Transgenic Mice in Carcinogenicity Testing

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Introduction

Cancer and carcinogenicity testing

Cancer is a collection of about 200 diseases grouped together because of their similar growth processes. Each cancer, regardless of the part of the body it affects, is believed to originate from a single »transformed cell«. A transformed cell, and its progeny, may grow and multiply to produce a tumour. Studies in human populations and in laboratory animals have linked cancer with exposures to certain substances. (Parke et al., 1993). On the molecular level the initiation of the cancer process lies in activation of protooncogenes, which are normal cellular genes, to oncogenes. Oncogenes are formed in human and animal tumours as a result of mutations leading to the abnormal expression or function of protooncogenes. Because cellular oncogenes are mutated forms of normal cellular genes, they provide a clear indication of the genetic targets that are altered due to exposure to mutagenic carcinogens. Therefore the applicability of oncogenes as markers of tumour development has been the subject of intensive research in the past decade and a clear connection has been shown between the oncogene activation and carcinogenesis (Cooper, 1992).

Carcinogens can be identified through epidemiology, the study of diseases and their determinants in human populations, and through various laboratory tests. Laboratory tests, which do not depend on human illness and death to produce data, have been developed to identify carcinogens. Currently, the testing of suspect chemicals in laboratory animals, generally rats and mice, is the backbone of carcinogen identification. As the animals which have been given the suspect chemical die, or when the survivors are killed at the end of the exposure period, a pathologist examines them for tumours. The number and types of tumours in the exposed animals is compared with the number and types of tumours in the »control« group of animals, which are treated exactly as the experimental group except that they are not exposed to the chemical under test. The finding of a significant excess of tumours in the exposed animals compared with the number found in controls in a well designed, well-executed animal test for carcinogenicity leads to the conclusion that the chemical is a carcinogen in that species.

The new developments in testing are short-term tests which require from a month to a few months to complete, as compared to long-term feeding experiments which may require a year or more. Such tests have been under development for 15 years and most of them depend on biologically measuring interactions between the suspect chemical and the genetic material, DNA.

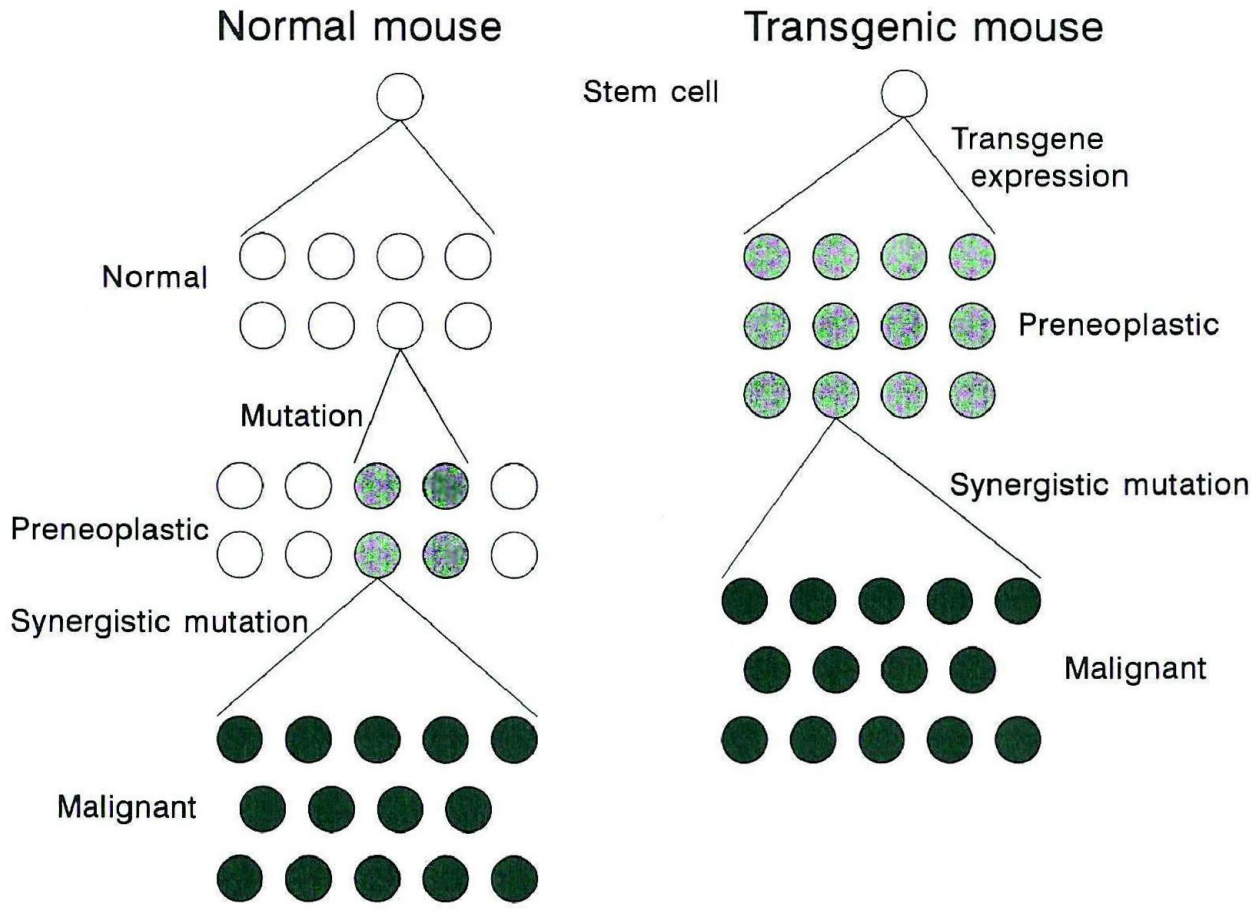
The best known test, the »Ames-test«, measures mutagenicity in bacteria. Many chemicals that have already been identified as carcinogens or non carcinogens in bioassays have also been assayed in short term tests to measure congruence between the two types of tests. Results from these »validation« studies vary but up to 90 percent of both carcinogens and non-carcinogens were correctly classified by short-term tests (Balls et al., 1991).

Potentials of transgenic technology

The ability to transfer oncogenes into the germ-line of animals, which became available over 10 years ago has contributed to our understanding of the function of oncogenes

and their role in genetic diseases and cancer. Although more than 40 oncogenes have been identified, not all of these are frequently encountered in human neoplasms (Reddy et al., 1988). Reproducible activation of about 20 oncogenes has been described in human tumours and some of these genes, which represent potential markers of human neoplasms, have been used in the construction of transgenic laboratory animal models for carcinogenicity studies.

Regardless of the technique used in constructing transgenic animals, the usual goal is to introduce new (foreign) DNA in the form of a defined gene (e.g. oncogene sequence) into the DNA of the host animal and have this new DNA retained in host somatic and germ cells so that it may be propagated across generations (Jaenish, 1988). Introduction of foreign DNA into a host animal can be accomplished in several ways. The most commonly used method was devised by Gordon and Ruddle (1981) and involves the direct micro-injection of purified DNA into the pronucleus of a single cell embryo which is then implanted into a pseudopregnant animal. Gene transfer efficiency with this method is high in mice, with up to 30% of the animals originating from microinjected embryos being transgenic.



Tumorigenesis is potentiated in the transgenic mouse by oncogene expression throughout a cell compartment, creating a preneoplastic population from which a malignant clone eventually evolves. In a normal mouse, mutation imposes significant risk only if the affected cell undergoes clonal expansion; expression of a transgene obviates both these steps. Open circles denote normal cells; grey circles, cells that have acquired one oncogenic alternation, either by transgenic expression or mutation; filled circles, malignant cells.

From: "Transgenic models of tumor development". (1991) Jerry M. Adams and Suzanne Cory. Science, Vol.254 p.1161- 1166.

Another method for directing foreign DNA into the host nucleus utilises retroviruses as a shuttle mechanism. In this system small (generally < 9000 bp) segments of foreign DNA are inserted into the retroviral genome. These retroviruses are then used to infect mouse embryos. This results in the production of single integrants which are minimally disruptive to host DNA but are rather inefficiently expressed. This, coupled with the size limitation for introduced DNA and the difficulty in making the recombinant retroviruses, has made this a less attractive approach for generating transgenic mice (Gordon, 1988).

A third method for generating transgenic mice has recently come to the forefront because it offers the possibility for targeting transgenes to particular sites in the genome. This technique involves use of embryonic stem (ES) cells. ES cells are pluripotent cells established from normal embryos at the blastocyst stage. These cells can be cultured and manipulated in vitro and will contribute to the embryo when implanted into normal blastocysts and transferred to foster mothers. Transfer of genetic material (e.g. oncogenes) is accomplished via microinjection, electroporation, or retroviral infection of the ES cells (Capecchi 1989). One advantage of the ES-cell based technique is that foreign genetic material can be transferred into ES cells and suitable clones selected before the generation of transgenic animals. Mice generated from ES-cell inoculated blastocysts are usually chimeric in both somatic and germ cells for the novel trait. Crosses of heterozygote transgenic permits the generation of homozygote transgenic and allows examination of the phenotypic expression of the trait.

Transgenic mice models based on oncogene insertion as models for carcinogenicity testing

The introduction of oncogenes into mice is a novel approach in investigating the cancer process. The ability to generate oncogene-driven tumours in transgenic mice holds great promise for providing us with a model that closely approaches a »spontaneous« tumour. Linking specific combinations of promoter and structural oncogene elements can result in reasonably predictable, site-specific development of tumours (*Figure 1).*

A breakthrough in rapid carcinogenicity testing in mice after treatment with a given carcinogen came in 1986 when the first transgenic mice – the so called »Onco-mouse« which contained specific human oncogenes – was developed and later patented by Philip Leder and his colleagues at Harvard (Leder et al., 1986). They produced multiple transgenic mouse strains containing a c-myc gene fused to the glucocorticoid sensitive promoter of mouse mammary tumour virus (MMTV). The majority of transgenic strains expressed the myc gene in breast tissue and developed mammary carcinomas.

The ras oncogenes in transgenic mice

The ras family of oncogenes has been implicated in the development of neoplasms in a wide variety of human and animal tissues. Studies using transgenic laboratory animals have demonstrated that activated ras oncogenes can exist in normal cells for long periods of time before the onset of neoplasia (Nielsen et al., 1991). More than 40 different individual protooncogenes (c-onc) exist in human genome (Cordaro 1989). They are divided into families according to their products. The ras family c-K-ras and c- H-ras code for the best known membrane-protein associated group of oncogenes. Transgenic animals carrying ras oncogenes have been produced which express ras in a variety of tissues with diverse effects. A transgenic mouse model of ras-associated oncogenesis offers several advantages for the study of human cancer. Unlike most animal models of tumourigenesis, transgenic c-H-ras mice have an intact immune system. This permits study of the role of immune system modulators in tumour progression (Suda et al., 1987).

An important contribution into understanding the effect of tumour inducers of different

potencies have been made by Momma et al. (1991). They have shown that transgenic mice carrying an epidermally expressed v-H-ras oncogene can serve as valuable model for evaluation of tumour inducers. These homozygotic transgenic mice were treated twice weekly by topical application on the skin for up to 20 weeks with inducers of different relative potencies, where the most potent inducer tested was TPA 2,5mg,> 2-butanone peroxide 5 mg > benzoyl peroxide 10 mg > acetic acid 30 mg or acetone (the last two served as controls). The study demonstrated a short latent period and high incidence of papilloma formation in all treatment groups except those treated with acetic acid or acetone, indicating that this transgenic mouse model is highly sensitive to chemicals with tumourigenic activity.

Another c-H-ras transgenic mouse model for carcinogenicity testing was developed by Katsuki et al. (1991). These c-H-ras transgenic mice were tested for papilloma formation after a single topical application of 7,12-dimethyl-benz[a]anthracene. Significant numbers of animals produced papillomas and carcinomas in as little as 6 weeks after treatment.The same fast formation of papillomas was obtained in the forestomach by the intraperitoneal injection of 660 µg MNU (N-methyl-N-nitrosourea) into the same strain of mice. In all 56 cases transgenic mice produced 2-12 papillomas each after 12 weeks.

Although the frequency of development of spontaneous tumours is rather high in the c-H-ras transgenic mice 50% develop tumours within 18 months (Griesemor & Tennant, 1992). Transgenic c-H-ras mice have proven useful in some carcinogenicity testing.

Transgenic models carrying activated v-H-ras, c-myc, and c-neu oncogenes

Three strains of transgenic mice carrying the v-H-ras, c-myc and c-neu activated oncogenes under the control of mouse mammary tumour virus (MMTV) regulatory signals LTR's were among the earliest models available for the study of carcinogenesis (Tennant et al., 1993). In order to determine if chemical carcinogens could alter the type of tumours, the pattern and rate of tumour induction or pathogenesis of tumour development, these three transgenic lines were exposed to two carcinogens. These carcinogens were chosen from among 150 chemicals that have demonstrated carcinogenic activity in long-term B6C3F1 mouse bioassays conducted by the National Toxicology Program, USA (Ashby & Tennant, 1991). One of the chemicals was reserpine, a non mutagenic mammary gland carcinogen. The other, para-cresidine is a mutagen which induces urinary bladder carcinomas in male and female B6C3F1 mice.

The carcinogens were both administered in the diet at doses of 2.500-5000 ppm for para-cresidine and 5-10 ppm for reserpine.

Exposure to para-cresidine did not appear to have any effect on the oncogenic effects of the transgene, or the resulting histopathology of induced mammary adenocarcinomas. However,although para-cresidine is mutagenic, did not induce tumours, even in tissues known to express the transgenes.

Unlike para-cresidine, reserpine is a relatively weak carcinogen and no tumours were induced in any of the non-transgenic FVB/N controls during the 9 month exposure period. The National Cancer Institute, USA established a 104 week bioassay in B6C3F1 mice for the mammary gland carcinogenicity of reserpine. The overall tumour incidence was only 15% and the tumours were detected during postmortem examination at the end of the 104 week of exposure (Bioassay for Reserpine for possible carcinogenicity 1982). In transgenic myc and neu mice treated with reserpine multiple mammary gland tumours were detected. There was also a high frequency of tumours in control animals. However, the incidence of multiple mammary gland tumours was significantly increased in treated females from both strains. Also the incidence of mammary gland adenocarcinomas was significantly increased in the transgenic mouse strains carrying the v-

H-ras oncogene treated with 5 ppm reserpine in the diet.
The results demonstrate, for these three transgenic strains and two carcinogens, the importance of the expression of the transgene. The results also suggest that the effects of a relatively strong carcinogen such as para-cresidine are not altered by transgenes that are transcriptionally targeted to specific tissues; however, the carcinogenic potential of a relatively weak carcinogen such as reserpine may be enhanced by oncogenes expressed in the target tissue (Rao et al., 1991).

pim-1 oncogene in transgenic mice
Anton Berns and his colleagues produced transgenic mice carrying the the pim-1 oncogene under the control of its own promoter, the Eμ enhancer and the Moloney murine leukemia virus(Mo-MuLV) LTR's (Berns et al., 1989). All of these transgenic pim-1 mice express high levels of the transgene in their hemopoietic tissues and eventually develop lymphoma. The idea was to use these transgenic mice to study synergism between cooperating oncogenes. These mice show no abnormalities in their hemopoietic or lymphoid organs in the early stages (van Lohvizen et al., 1988). Only after a latency period, of up to 8 months, do 10% of the transgenic mice develop T-cell lymphomas, which is a rather low tumour incidence. These mice are ideal for identification of other genes whose expression or suppression will synergise with pim-1 expression and lead to tumour formation. The mechanism by which over-expression of pim-1 mediates the enhanced susceptibility to tumourigenesis is still unknown.
Eμ pim-1 mice also have general applicability in carcinogenicity testing. When Eμ pim-1 transgenic mice were injected with single dose of the alkylating agent N-ethyl-N-nitrosourea (ENU) at 60 mg/kg body weight, all of them developed T cell lymphomas, compared to only 20% of non-transgenic control mice (Breuer et al., 1989). This indicates that Eμ pim-1 transgenic mice are tumour prone and that carcinogen treatment can accelerate lymphomagenesis. Also all ENU -induced lymphomas showed high levels of c-myc oncogene m-RNA, supporting the notion that pim-1 and c-myc cooperate in lymphomagenesis. Eμ pim-1 mice were up to 25 times more susceptible to ENU-induced lymphomagenesis than nontransgenic mice and so represent a highly sensitive in vivo system for ENU induced lymphomagenesis. The suitability of Eμ pim-1 for studying the carcinogenicity of other chemicals was tested at the National Institute of Public Health and Environmental Protection(RIVM) Holland by performing short-term in vivo carcinogenicity tests. Eμ pim-1 mice were given by gavage a dose of 13 mg/kg body weight of benzo[a]pyrene 3 times weekly for 13 weeks (Kroese D. personal communication). This treatment was reported to be non-carcinogenic in previous experiments using several non-transgenic mouse strains (Montizaan et al., 1989). A remarkable difference in the response between transgenic pim-1 mice and the controls was observed after only 100 days. Transgenic mice developed lymphomas much faster and 60% of Eμ pim-1 animals got lymphoma within 100 days. If this assay had been carried out only with the mice of wild type mice, benzo[a]pyrene would not have been identified as a carcinogenic agent in this test. Further examination of the applicability of Eμ pim-1 mice for carcinogenicity testing is in progress at the Institute of Toxicology, National Food Agency of Denmark. Transgenic Eμ pim-1 mice have been fed for about 7 months with 0,03% of PhIP, a food derived heterocyclic amine 2-amino-1-methyl-6-phenylimidazo(4,5-b)pyridine, which is a potent mutagen. So far this experiment shows that 90 percent of transgenic Eμ pim-1 females and 36 percent of males have developed lymphoma within 200 days (I.K. Sørensen et.al., 1994 unpublished results). An obvious indication of these experiments is that this system, once further characterised and validated, may be useful for the screening of industrial or environmental carcinogens.

P-53 deficient mice as a potential model for carcinogenicity testing

Two classes of genes have been identified that influence tumour formation; tumour suppressor genes, which act in a negative manner to control cell growth, and oncogenes, which appear to function in a positive fashion. One tumour suppressor gene being intensively studied is the p53 gene, whose product appears to be involved in maintaining genomic stability and control of cell division (Hollstein et al., 1991). Mutations that inactivate the p-53 gene are the most common genetic alternation observed in a wide variety of human cancers. Compared to normal animals, transgenic mice that carry a mutant p-53 transgene are much more susceptible to tumour formation after treatment with chemical carcinogens (Donehower et al., 1992). To investigate the role of p-53 in tumourigenesis a null mutation was introduced into the p-53 gene by homologous recombination (knock-out mutation) in murine embryonic stem cells (Donehower et al., 1992). Mice homozygotes for the null allele appear normal but are prone to the spontaneous development of variety of neoplasms by 6 months of age. Some tumours develop very early, within the first 10 weeks of life and tumour occurrence increases rapidly between 15 and 25 week of age. The increased probability of these mice to develop a wide range of tumours makes them valuable for testing suspected carcinogens. There is, however, to date a lack of well performed carcinogenicity studies in this transgenic mouse model.

Conclusion and Perspectives

In the examples quoted above, mice expressing an inserted oncogene in their tissues almost invariably develop tumours. The latency of such tumours is sometimes short and the tumour can cause physiological disturbances which make maintenance and breeding of these animals problematic. However, transgenic animals are an excellent tool in studying cooperation between oncogenes and the process of carcinogenesis and have already made an important contribution to our understanding of cancer. Experiments have shown that activation of a single oncogene is not always sufficient for transformation. Studies of oncogenes in transgenic animals support the concept that activation of multiple oncogenes and/or the inactivation of oncogene suppressors represent one of the steps in the process of tumourigenesis.

When an oncogene such as myc is transferred to the mouse germ line under the control of breast-cell specific promoter, the transgenic animals develop breast tumours. However, of thousands of breast stem cells in the mouse only one or two become neoplastic. This suggests that the presence of a single oncogene is not sufficient for tumourigenesis. However, doubly transgenic mice, made by breeding a myc transgenic with a mouse carrying a second breast-specific oncogene, develop tumours much earlier and more frequently. Two oncogenes are more efficient than one (Adams & Cory 1991). Carcinogens can directly activate oncogenes. There is considerable chemical specificity in this activation, such that one chemical will produce a characteristic mutation in the activated oncogene, while another carcinogen will produce a different mutation. Therefore transgenic animal models containing only one activated oncogene can not be broadly used in testing of the different carcinogenic agents. As yet too few studies have been conducted with double transgenics to assess the usefulness of transgenic mice in carcinogenicity testing. So far over two dozen tumour types have been modelled in transgenic mice but only a couple of them have been tested in carcinogenicity studies (Eddy, 1993).

A high-light of the last decade was the discovery of new genes that are responsible for human cancers. The next decade should bring about the characterisation of transgenic animal models that are susceptible to carcinogens leading to a more rapid and precise categorisation of genotoxic carcinogens and to a better understanding of the role of genetic events in the process that results in cancer caused by chemical carcinogens.

Summary:
The ability to transfer oncogenes into the germline of animals, which became available over 10 years ago has contributed to our understanding of the function of oncogenes and their role in genetic diseases and cancer.
Studies have been conducted with transgenics to assess the usefulness of transgenic mice in short term carcinogenicity testing. The next decade should bring about the characterisation of transgenic animal models that are susceptible to carcinogens leading to a more rapid and precise categorisation of genotoxic carcinogens and to a better understanding of the role of genetic events in the process that results in cancer caused by chemical carcinogens.

Resumé
I løbet af det sidste årti er det lykkedes at fremstille en række nye transgene laboratoriedyr med forskellige onkogener (kræftgener) indsat i deres arvemateriale. Disse transgene dyr, har allerede bidraget til bedre forståelse af onkogenernes rolle i udvikling af kræft. Brug af disse transgene dyr, bl.a. mus, til in vivo testning af kræftfremkaldende stoffer (carcinogener) er lige begyndt og de fremstillede dyre-modeller er under evaluering. Disse dyr har vist sig til at være prædisponeret til hurtigere udvikling af kræft efter behandling med carcinogener. De opnåede resultater åbner nye veje til udvikling af transgene dyre-modeller til korttids testning af kræftfremkaldende stoffer. Transgene dyre-modeller vil endvidere bidrage til præcis kategorisering af kemiske carcinogener, og til bedre forståelse af onkogenernes rolle i udviklingen af kræft herunder betydningen af sammenspillet mellem de forskellige onkogener i kemisk induceret carcinogenese. I artiklen diskuteres både de hidtil fremstillede transgene muse-modeller til brug for testning af kræftfremkaldende stoffer samt de transgene dyrs fremtidige perspektiver som modeller til korttids in vivo testning af potentielt carcinogene stoffer.

References

Adams JM & S Cory: Transgenic models of tumor development. Science 1991, 254, 1161-1167.

Ashby J & RW Tennant: Definitive relationships among chemical structure, carcinogenicity and mutagenicity for 301 chemicals tested by the US NTP. Mutat. Res. 1991, 257, 229-306.

Balls M, J Bridges & J Southee: Animals and Alternatives in Toxicology. Present status and Future Prospects (eds.) Macmillan Press, Frame 1991.

Berns A, M Breuer, S Verbeek & M van Lohvizen: Transgenic mice as a means to study synergism between oncogenes. Int. J. Cancer, 1989 Supp. 4, 22-25.

Bioassay for Reserpine for possible carcinogenicity. National Cancer Institute. Carcinogenesis Technical Report Series, no 193.1982, National Technical Information Service, Springfield, VA.

Breuer M, R Slebos, S Verbeek, M van Lohvizen, E Wientjens & Berns A: A very high frequency of lymphoma induction by a chemical carcinogen in pim-1 transgenic mice. Nature 1989, 340, 61-63.

Capecchi MR: The new mouse genetics: Altering the genome by gene targeting. Trends in Genetics 1989, 5, 70-76.

Cooper GM: Oncogenes as markers for early detection of cancer. J. Cell. Biochem. *1992,* Supp. 16G, 131-136.

Cordaro JC: Transgenic mice as future tools in risk assessment. Risk Anal. 1989, 9(2), 157-168.

Donehower LA, M Harvey, L Slagle, M McArthvr, CA Montgomery, JS Butel, A Bradley: Mice deficient for p-53 are developmentaly normal but susceptible to spontaneous tumors. Nature 1992, 356, 215-221.

Eddy M: The use of transgenic mice for environmental health research. Environ. Health Perspect. 1993, 101(4), 298-300.

Gordon JN: State of art transgenic animals. ILAR NEWS 1988, XXX (3), 8-17.

Gordon JW & FM Ruddle: Integration and stable germ line transmission of genes injected into mouse pronucleus. Science 1981, 214, 1244-1246.

Griesemor R & R Tennant: Transgenic mice in carcinogenicity testing. Mechanism of carcinogenesis in risk identification. IARC 1992, 429-436.Hollstein M, D Sidransky, B Vogelstein, C Harris: P-53 Mutations in human cancers. Science 1991, 253, 49-53.

Jaenish R: Transgenic animals. Science 1988, 240, 1468-1474.

Katsuki M, M Kimura, M Izawa, N Shindo-Okada & S Nishimura: Use of transgenic mice carrying normal human c-Ha-ras for studies on chemical carcinogenesis. In: Transgenic Mice in Developmental Biology and Toxicology, Research Triangle Park, NC, National Institute of Environemental Health Sciences 1991, 30-46.

Leder A, PK Pattengale, A Kuo, TA Stewart & P Leder: Consequences of widespread deregulation of the c-myc gene in transgenic mice: Multiple neoplasmas and normal development. Cell 1986, 45, 485-495.

Momma J, JW Spalding, RW Tennant & MR Elwell: Sensivity to tumor promoters of transgenic mice carrying an epidermally expressed v-H-ras oncogene. In: Transgenic Mice in Developmental Biology and Toxicology, Research Triangle Park, NC, National Institute of Environmental Health Sciences 1991, 49-54.

Montizaan GK, PGN Kramers, JA Janus & T Posthumus: Effects of 10 selected compounds, Appendix to Integrated Criteria Document PAH, report no 75847011, National Institute of Public Health and Environmental Protection, The Netherlands 1989.

Nielsen LL, CM Discafani, M Gurnani & RD Tyler: Histopathology of salivary and mammary gland tumors in transgenic mice expressing a human Ha-ras oncogene. Cancer Res. 1991, 51, 3762-3767.

Parke DV, C Joannides, R Walker: Food, Nutrition and Chemical Toxicity. In: Eldved Smith-Gordon (eds.) 1993.

Rao GN, RW Tennant, AG Braun, A Russfield & P Leder: Transgenic mouse models for assesment of carcinogenic potential of chemicals. In: Transgenic Mice in Development Biology and Toxicology. Research Triangle Park, NC, National Institute of Environmental Health Sciences 1991, 46-52.

Reddy EP, AM Skalka & T Curran: The Oncogene Handbook: (eds.) Elsevier 1988.

Suda Y, S Aizawa, S Hirai, T Narone, Y Furuta, M Suzuki, S S Hirohashi & Y Ikawa: Driven by the same Ig enhancer and SV40 T Promoter RAS induced lung adenomatous tumours, myc induced pre-β cell lymphomas and SV40 large T gene a variety of tumors in transgenic mice. EMBO J. 1987, 6, 4055-4065.

Tennant RW, NG Roa, A Russfield, S Scilkop & AG Braun: Chemical effects in transgenic mice bearing oncogenes expressed in mammary tissue. Carcinogenesis 1993, 14(1), 29-35.

van Lohvizen M, S Verbech, P Krimpenfort, J Domen, C Saris, T Radaszkiewicz & A Berns: Predisposition to lymphomagenesis in pim-1 transgenic mice: Cooperation with c-myc and N-myc in murine leukemia virus-induced tumors. Cell 1988, 56, 673-682.