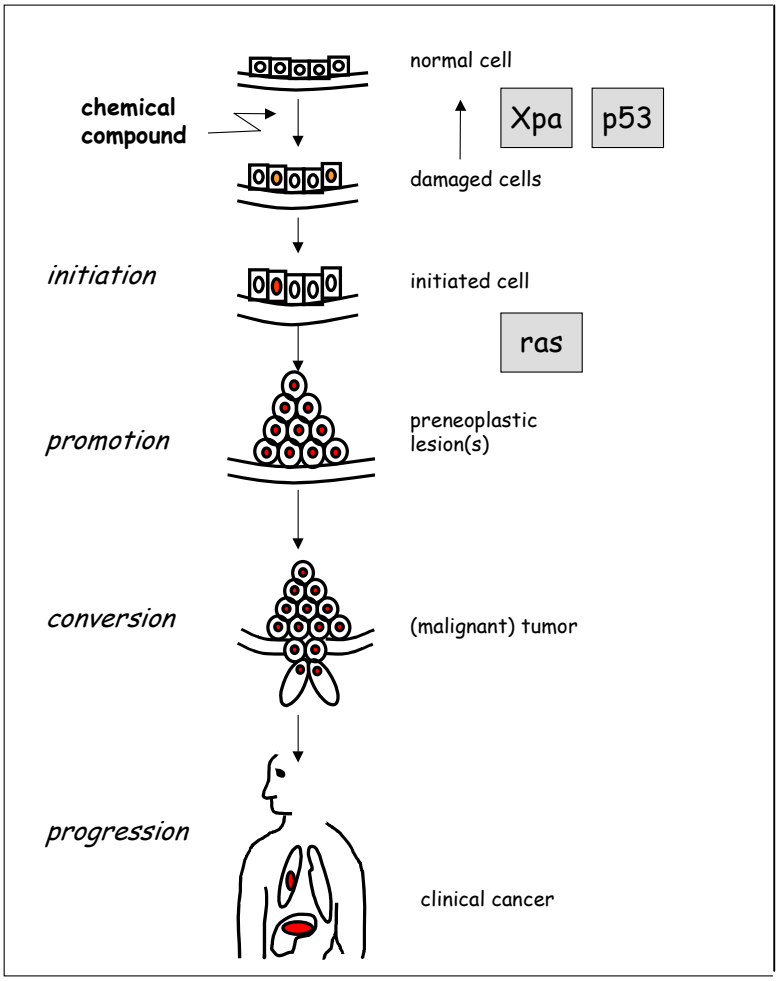

Figure 1: Initiation-promotion model for cancer development. Genes inactivated/activated in the transgenic models currently frequently used in carcinogenicity testing are depicted at the specific stages.

4 ILSI/HESI Alternatives to Carcinogenicity Testing project

Scientists involved in basic carcinogenicity research, regulatory carcinogenicity testing and risk assessment have suggested for quite a long time that transgenic mice might provide useful tools in the identification of human carcinogens (Tennant 1993; Cohen et al. 2001). A major step forward was achieved when the International Conference on Harmonisation (ICH) Expert Working Group on Safety suggested that, under certain circumstances, data from alternative transgenic assays could be used in safety evaluation instead of a second rodent bioassay (i.e. mouse). This led to the publication of a new ICH guidance on testing of new pharmaceuticals for carcinogenicity in 1998, and international regulatory guidelines now allow for use of a short-term, alternative bioassay in transgenic mice as a substitute for a second long-term rodent bioassay (International Conference on Harmonization, Expert Working group on Safety. Draft guideline on testing for carcinogenicity of pharmaceuticals).
This important, significant change in regulatory guidelines for testing of carcinogenicity was preceded and greatly facilitated by an initiative of the Health and Environmental Sciences Institute (HESI) branch of the International Life Sciences Institute (ILSI), to evaluate transgenic models as such alternatives. Within this initiative, named the ILSI/HESI Alternatives to Carcinogenicity Testing (ACT) Project, several academic laboratories, the National Toxicology Program (NIEHS/NTP) in the U.S.A., the Central Institute for Experimental Animals (CIEA) in Japan, and the National Institute for Public Health and the Environment (RIVM) in the Netherlands collaborated in both developing but more importantly, in evaluating new transgenic models (Robinson and MacDonald 2001). The NTP, CIEA and RIVM all collaborated with pharmaceutical and chemical companies in testing primarily pharmaceutical agents (see for detailed overview of membership of ILSI/HESI ACT project Toxicologic Pathology, supplement volume 29, 2001, pages 2-4). In total, over 50 laboratories worldwide have participated in this collaborative research program. It was believed that the ILSI/HESI initiative might provide a better basis for understanding and interpretation of rodent bioassays and alternative short-term assays, with the ultimate goal to provide more reliable rodent assay results that accurately predict the carcinogenic feature of a certain compound in humans.

The specific aims of the ILSI/HESI ACT project were (Omenn 2001):

1. Develop and apply new methods for assessment of potential carcinogenic risk to humans from a great variety of chemicals;
2. Stimulate a cooperative scientific effort;
3. Promote consideration of use of new tests and data in risk assessment.

A total of five transgenic models were evaluated in a collaborative effort; namely the $p53^{+/-}$, rasH2, Tg.AC, $Xpa^{-/-}$ (further referred to as Xpa model) and $Xpa^{-/-}p53^{+/-}$ (cross between $Xpa^{-/-}$ and $p53^{+/-}$ mice, further referred to as Xpa/p53 model) transgenic models. In addition, two non-transgenic alternatives were evaluated, the Syrian Hamster Embryo (SHE) transformation assay and the neonatal mouse models. Given the objectives of this report, the latter two will not be further discussed, but detailed information can be found in Toxicologic Pathology, supplement volume 29, 2001.
Uniform standard protocols, pathology review and statistical evaluations were developed. A total of 21, predominantly pharmaceutical, chemicals were evaluated, including genotoxic

carcinogens, non-genotoxic carcinogens and non-carcinogenic chemicals (see for a complete list of chemicals tested and information on mechanism of action Table 1 at page 15, and see for more extensive background information the following webpage: www.hesi.ilsi.org). For all the pharmaceuticals analyzed, extensive data, highly relevant to human risk assessment, were available on exposure, pharmacodynamics and pharmacokinetics in humans. Additional compound selection criteria included the availability of long-term carcinogenicity data from the lifetime bioassay (1 or 2 species), characterization of genotoxic potential and information on mode of action. In addition to the pharmaceutical compounds, a group of non-pharmaceuticals with the same information available was included.
The ILSI/HESI ACT project turned out to be very successful, in that the project provided information that can be used as a start to evaluate the use of transgenic mice in regulatory carcinogenicity testing. A summary of the responses of the individual transgenic mouse models to the different compounds is depicted in Table 2 at page 16, and detailed information can be found in: Eastin et al. 2001; Storer et al. 2001; Usui et al. 2001; Van Kreijl et al. 2001.

A number of conclusions can be drawn from the data obtained in the ACT project. First, all non-carcinogens tested are indeed negative in all 5 transgenic models, providing evidence against the concern of "oversensitivity" of transgenic mice with modifications in cancer-related genes. Second, the majority of rodent carcinogens/putative human non-carcinogens that were scored positively in the lifetime bioassay are negative in the transgenic models. However, the exact number of rodent carcinogens negatively identified appeared dependent on the specific model. For example, in $p53^{+/-}$ mice, 10 out of 12 rodent carcinogens tested were negative, and the remaining 2 showed only an equivocal response. For the rasH2 model, the response was highly comparable, i.e. 2 out of 11 scored positive. Only in the Xpa/p53 double transgenic mouse model none of the rodent carcinogens were positive, however, the amount of rodent carcinogens tested in this model (5 total) is too limited to make firm conclusions. But in general, the transgenic mice appeared not overly sensitive and not more subject to false positives, but rather reduced the number of false positives. In contrast, it was disappointing and quite unexpected that the transgenic mouse models were not capable of detecting all known human carcinogens, thereby leading to false negatives (see Table 2). For example, none of the models, except the rasH2 strain, was capable of detecting the carcinogenic property of the human carcinogen phenacetin. The explanation for this could be that phenacetin is a rather weak carcinogen, and that the time period in which the compound is analyzed in the transgenic assay (generally 6 months) is not long enough to detect a significant tumor induction (see also section 5 for information about exposure times). Also estradiol, a genotoxic human carcinogen, was not positively identified in all assays. Interestingly however, the non-genotoxic immunosuppressive compound cyclosporine A was positive in all models, arguing against the idea that some of these models ($p53^{+/-}$, Xpa) are only capable of detecting genotoxic carcinogens.
In conclusion, the ILSI/HESI ACT project showed that these specific transgenic mouse models provide good alternative assay systems for the lifetime bioassay to detect carcinogens and non-carcinogens, but the models appear to have shortcomings that need to be addressed. In particular the negative response to known human carcinogens is of concern. However, the advantages of these models that are evident have resulted in the fact that the FDA and the European CPMP now allow the use of transgenic mice as an alternative for a second lifetime bioassay, when a 2-year bioassay with rats has been carried out. Nonetheless, a more extensive validation of these models using more compounds with different modes of action needs to be carried out in the future.

Table 1: Compounds used in the ILSI/HESI Alternatives to Carcinogenicity Testing Project

class	*compound*	*genotoxic*	*application*	*target tissue lifetime bioassay*
Genotoxic human carcinogens	Cyclophosphamide	+	anti-neoplastic	heamatopoietic/urinary bladder/various
	Melphalan	+	anti-neoplastic	haematopoietic/lung/peritoneal
	Phenacetin	+	analgesic/antipyretic	urinary tract/lung/liver/various*
Immunosuppressant human carcinogens	Cyclosporin A	-/eq[1]	anti-rejection	heamatopoietic
Hormones	Diethylstilbestrol	+/eq[3]	synthetic estrogen	mammary/uterus/ovarian
	Estradiol	+/eq[4]	natural estrogen	mammary/cervix/uterus/renal
Rodent carcinogens/putative human non-carcinogens (epidemiology)	Phenobarbital	-	sedative	liver
	Clofibrate	-	hypolipidemic	liver
	Reserpine	-/eq[6]	antihypertensive	adrenal/seminal vesicle/mammary
	Dieldrin	-	insecticide	liver
	Methapyrilene	+/-[5]	antihistamine	liver
Rodent carcinogens/putative human non-carcinogens (mechanism)	Haloperidol	-	antagonist dopamine rec.	pituitary/mammary
	Chlorpromazine	-	antipsychotic	liver
	Chloroform	-	solvent	liver/kidney
	Metaproterenol	-	adrenergic agonist	ovary/mesovarium/liver
	WY-14643	-	hypolipidemic	liver
	DEHP	-/eq[2]	plasticizer	liver
	Sulfamethoxazole	-	antibacterial agent	thyroid
Non-carcinogens	Ampicillin	-	anti-bacterial agent	-
	D-Mannitol	-	flavor enhancer food	-
	Sulfisoxazole	+/-[7]	anti-bacterial agent	-

[1]only pos. in SCE assay with human peripheral lymphocytes.
[2]pos. only in inducing dominant-lethal mutations in mice after systemic administration.
[3]Ames/Salmonella neg., *in vitro* aneuploidy pos., SHE assay pos., UDS and DNA adducts *in vivo* pos
[4]Ames/V79 neg., SCE *in vitro* neg., structural CA pos *in vitro*, aneuploidy pos., SHE assay pos. micronuclei *in vitro* pos.
[5]indirect genotoxic mechanism
[6]only pos. in SHE transformation assay.
[7]pos. in mutations in mouse lymphoma cells, pos. for SCE in CHO cells
*results controversial

Table 2: Response of different transgenic mouse models to compounds tested in ILSI/HESI ACT Project

Compound	*IARC Class.*	*p53*$^{+/-}$	*Xpa*	*Xpa/p53*	*rasH2*	*Tg.AC*	
						Topical	*diet/gavage*
Cyclophosphamide	1	+	nd	nd	+, +, eq	eq	+
Melphalan	1	+	nd	nd	nd	eq	+
Phenacetin	2A	-,-	-	-	+	-	-
Cyclosporin A	1	-,+,+	+	+	eq	+	eq
Diethylstilbestrol	1	-,+	+	+	+	+	-
Estradiol	1	-,eq	-	+	-	+	-
Phenobarbital	2B	-,-	-	-	-	inadeq	inadeq,inadeq
Clofibrate	3	-,-	inadeq	nd	eq,+	+	nd
Reserpine	3	-	-	-	-	-	-
Dieldrin	3	-	nd	nd	-	nd	nd
Methapyrilene	NE	-,-	nd	nd	-	-	nd
Haloperidol	NE	-	-	-	-	nd	nd
Chlorpromazine	NE	-,-	nd	nd	-	nd	nd
Chloroform	2B	eq	nd	nd	-	nd	nd
Metaproterenol	NE	-,-	nd	nd	-	nd	nd
WY-14,643	NE	-	+	nd	nd	-	eq
DEHP	3	eq	-	-	+	-	-
Sulfamethoxazole	3	-	-	-	-	-	-
Ampicillin	3	-	-	nd	-	nd	nd
D-Mannitol	NE	-	-	-	-	nd	nd
Sulfisoxazole	3	-	nd	nd	-	-	-

5 Comparison of guidelines short-term transgenic carcinogenicity assay with lifetime bioassay

As was shown by the ILSI/HESI ACT project, transgenic models are well capable of identifying carcinogens and non-carcinogens. Moreover, the models analyzed appeared less sensitive towards the positive detection of rodent carcinogens. Comparing the transgenic carcinogenicity assay with the standard lifetime bioassay also reveals more practical advantages that will be briefly discussed below.

5.1 Time period of the assays

The lifetime bioassays (both mice and rats) are carried out following OECD guidelines. The length of the assay comprises approximately the normal life span of the animals. For mice, this generally means termination of the study at 18-24 months (OECD 1996). The alternative carcinogenicity assays with transgenic mice as carried out in the ILSI/HESI ACT project originally lasted 6 months (Petitt 2001). However, during the course of the project, it became evident that this was probably too short since some of the positive controls in the studies were not in all cases positive (for example lack of positive response after oral exposure to benzo[a]pyrene in the Xpa/p53 model). This resulted in inadequate studies, and moreover, might provide an explanation for the troublesome observation that some carcinogenic compounds were negative in the transgenic models (see previous section). Therefore, through an initiative of the RIVM participants, it is now recommended to expose transgenic mice 9 months to the compound of interest. If routinely applied, using transgenic mice would result in a 2 to 3 fold reduction in time needed to perform a carcinogenicity assay.

5.2 Number of animals

In the lifetime two-year bioassay approximately 1000 animals (500 rats; 500 mice) are used (including sentinel animals). For the mouse bioassay the following groups are used according to NTP guidelines:

	animals		*sexes*		*test-groups*		*total*
treatment	50	x	2	x	3	=	300
controls	50	x	2	x	1	=	100
							400

For a short-term carcinogenicity assay with transgenic mice, the following groups are now recommended according to the ILSI/HESI ACT project guidelines:

	animals		*sexes*		*test-groups*		*total*
wild type							
treatment	15	x	2	x	1	=	30
controls	15	x	2	x	1	=	30
transgenics							
treatment	15	x	2	x	3	=	90
controls	15	x	2	x	2*	=	60
							210

*including positive compound control

However, if short-term carcinogenicity assays with transgenic mice are fully validated, it is envisioned not to be necessary to include wild type mice in the assay, since the analysis of these mice does not provide additional information not obtained with the transgenic mice. Furthermore, including a positive control compound can be omitted. Therefore, 120 transgenic mice would be sufficient to demonstrate the carcinogenic potential of a compound, and as a result, the number of mice used has decreased 3 to 4 fold compared to the lifetime bioassay.

5.3 Costs

The costs associated with the lifetime bioassay are enormous, namely ~ 1.000.000 euro per compound. This is, amongst others, due to the high numbers of animals used (housing costs), extensive pathology of all tissues, the long time period and finally the organization of such enormous experiments. Given the reductions associated with the use of transgenic mice as outlined above (3 to 4 fold in number of animals and 2 to 3 fold in exposure time), and the associated reduction in histopathological analyses that need to be performed, the costs of a short-term carcinogenicity assay with transgenic animals could, roughly estimated, be reduced by approximately 50% compared to the lifetime bioassay.

6 Accuracy of short-term transgenic carcinogenicity assays compared to the lifetime bioassay

As outlined in chapter 4, the ILSI-HESI ACT project made it possible to compare five transgenic mouse models with respect to their response to a panel of compounds with defined mode of action. However, for a proper validation of the usefulness of transgenic mice in routine carcinogenicity testing, the amount of compounds analyzed in this project is limited. Moreover, the choice of compounds analyzed was primarily restricted to pharmaceuticals, whereas for regulatory purposes it is of course also important to analyze the response of transgenic mice to chemical compounds with broader application.

A much more extensive examination of data available on the response of transgenic mice to chemical, physical as well as pharmaceutical compounds was carried out by the NIEHS in collaboration with the National Toxicology Program (NTP) (Eastin et al. 1998; Bucher 1998; Pritchard et al. 2003). The NIEHS has been involved in the effort to develop and analyze alternatives for carcinogenicity testing for more than a decade, including the evaluation of several different transgenic and knockout mouse models (Tennant et al. 1993; Tennant et al. 1996; Tennant et al. 1999). In the examination described here, existing and predominantly published data on the use of transgenic mouse models for identification of human carcinogens was evaluated, with the focus on the three most extensively studied and used models. These are the $p53^{+/-}$, Tg.AC and RasH2 models. In addition to the analysis by NIEHS of these models, we have included data obtained with the Xpa and Xpa/p53 model in this Chapter, mostly obtained in our own laboratory and through collaborations of our laboratory with others (De Vries and Van Steeg 1996; Van Steeg et al. 2000).

In total, data on 99 chemicals were evaluated. Unique in this evaluation is the comparison that is made between the response of the different transgenic models to the compounds and the data obtained with the lifetime bioassay with both rats and mice with the same compounds. Also, IARC classification of all chemicals is provided (see for information on IARC classification Chapter 2), allowing the analysis of specific subgroups of chemicals with regard to their carcinogenic potential. For the group of chemicals, 14 known human carcinogens (IARC classification I), 32 probable or possible human carcinogens (IARC classification 2A or 2B) and 53 non-carcinogens (IARC classification 3 or not evaluated) were analyzed. A list of compounds analyzed is provided in Appendix III, and detailed information on the specific compounds can be found at the following webpage: http://ehp.niehs.nih.gov/roc/toc10.html.

Table 3: Performance of single transgenic mice versus likelyhood of human cancer (compounds with IARC classification 1, 2A or 2B, classification 3 as true non-carcinogens)

strategy	*positive for carcinogens*	*negative for non-carcinogens*	*positive for non-carcinogens*	*negative for carcinogens*	*overall accuracy*
$p53^{+/-}$	21	27	1	10	81% (48/59)
$p53^{+/-}$ (g)	16	6	0	4	85% (22/26)
Tg.AC	17	29	10	6	74% (46/62)
rasH2	21	18	5	7	76% (39/51)
Xpa and/or Xpa/p53*	7	8	1	2	83% (15/18)
NTP lifetime bioassay	**23**	**17**	**18**	**0**	**69% (40/58)**

(g); genotoxic compounds only *adapted from: Pritchard 2003*

*; depicted response detected in either one of the two models or both

It is clear from Table 3 that on the whole all transgenic models are capable of discriminating carcinogens from non-carcinogens. The models display an overall accuracy, defined as correct prediction of carcinogens and non-carcinogens, ranging from 74 to 83%. The $p53^{+/-}$ and Xpa or Xpa/p53 models display the highest prediction, with 81% and 83 % respectively. However, as was the case within the ILSI/HESI ACT project, the number of chemicals analyzed in the latter two models is limited. When the analysis of the responsiveness of the $p53^{+/-}$ model is restricted to genotoxic compounds only, the prediction is even higher, i.e. 85%. This indicates that the $p53^{+/-}$ model is slightly more sensitive to genotoxic compounds than to non-genotoxic compounds. The two transgenic models with modified ras alleles both show a lower accuracy, namely 74% and 76%. This is mainly due to the relatively high number of non-carcinogens (IARC classification 3 or not evaluated) that are positively identified in these models, see also Table 4.

Table 4: Positive responses of transgenic models as a function of the IARC classification of the compounds

IARC classification	*$p53^{+/-}$*	*Tg.AC*	*rasH2*	*Xpa and/or Xpa/p53*	*Overall*
1	83% (10/12)	89% (8/9)	57% (4/7)[1]	100% (4/4)	81% (26/32)
2A	62% (5/8)	50% (2/4)	100% (9/9)	50% (1/2)	74% (17/23)
2B[2]	55% (6/11)	64% (7/11)	69% (9/13)	67% (2/3)	63% (24/38)
3	0% (0/13)	21% (3/14)	29% (4/14)	0% (0/6)	15% (7/47)
not evaluated	7% (1/15)	29% (7/24)	0% (0/8)	33% (1/3)	18% (9/50)

[1]two equivocal results *adapted from: Pritchard 2003*

[2]includes 7,12-dimethylbenzanthracene, 4-nitroquinoline*N*-oxide, 4-hydroxyaminoquinoline-1-oxide

Apparently, the ras models still display positive tumor responses upon exposure to these so-called rodent carcinogens, a major drawback of the lifetime bioassay as discussed before. In contrast, it is clear from Table 4 that the $p53^{+/-}$ and Xpa/p53 models mainly show negative results when exposed to compounds with IARC classification 3 or compounds not evaluated. Looking at the results obtained with the $p53^{+/-}$ model for example, all not evaluated compounds are negative,

and only the introduction of a foreign body under the skin, i.e. a transponder, results in the development of tumors at the site of incision, presumably through the induction of cell proliferation (Pritchard et al. 2003). In Xpa/p53 mice, all IARC group 3 chemicals tested are negative, and for the chemicals not classified only WY-14643 appears (weakly) positive (see also Van Kreijl et al. 2001). However, although WY-14643 is not classified, carcinogenicity assays with rodents have shown an increased carcinogenic response (hepatocellular adenomas) in both rats and mice (Reddy et al. 1979). Based on this, WY-14643 is now generally considered to be a non-genotoxic rodent carcinogen, and therefore, could also be considered as an IARC group 3 compound.
Comparing all these results obtained with the transgenic models with an overall accuracy of 69% obtained with the lifetime bioassay as currently applied (Pritchard et al. 2003), clearly shows the increase in accuracy reached when transgenic models would have been used. However, for all transgenic models tested, it is also clear from Tables 3 and 4 that not all known (human) carcinogens are scored positively, whereas the rodent lifetime bioassay detects all these carcinogens (when tested). For example, in the $p53^{+/-}$ model, 2 out of 12 human carcinogens (TCDD and estradiol, both positive in the lifetime assay) are not identified. In the Tg.AC model, 1 out of 9 carcinogens tested (sodium arsenate) is negative, for the rasH2 model this number is 3 out of 7, with the comment that 2 of the 3 negative assays were in fact equivocal. Only in the Xpa/p53 model, all human carcinogens tested so far are positive (4/4, see Table 4). For all models, the negative responses to known carcinogens as depicted in Table 3, are most commonly obtained with group 2A and 2B chemicals. Since these compounds are mainly classified in these groups according to their positive response in the lifetime bioassays, and direct evidence of carcinogenicity in humans is not present, it remains to be seen whether all these compounds are indeed true carcinogens. However, for a routine regulatory assay to detect the carcinogenic property of unknown compounds, it is of course of concern that transgenic models do not detect all true carcinogens.
Another drawback of a standard test system would be the positive scoring of non-carcinogens (so-called rodent carcinogens), as is currently frequently observed in the lifetime rodent bioassay (18 non-carcinogenic compounds scored positive in this assay, see also Table 3). Although the transgenic models in general perform much better in this respect, in that the majority of non-carcinogens scored positive in the rodent bioassay are negative, some compounds are still detected as positive. Especially the Tg.AC and RasH2 models still respond to these chemicals (frequently compounds with promoting effects), which is probably intrinsic to the initiated nature of these models. In $p53^{+/-}$ and Xpa/p53 mice the number of non-carcinogens scored positively has decreased dramatically; in both models only 1 compound is scored positive.

But clearly, if short-term carcinogenicity assays with transgenic models would be applied as the only carcinogenicity assay, not all compounds potentially carcinogenic to humans would be identified. Therefore, the prediction level of the assays is also analyzed if transgenic assays would be used in combinations, either with each other (not shown, see ref Pritchard)) or with the rat lifetime bioassay. Results of these analyses are shown in Table 5, with the note that these comparisons are not calculated for the Xpa/p53 model given the limited amount of chemicals tested.

Table 5: Performance of transgenic mice in combination with the lifetime bioassay versus likelyhood of human cancer(compounds with IARC classification 1, 2A or 2B carcinogens, classification 3 as true non-carcinogens)

strategy	*positive for carcinogens*	*negative for non-carcinogen*	*positive for non-carcinogens*	*negative for carcinogens*	*overall accuracy*
NTP rat & Tg.AC (n-g) or $p53^{+/-}$ (g)	35	13	9	0	84% (48/57)
NTP rat & RasH2 (n-g) or $p53^{+/-}$ (g)	33	12	8	0	85% (45/53)
NTP rodent bioassay	23	17	18	0	69% (40/58)

(g); genotoxic compounds only *adapted from: Pritchard 2003*
(n-g); non-genotoxic compounds only

Clearly, overall performance improved significantly when a combination of the rat bioassay with a transgenic model would be used. The prediction was ~84 % for the two combinations calculated, a significant increase compared to the prediction accuracy of 69% of the bioassay alone. Importantly, the negative results for known carcinogens as observed with the transgenic models and discussed above were completely eliminated. In addition, positive findings for non-carcinogens in the lifetime bioassay (31%) were reduced to ~15%. Therefore, based on these calculations with 99 chemicals, the most promising test strategy would be to, when a lifetime bioassay with rats has been performed, substitute the standard mouse bioassay with a transgenic model. The specific transgenic model to be used for this will be dependent on the genotoxic properties of the specific compound, as analyzed in standard, currently routinely applied, genotoxicity assays (both *in vitro* as well as *in vivo*).

7 Discussion and future perspectives

Taken all results described in this report into account, one can conclude that transgenic mouse models indisputably have the potential to play an important role in the identification of potential human carcinogens. Overall, a strategy employing both conventional assay systems, such as the rat lifetime bioassay, and short-term assays with transgenic mouse models, provides the best strategy to identify the potential carcinogenic risk of chemicals, and is therefore favorable to implement in regulatory toxicity testing (Pritchard et al. 2003). For this, the $p53^{+/-}$ and RasH2 model are now both most acceptable for use in a regulatory context, with the first model best applied for compounds with clear/equivocal genotoxicity (French et al. 2001; Storer et al. 2003). The Tg.AC model is also a frequently requested assay, but is most appropriate for dermal applied products since this is the exposure route most extensively validated in this model (Eastin et al. 2001). However, using the Tg.AC model it is not possible to discriminate between promoters and complete carcinogens, possibly providing difficulties in classifying compounds. The Xpa/p53 model seems very promising to routinely use, but the number of compounds tested is too limited to allow regulatory use at the moment. Therefore, further research is needed to produce a more valid evaluation of this model, using more compounds with known carcinogenic features.
Despite all the clear advantages and strengths of transgenic models compared to the lifetime bioassay, such as: I) a reduction in number of animals needed; II) a reduction of the time period of the assay; III) a reduction of associated costs; IV) less false positive rodent carcinogens identified and V) more information on the mode of action of the chemical (genotoxic property); it is also clear that several issues and data gaps need to be addressed before these assays can be routinely applied (Ashby 2001; Petitt 2001). Also, not all disadvantages associated with the lifetime bioassay as described in the Introduction have been fully eliminated. For example, although the number of false positive rodent carcinogens positively identified with the transgenic models has decreased significantly, the majority of compounds were still analyzed at the MTD dose in transgenic assays. As was the drawback with the lifetime bioassay, this might still result in possible confounding effects of toxicity.
Importantly, optimal protocol designs for transgenic carcinogenicity assays have not been fully identified and evaluated. For example, number of animals, duration of dosing, panel of tissues analyzed by histopathology, total time period of the assay and interpretation of results (classifying an assay as positive/negative/equivocal) varied amongst the assays analyzed in this report. Therefore, the first step now would be to develop an optimal design for a short-term transgenic carcinogenicity assay, with the emphasis on the elimination of the occurrence of false negatives for known human carcinogens in carcinogen identification as currently observed with these models. To achieve this, the amount of animals per experimental group can be increased (25 mice per group instead of 15), to enhance the accuracy of the study. In fact, the FDA recently started requesting 25 mice per group in testing of pharmaceuticals with transgenic models. Changing this guidance for all transgenic models still reduces the number of animals needed ~2 fold compared to the lifetime bioassay. Alternatively, a so-called benchmark approach could be introduced, characterized by a multi-dose study design with smaller number of animals per dose (Slob 2002). This approach has been used before in toxicity tests, and was shown to be a more accurate method for analyzing dose-response data compared to the NOAEL approach (Piersma 2000). Whether the benchmark approach is also useful in the lifetime bioassay needs to be tested of course.

Secondly, the time period of the study can be increased to 9 months instead of the time period of 6 months frequently used at this moment. As was also discussed in Chapter 5, this was already recommended while in the process of evaluating these models, and the 9 months time period is currently routinely applied in the transgenic assays. New initiatives by researchers from several countries, such as that by the International Workshop on Genotoxicity Testing (IWGT, RIVM participates), are aimed on the development of standard, optimized protocols that should, when available, greatly facilitate the implementation of short-term transgenic assays for regulatory testing of all chemicals (Storer et al. 2003).

Finally, the evaluation performed thus far has been restricted to three to five transgenic mouse models. In addition to evaluating existing alternatives in regulatory testing, it is of great importance to also support research programs aimed at developing (even better) alternatives. Over the past few years, our knowledge regarding the development of cancer and the identification of genes involved in cancer development has evolved enormously. As a result, a considerable number of new transgenic mouse models, that are potentially more useful in detecting human carcinogens than the models evaluated up to now, have been developed (Marx 2003). For example mouse models with an inducible expression of genes and transgenic models with subtle point mutations instead of crude deletions are now available (Liu et al. 2000; Bruins et al. 2003). Promising are also the transgenic models that allow tissue-specific deletion of cancer genes, for example the tissue-specific p53 models currently also used in our own laboratory to analyze their potential application in carcinogenicity testing. (Berns 2001; Wijnhoven et al. 2003). Using these models allows the analysis of tumor development by chemicals in specific tissues, that could be used in regulatory testing of compounds with already known (exposure) target sites *in vivo*. Most ideally, a whole arsenal of models will be available in the future to test chemical compounds. Depending on the intrinsic properties of the compound a choice can be made for the best model to be used. Also the genetic background of the transgenic model can be changed, which has been shown to have quite a dramatic effect on tumor development (Reilly et al. 2000). Importantly, new models should be generated that potentially are useful for the testing of non-genotoxic compounds, since the models discussed here appear most sensitive to genotoxic compounds.

Next, combining transgenic mouse models with other alternative approaches for carcinogenicity testing could be a powerful strategy. An alternative potentially useful for this could be the use of genomics, the whole-genome gene expression analysis by micro-array technology, to detect (geno) toxicity and/or carcinogenicity (MacGregor 2003). The rationale for this strategy is that classes of compounds induce common mechanisms such as cellular toxicity or DNA damage, and that these mechanisms can be identified at the level of gene usage, i.e. gene expression. If so, the identification of toxic and/or carcinogenic compounds can be performed much faster (within weeks), using much less animals and lower (more human relevant) doses. Applying genomics in conjunction with transgenic mice is envisioned to result in a very sensitive assay system to detect carcinogens. Research addressing this issue is currently ongoing in our laboratory.

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Appendix I: Abbreviations

2-AAF	2-Acetylaminofluorene
DMBA	Dimethylbenz[a]anthracene
DNA	Desoxyribo Nucleic Acid
ES	Embryonic Stem cell
LOH	Loss Of Heterozygosity
MTD	Maximal Tolerated Dose
NER	Nucleotide Excision Repair
SHE	Syrian Hamster Embryo
UV	Ultraviolet
XP	Xeroderma Pigmentosum
XPA	Xeroderma Pigmentosum Complementation group A

CIEA	Central Institute for Experimental Animals
CPMP	Committee for Proprietary Medicinal Products
EMEA	European Agency for the Evaluation of Medicinal Products
EU	European Union
FDA	Federal Drug Administration
GHS	Globally Harmonised System
IARC	International Agency for Research on Cancer
ICH	International Conference on Harmonisation
ILSI/HESI ACT	International Life Sciences Institute/Health and Environmental Sciences Institute Alternatives to Carcinogenicity Testing
IWGT	International Workshop on Genotoxicity Testing
NIEHS	National Institute of Environmental Health Sciences
NTP	National Toxicology Program
OECD	Organisation for Economic Co-operation and Development