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Review

Transgenic mice as research tools in neurocarcinogenesis

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Transgenic animal models for neurocarcinogenesis have provided significant insights into the molecular mechanisms underlying carcinogenic processes, including those which affect the nervous system. In view of the very rapid pace of acquisition of knowledge, it is not possible to cover all transgenic mouse models for neural tumors. Instead, this article discusses some of the most important technical innovations for manipulation of the mammalian genome (notably the various methods for targeted genome modifications, as well as the technology for introducing large DNA fragments into the germ line of mice), and presents a selection of the transgenic mouse models which are proving most promising for furthering our understanding of the pathogenetic basis of cancer in the nervous system.

Keywords: transgenic mice; neurocarcinogenesis models; knock out mice

The new tools of transgenesis

Although transgenic mice continue to be highly complex systems (Hanahan, 1989), the last years have witnessed significant technical improvements in the field of transgenesis, and many long-standing problems can now be overcome. The conventional method of generation of transgenic mice by pronuclear microinjection leads to random integration of an undefined number of copies of the construct of interest. While fast and often very efficient, this approach is fraught with significant drawbacks, notably poor or unpredictable expression levels, and ectopic expression of the transgene in undesired tissue compartments. These problems have been solved by utilizing embryonic stem cell lines (ES), which are derived from pre-implantation mouse embryos. ES cells are totipotent: when introduced into a host blastocyst, or aggregated within a morula-stage embryo, they fully participate to embryogenesis upon introduction into foster mothers and give rise to all tissues normally encountered in the mouse.

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While the most popular use of ES cells has been to generate knockout mice by ablating specific segments of the mouse genome, one can also introduce transgenes into mice using ES cells. Animals hosting ES cells are chimeras, consisting only partially of transgenic cells. While fully transgenic animals can be produced by cross-breedings, lethal dominant mutations, which would not allow for development of a transgenic mouse, can be often studied in chimeric transgenic animals (Boulter *et al*, 1991; Hilberg *et al*, 1993).

An exciting example use of this technology was recently exemplified by the fusion of AF9 sequences with the mouse Mll gene, which mimics a fusion gene produced by a chromosomal translocation in human acute myeloid leukemias (AML). Chimaeric mice carrying the Mll-AF9 fusion developed AML, despite expression of the transgene in many tissues. Beside providing a useful tool for directing a transgene to a precise chromosomal location, this study formally proves the causal role of the Mll-AF9 sequence translocation in myeloid leukemogenesis (Corral *et al*, 1996).

A further refinement of the homologous recombination technology is Cre/*lox*P-mediated site-speci-

fic recombination. The bacterial Cre protein is capable of mediating sequence-specific recombination of *lox*P sites, resulting in excision of intervening DNA sequences. The distance between the two loxP sites can be at least as large as 400 kb (Li *et al*, 1996), allowing for ablation of extremely large loci. Not only with ES cells exposed to Cre protein *in vitro* undergo *lox*P recombination: also transgenic mice expressing the Cre protein in specific tissues will ablate genes included between *lox*P sites (DiSanto *et al*, 1995; Gu *et al*, 1993; Rickert *et al*, 1997; Zou *et al*, 1994).

Another problem related to transgene integration concerns the presence of regulatory sequences driving a selectable marker (which, for example, encodes resistance to a certain drug), which may interfere with expression of the transgene that is being studied or with genes adjacent to it. The CRE-*lox*P system can eliminate such additional sequences as long as the two *lox*P sites are in the same orientation. Meanwhile, the *Hprt* (hypoxanthine phosphoribosyl transferase) locus of the mouse has become a popular site of gene targeting. This locus has been extensively characterized, and allows selection for presence or for absence of enzymatic activity. Moreover, being located on the X chromosome, it is hermizygous in male animals. In the model of Bronson *et al*. (Bronson *et al*, 1996), the mutated *Hprt* becomes the target for correction of the gene, and the correcting transgene is placed upstream of the locus. This procedure avoids the uncertainties of random integration events: since *Hprt* is widely expressed in the mouse, ES clones display very reproducible, high expression levels. The 'upstream targeting' characteristic of this model seems to be less prone to promoter occlusion effects that had biased other vector systems.

Several approaches have been developed to achieve transgene inducibility. This can be achieved using expression vectors controlled by derivatives of tetracycline (Kistner *et al*, 1996; Shockett *et al*, 1995). Doxycycline is very stable and can be even added to the water supply of the animals, and subtle modifications of the *tet*-responsive site allow for *tet*-based repression as well as induction of the transgene. Since the concentration range for induction is different from that needed for repression, one can even express two transgenes *in vivo* which will be independently controlled by variations in doxycycline concentration (Baron *et al*, 1997; Bohl and Heard, 1997).

Linkage of the Cre gene to the *tet*-inducible construct allows recombination to be induced *in vivo* upon administration of doxycycline (St Onge *et al*, 1996). Several groups have developed inducible systems utilizing an insect steroid hormone (ecdysone) transduction pathway system (Li *et al*, 1997a; No *et al*, 1996). However, their applicability to a wider range of transgenic models remains to be determined.

The expression of many genes is crucially determined by *locus control regions*, which may be located very far away from the coding sequences. This problem can be addressed by the use of very large vectors, such as PACs (P1 artificial chromosomes), BACs (bacterial artificial chromosomes), YACs (yeast artificial chromosomes) and HACs (human artificial chromosomes). Although both PACs and BACs can clone insert sizes up to 350 kb, transgenesis has so far only been achieved with YACs and, in very few instances, with HACs.

Various methods have been successfully utilized for transfecting YAC DNA into ES cells. Initial attempts exploited PEG-induced fusion between ES cells and YAC-containing yeast spheroblasts and selection markers (such as neomycin phosphotransferase), albeit with moderate efficiency. YAC DNA could also be transfected into ES cells upon complexing with liposomes. This method probably offers the best results in terms of DNA integrity. The most widely utilized method for YAC transduction, however, is pronuclear microinjection. Microinjection of YACs is more difficult than the generation of conventional transgenic mice, because very large molecules can easily break, and calls for the use of agents which protect DNA from shearing. The efficiency of transgenesis with YACs is not always lower than that of conventional fragment microinjection: transgene integration was seen in 10–14% of embryos microinjected with a 248 kb human β-globin YAC (Peterson *et al*, 1993) and in up to 17% with a 250 kb mouse tyrosinase YAC (Schedl *et al*, 1993).

Additional strategies for creating YAC transgenes include the creation of several lines with a series of overlapping clones, as well as 'binary systems' in which animals containing specific *cis*-controlling sequences are bred with animals containing specific *trans*-acting factors (Li *et al*., 1997b). Some of the best studied models relate to the introduction of the human β-globin locus into transgenic mice (Peterson, 1993, 1995, 1996). The original 248 kb YAC contained the five functional globin genes as well as upstream (39 kb) and downstream (82 kb) flanking sequences. The upstream flanking sequences contain the locus control region (LCR). The globin transgene appears to operate very similarly to the human locus: the ε-globin gene is expressed very early during gestation. At this time, a first switch occurs which induces the synthesis of γ-globin in the yolk sac, which lasts till the second switch, which stimulates β-globin expression. The latter is maintained in adult life. This technology has allowed to study the hereditary persistence of fetal hemoglobin (HPFH), which is not associated with large deletions of δ-β genes and surroundings. Introduction of a base pair substitution which had been identified in HPFH produced a delayed switch in fetal liver as well as persistent expression of γ-HB in adult life. Also, β-globin YAC transgenic mice

were mated with mice expressing high levels of the GATA-1 transcription factor. The results indicate that the latter is essential not only for the activation but also for proper repression of globin genes.

Models involving dominant oncogenes

Several transgenic neuro-oncological models have been developed in the past. In the following, we will limit our review to the models developed after 1995: for earlier work the reader is referred to our previous article (Aguzzi *et al*, 1995). We will particularly emphasize those models which employed technical innovations as well as those which seem to open new horizons in the field of neurooncology.

Pinealoblastomas

Pinealocytes have been extensively studied because they can originate neuroectodermal tumors, and also for their role in the circadian clock. The promoter of tryptophan hydroxylase (TPH) has been also employed to specifically direct transgene expression to neuroendocrine cells. A transgene encompassing a 6.1 kb TPH promoter fragment directed expression of *lac*Z to the raphe nucleus and the pineal gland (Huh *et al*, 1994; Son *et al*, 1996). Fusion of the TPH promoter to the SV40 large T antigen caused the appearance of very aggressive and invasive pineal tumors in transgenic animals causing their death at 12–15 weeks of age (Son *et al*, 1996).

Neuroblastoma

Neuroblastoma, the most common extracranial tumor of early childhood, often shows typical genetic lesions, such as deletion of the 1p36.1-2 locus (Δ1p), where a tumor suppressor gene for human neuroblastoma is presumed to reside. Several attampts to identify the latter gene among at least seven different candidates have failed so far (Brodeur 1995; Brodeur *et al*, 1997). Furthermore, susceptibility to familial neuroblastoma is not linked to 1p36.1-2 (lod scores of *ca* −4) (Maris *et al*, 1996; Rovigatti 1997). The N-*myc* proto-oncogene seems to play an important role in the ontogenesis of this tumor. Typically, N-*myc* amplification (NMA) is found in 25–30% of neuroblastomas, and involves aggressive, higher-stage disease (Brodeur *et al*, 1984, 1985, 1988a; Shimada *et al*, 1995). N-*myc* may therefore be a marker of progression. Alternatively, two distinct types of neuroblastoma may exist, and the more aggressive form is characterized by NMA (Brodeur 1995; Rovigatti 1992; Tsuchida *et al*., 1996), particularly since patients with lower stage tumors (characterized by the presence of aneuploid karyotypes and absence of NMA and Δ1p) never progress to tumors with NMA and Δ1p (Brodeur *et al*, 1997). More than 90% of high stage neuroblastomas display both NMA and Δ1p (Brodeur *et al*, 1988b; Fong *et al*, 1989, 1992).

Although animal models of neuroblastoma have been presented in the past, they did not reflect all peculiarities of the human condition (Aguzzi *et al*, 1990, 1992). Despite considerable overexpression of N-*myc* in tumors arising in these mice, the characteristic genetic aberrations described in human neuroblastoma, i.e. amplification of N-*myc* (NMA) and deletions on the short arm of chromosome 1 (Δ1p, where a tumor suppressor gene for human neuroblastoma is presumed to reside), were not faithfully reproduced in the available models. A recent transgenic model has provided the first formal proof of the tumorigenic potential of N-*myc in vivo* (Weiss *et al*, 1997). Here, the promoter of the tyrosine hydroxylase gene was linked to the cDNA of N-*myc*. The resulting transgenic lines developed neuroblastomas with an incidence of 5–20% at 3–6 months of age. Frequency of tumorigenesis was increased to 75% at 10 months by crossing these transgenics with mice hemizygous for either *Nf-1* or *RB1*, and approached 100% at 4 months when the N-*myc* transgene was bred to homozygosity. However, also in this mouse model, secondary genetic lesions did not always coincide with those of human neuroblastoma. Significant chromosomal additions were found in regions syntenic with human chromosomes 6 and 17p (while most of the chromosomal gains in human neuroblastoma are on 17q21.3-qter) and losses for regions syntenic with chromosomes 4, 11, 3 and X. Deletions in regions syntenic with human chromosome 1p36.2-3 were conspicuously absent (Weiss *et al*, 1997). Intriguingly, secondary chromosomal changes were not documented in all mice. This suggests that the identified additional lesions may not always be necessary for tumor progression, and that they may be complemented for by higher by N-myc expression.

Astrocytic tumors

Mice expressing the SV-40 large T antigen from the regulatory region of the glial fibrillary acidic protein (GFAP) gene (Danks *et al*, 1995) developed brain tumors at an extremely early age and rapidly progressed to death within 3 weeks after birth. Two animals developed somewhat milder symptoms and progressed to death at about 28–30 days of age: they were defined as 'late' tumors. The tumors were described as composed by atypical cells in the periventricular subependymal zone. SV40 large T antigen-expressing cells were scat-

tered throughout the brain: they also appeared atypical and frequently mitotic. An additional abnormality was hyperplasia of the choroid plexus.

T-antigen positive cells were detached in the peripheral nervous system (PNS) in cells identified as non-myelinating Schwann cells. When cell lines were derived from transgenic brains, two distinct cell populations were identified: one contained astrocyte-like cells, while the other cellular population had an epithelial phenotype. With passaging and starting at about passage 8–10, the latter cells appeared to become the predominant type in culture, did not consistently express GFAP (although some of them could be induced to express this marker by growth arrest), and did not express intermediate filaments *in vitro*, as detected by immunofluorescence and electron microscopy. It is therefore questionable whether such tumors, and the cell lines derived from them, can be described as astrocytomas. High expression of the transgene was detected in the choroid plexus, and led to hyperplasia (a typical phenotype of T-antigen transgenic mice) (Messing *et al*, 1985). The latter, rather than hyperproliferation of astrocytes, was probably responsible for death by occlusive hydrocephalus. GFAP expression is not normally detected in choroid plexus of human or mouse brain, although it can occur ectopically in choroid plexus papillomas (Aguzzi *et al*, 1997).

In summary, the mice discussed above (Danks *et al*, 1995) provide an interesting model for neoplastic transformation of astrocytic precursors. However, the life span of the mice is severely limited and this has impeded the establishment of transgenic families. This problem may be related to the oncogene employed (SV40 large T antigen) which seems to induce transformation of cells at an extremely early stage. In addition, the chosen GFAP regulatory fragment (proximal 2.3 kb flanking sequence at the 5′ of the gene) may provide a pattern of expression not completely superimposable to that of the endogenous GFAP gene.

In an effort to overcome the problems discussed above, we inserted the v-*src* oncogene inside the first exon of a GFAP minigene which maintains a large portion of upstream regulatory sequences and the intact structure of the gene with 9 exons, 8 introns and the 3′-untranslated region (Mucke *et al*, 1991). This structure allows for a very precise transgenic recapitulation of the endogenous GFAP expression pattern. *Src* has been linked to glioma/astrocytoma carcinogenesis by several lines of evidence, since gliomas were induced by intracerebral injection of RSV in dogs, and previous studies (Aguzzi *et al*, 1991) showed that *src* induces neuroectodermal tumors in rats. *Src* seems to transform astrocytes with a longer latency period than T-antigen, inducing astrocytoma, anaplastic astrocytoma and finally to glioblastoma multiforme.

Two lines expressing an intact *src* transgene were obtained. We detected transgene expression in lung, heart, thymus, spleen, ovary and kidneys in addition to the brain (as is the case for the endogenous GFAP gene). On the other hand, Northern blotting analysis revealed *src* expression only in the brain and in testes, as previously described (Holash *et al*, 1993). Pathological astrogliosis was detected at 2.5–3 weeks of age. Further steps of carcinogenesis such as dysplasia, pre-neoplasia and neoplasia appeared in the following weeks. Animals with large brain tumors occasionally died very suddenly, perhaps due to sudden transtentorial herniation. The majority of the tumors arose in the brain stem and thalamus, where the highest expression of GFAP and transgene, as well as the first signs of dysplasia, were detected. Some tumors with the morphology of schwannomas arose in the PNS (Weissenberger *et al*, 1997).

Large malignant tumors displayed areas of necrosis associated with perifocal overexpression of vascular endothelial growth factor (VEGF), a potent inducer of angiogenesis expressed in human glioblastomas (Plate *et al*, 1993). The gene encoding the cognate VEGF receptor *flk-1* was also highly expressed in the endothelial comportment around necrotic lesions.

Even after 1 year, the total frequency of overt tumors did not exceed 15–20%. While this low incidence is a drawback for therapeutical studies, it provides a realistic model of human astrocytoma progression. The *src* transgene apparently provides only an initiating step of carcinogenesis and additional genetic lesions contribute to neurocarcinogenesis. In an effort to identify such candidate lesions, we are currently intercrossing heterozygous transgenic *src* mice with $Rb^{+/-}$ (Saenz Robles *et al*, 1994) and with $p53^{+/-}$ mice ** (Jacks *et al*, 1994a,b), since both genes have been implicated in human astrocytoma progression.

p53 deficient mice have been recently exploited to simulate a multistep model for human gliomas (Donehower, 1996, 1977; Harvey *et al*, 1995). Astocyte cultures from $p53^{+/-}$ and $p53^{-/-}$ transgenes were compared to cultures obtained from wild-type $p53^{+/+}$ mice (Yahanda *et al*, 1995). Since early passages, $p53^{-/-}$ astrocytes displayed very rapid growth. At late passages these cells obtained extremely high saturation densities and gave rise to large, vascularized tumors in nude mice. A similar pattern was observed with cells derived from $p53^{+/-}$ animals once they had lost the intact germ line allele. In sharp contrast, cells from $p534^{+/+}$ animals never underwent such changes, rapidly senesced, and died after 7–10 pasages. The changes in the heterozygous mutants were associated with dramatic karyotypic and ploidy alterations, suggesting that p53 is capable to prevent gross genetic aberrations (Yahanda *et al*, 1995).

Olfactory neuroblastomas

Early olfactory neuroblastoma (ONB, also called esthesioneuroblastoma) models have been covered in our previous review article (Aguzzi *et al*, 1995). Servenius and colleagues (Servenius *et al*, 1994) used the olfactory marker protein (OMP) promoter to drive SV40 large T antigen expression: however, transgenic mice developed peripheral tumors resembling neuroblastomas. This finding was surprising since OMP was not known to be expressed in anatomic sites other than olfactory neurons. On the other hand (Carney *et al*, 1995) showed that OMP is not expressed in human ONB, while a marker of immature olfactory neurons, the Achaete-scute 1 gene HASH-1, is consistently present in human primary and metastatic ONB.

Transgenic mice expressing the early region of Adenovirus E1 from the renin or the angiotensinogen gene promoter developed ONB-like tumors, but also neuroectodermal tumors in the retroperitoneal and pelvic regions (Sugiyama *et al*, 1995). One further transgenic line developed carcinoid tumors, suggesting that the phenotype of carcinogenesis was highly dependent upon the integration site of the transgene within the host genome (Sagara *et al*, 1995).

Primitive neuroectodermal tumors (PNET)

Transgenic mice in which the SV40 T-antigen is driven by the tyrosine hydroxylase promoter (rTH-Tag mice) developed primitive neuroectodermal tumors (PNETs) essentially undistinguishable from their human counterpart (Fung *et al*, 1994). The actual mechanism of tumorigenesis is associated with a defect in developmental apoptosis, which prevents the regression of a group of neuroepithelial progenitor stem cells located ventrally to the median eminence (Fung *et al*, 1995). In normal age-matched controls these progenitor cells spontaneously regress two weeks postnatally, while rTH-Tag transgenics presented microscopic lesions consisting of packed small blue cells, and later actual PNET. Apoptosis was present but obviously not sufficient to balance the excessive proliferative stimulus. A similar phenomenon occurs in mice expressing SV40 large T antigen from the insulin promoter, which develop islet cell tumors only if insulin like growth factor II (IGF-II) is produced and counteracts spontaneous apoptosis. Tumor growth is strongly reduced in IGF-II$^{-/-}$ mice (Christofori *et al*, 1995): it may therefore be interesting to determine the effect of IGF-II ablation in rTH-Tag mice.

JCV is the etiologic agent of progressive multifocal leukoencephalopathy (Jochum *et al*, 1997) and its early region induces demyelination in transgenic mice (Small *et al*, 1985, 1986a,b. However, these early studies also documented abdominal PNET-like tumors in the founder mice. More recently, heritable PNETs were induced in mice expressing a similar construct (Franks *et al*, 1996). JCV T antigen probably causes carcinogenesis by binding endogenous p53 and *Rb*, as it has been shown in other systems. Finally, the *dbl* oncogene driven by the neuron specific enolase (NSE) promoter did not induce neuroectodermal tumors in wild type mice but did so in p53$^{+/-}$ mice (Colucci *et al*, 1995).

Ablation of 'caretaker' and 'gatekeeper' genes

We have discussed the complex tumor phenotype of p53$^{-/-}$ mice in our earlier review article (Aguzzi *et al*, 1995). Many cellular factors, besides p53, have been recognized to feed into the pathways of cell cycle, transformation, apoptosis, and senescence. The terms *gatekeepers* and *caretakers* for such regulatory proteins was introduced as a conceptualization of these interactions (Kinzler and Vogelstein, 1997). Gatekeepers are master regulators which interact directly with the different phases of the cell cycle, while caretakers affect the cellular machinery which senses cellular 'well-being'. While carcinogenesis seems to be strongly enhanced by loss-of-function of single gatekeeper genes (with an increase of frequency of up to 1000 times), carcinogenesis due to gatekeeper loss of function seems to progress more slowly. p53 is a well-recognized 'gatekeeper' capable of inducing apoptosis if DNA damage occurs. But p53 might be more than a mere 'guardian of genome' (Norimura *et al*, 1996), and may control several differentiation pathways (Ferreira and Kosik, 1996).

Another gatekeeper prototype, the retinoblastoma tumor suppressor gene *Rb*, plays a crucial role during embryogenesis: its ablation leads to embryonic death, while restortion of expression in Rb-deficient embryos allows to prolong the embryonic life up till birth - despite major defects in nervous system, liver and skeletal muscle (Jiang *et al*, 1997; Zacksenhaus *et al*, 1996).

Transgenic mice have allowed to study the interaction between *Rb*, p53, and other gatekeeper. For example, mice expressing a truncated SV40 large T antigen which maintains the capability of interacting with p*Rb* and with p107, but has lost the capacity of binding p53, do not display the lymphoid abnormalities typically detected in transgenic mice expressing SV40 large T antigen (McCarthy *et al*, 1994), yet develop choroid plexus tumors. However, the tumors develop much more slowly than in transgenic mice expressing full length SV40 large T antigen, with maximum incidence at approximately 8 months instead of the typical 1–2 months (Bowman *et al*, 1996). This indicates that the p53 binding region of SV40 large T antigen contributes to tumorigenesis. Attenuation

of carcinogenesis in the deleted SV40 transgenes probably involves p53-induced apoptosis, which seems to be mediated by the apoptogenic protein *Bax*. In animals hemizygous for *Bax*, apoptosis dropped by 50% and tumor growth was accelerated (Yin *et al*, 1997). However, if dominant negative mutants of p53 are expressed in these mice, very aggressive tumors develop, indicating that p53 is capable of inhibiting tumor growth, and of exerting a crucial gatekeeping function. Further, p53 is induced in the CNS of *Rb* knockout mice and may stimulate apoptosis. Loss of p53 prevents cell death in *Rb* knockout mice, but does not suffice to restore normal proliferation (Macleod *et al*, 1996).

The majority of the mouse models in which caretakers have been disrupted or modified have targeted sites other than the nervous system. Disruption of the gene responsible for ataxia teleangiectasia (ATM) reproduced several of the ATM-associated deficits, such as neurologic dysfunction due to degeneration of cerebellar and other neurons, retarded growth, T-cell deficits and extreme sensitivity to γ-radiation (Barlow *et al*, 1996). We can therefore expect that ATM-deficient mice will become an important model for oncology.

Other transgenic models have focused on genes involved in apoptosis. *Bcl*-2 was initially discovered in B-cell lymphoma, and was soon recognized to be an ubiquitous cellular regulator capable of blocking programmed cell death. *Bcl*-2 is the prototype of a growing family of proteins involved as positive and negative regulators in the control of cell survival and death (Merry and Korsmeyer, 1997). *Bcl*-2 is highly expressed during development, but its expression ceases in adult life. However, *Bcl*-2 may have a physiological role in the maintenance and repair of nervous circuitry (Chen *et al*, 1997). On the other hand, expression of *Bcl*-2, as well as its associated proteins *Bcl*-X, *Mcl*-1 and *Bax*, is often dysregulated in brain tumors (Krajewski 1994, 1995, 1997; Weller *et al*, 1995).

The BRCA1 and BRCA2 genes were identified as susceptibility genes for breast and ovarian cancer (Brugarolas and Jacks, 1997). Several domains of these proteins have been tentatively associated with various functions, but their mode of action is still quite unclear. Mice deficient for BRCA die in utero at approximately E5–6 in the case of BRCA1 and at approximately E7.5–8.5 in the case of BRCA2, probably because of insufficient cellular proliferation. These mice show abnormalities of the neural tube, such as exencephaly and spina bifida, and disorganized development of neuroendocrine cells (Gowen *et al*, 1996; Hakem *et al*, 1996, 1997; Liu *et al*, 1996; Ludwig *et al*, 1997; Sharan *et al*, 1997; Suzuki *et al.*, 1997).

Both BRCA-1 and BRCA-2 bind to Rad51, which is the eukaryotic homologue of RecA, one of the most important portein in *E. Coli* recombination, and co-localize in nuclear structures during mitosis and in meiotic cells. Both BRCA-2 and Rad51 deficient mice show dramatic hypersensitivity to γ-radiation. Therefore, both BRCA1 and 2 may involved in repair of serious DNA damage, such as double strand breakage. Therefore, these caretaker molecules may exert an important function in DNA repair, and are prime candidate caretakers in tumorigenesis. The paradoxical effects witnessed in BRCA-deficient mutants are probably due to essential functions associated with rapid cellular proliferation during embryogenesis.

Neurofibromatosis

The *Nf*-1 gene, whose product is called neurofibromin, was cloned in 1990 as the susceptibility gene for neurofibromatosis 1 (Viskochil *et al*, 1990; Wallace *et al*, 1990) and subsequently discovered to encode a member of the GTPase activating proteins (GAP) (Xu *et al*, 1990). GAPs are capable of terminating *ras*-mediated signal transduction by triggering the hydrolysis of *ras*-bound GTP. *Nf*-1 deficient mice die at midgestation with gross malformations in neural crest-derived structures (Jacks *et al*, 1994b). Embryonic death is due to major cardiac defects: these abnormalities are likely to be due to impaired migration of neural crest cells, which normally populate the outflow tracts of the heart. Interestingly, in a variant form of *Nf*-1, referred to as Watson's syndrome or Noonan-fibromatosis syndrome, patients suffer from severe cardiac defects involving pulmonary valvular stenosis (Leao and da Silva, 1995).

Hemizygous *Nf*-$1^{+/-}$ mice show a high incidence of tumorigenesis. Most tumors fall into the classical spectrum of *Nf*-1: neurofibrosarcomas, adrenal tumors, and particularly pheochromocytomas, which are exceedingly rare in mice. Myeloid leukemias were also observed: this is consistent with the typical occurrence of myeloid leukemias (particularly juvenile CML) in human *Nf*1. The great majority of tumors show very frequently loss of the non-targeted allele. In addition, adoptive transfer of fetal liver from homozygous *Nf*-$1^{-/-}$ embryos induces an aggressive form of myeloid leukemia in the recipients, which appears to be due to hypersensitivity to granulocyte/macrophage colony stimulating factor (GM-CSF) (Largaespada *et al*, 1996). The latter is apparently due to the activation of *RAS* signal transduction pathway (STP) (Bollag *et al*, 1996), suggesting that neurofibromin may be a negative regulator of GM-CSF through RAS-STP.

Nf-1 knockout mice have also been used to study the role of neurofibromin in the hyperproliferation of Schwann cells characteristic of *Nf*-1 lesions (Kim *et al*, 1995). The changes induced in Schwann cells resemble the effects of v-*ras*. Ras-GAP deficient mice show a phenotype similar to that of *Nf*-$1^{-/-}$ mutants: endothelial cells seem to be particularly affected and

incapable of organizing a vascular network. Furthermore, double mutants mice show an exacerbated phenotype, as would be expected of genes acting in parallel pathways of signal transduction (Henkemeyer *et al*, 1995). In Gap/*Nf*-1 double mutants the neural tube hyperproliferates and invades the head mesenchyme (Henkemeyer *et al*, 1995).

The *tax* gene of HTLV is also capable of inducing multiple neurofibromas in transgenic animals resembling those of human *Nf*-1 (Hinrichs *et al*, 1987). Further studies have showed that the *tax* gene is indeed a negative trans-regulator of the neurofibromin gene (Feigenbaum *et al*, 1996). This mechanism may modify *Nf*-1 gene expression and contribute to tumorigenesis.

A wealth of information about the signal transduction pathways to which *Nf*-1 participates is coming from studies with transgenic flies. In *Nf*-1-deficient flies, the most obvious phenotype was a reduction in the sizes of larvae, pupae and adults (Guo *et al*, 1997; The *et al*, 1997). Heterozygous loss of the Gap1 gene did not exacerbate the phenotype, but homozygous loss of both *Nf*-1 and Gap1 resulted in lethality during larval development. However, manipulation of other effectors of the same pathway, such as Ras1 or SOS or Raf^{gof} did not dramatically change the phenotype, thus suggesting that *Nf*-1 acts through a pathway slightly different from Ras and which may involve kinase A (Guo *et al*, 1997; The *et al*, 1997).

Neuroectodermal tumors and *hedgehog* signalling

The *patched* and *sonic hedgehog (shh)* proteins participate to a signal transduction pathway that includes *gli*, the family of *wnt* genes, that of transforming growth factors β (TGF-β), DPC4, APC, β-catenin, GSK3B, the nuclear regulators p15 and p16, the cyclin-dependent kinase inhibitors p21 and p27, E2F as well p53 and RB. *Hedgehog* belongs to a family of regulatory genes which were initially dissected by in *Drosophila Melanogaster*. An increasing number of such genes has been identified from vertebrate species such as mouse and man. The mouse *sonic hedgehog (shh)* gene product can function as a morphogen in the patterning of the developing limb. Further, it can induce somitic cells to acquire a sclerotomal fate, and it induces patterning of the ventral neural tube. Floor plate induction occurs through surface-bound SHH molecules in direct physical contact with the neural plate, whereas the other two functions require a long-range patterning signal. Accordingly, mice deficient for *shh* die at birth with malformations of the frontal brain (Chiang *et al*, 1996) similar to the human condition known as holoprosencephaly (HPE). The latter occurs in 1/16000 of live birth and in 1/250 aborted fetuses, and was mapped to at least four loci (21q22.3, 2p21, 7q36 and 18p11.3). Recently, one of these loci was mapped to the *shh* locus (Belloni *et al*, 1996). The alterations detected in this gene consist of chromosomal translocations affecting its regulatory elements, or of nonsense and missense mutations (Belloni *et al*, 1996; Roessler *et al*, 1996).

Patched (*ptc*), a 12-transmembrane protein receptor, is the receptor for HH (Marigo *et al*, 1996; Stone *et al*, 1996). Another receptor called *smoothened* or *smo* participates to the signal transduction pathway. *Ptc* gene expression is normally inhibited and becomes activated only in the presence of HH induction. PTC represses this signal by binding and inactivating SMO. In the presence of HH, PTC releases SMO, which induces target genes including *wg (wingless)* and *dpp (decapentaplegic)*. The latter gene, *dpp*, shares homology with the TGF-β gene family, and interacts with TGF-β receptors. Signal transduction of the *ptc/dpp* pathway involves a gene which had been identified as a maternal effect enhancer of Drosophila *dpp* embryonic patterning mutants: this gene was therefore called 'mothers against *dpp*' or *mad*. There seems to be a very high specificity in the interaction of the TGF family receptors with their ligand and in the interaction of MAD related proteins with their receptor.

Expression of mutant genes belonging to the three hedgehog pathways (*wnt, hedgehog,* and *dpp*) has helped characterizing the effects of p53 deficiency on mammary carcinogenesis induced by *wnt* family members (Donehower *et al*, 1995; Frank *et al*, 1997; Gunther *et al*, 1994; Lagna *et al*, 1996; Lee *et al*, 1995). Ectopic expression of Wnt-1 could be induced by the *Hoxb*-4 region A enhancer (Dickinson *et al*, 1994), and caused a great increase in mitotic rate and expansion of the cells of the ventricular region. Interestingly, wnt-1 acted solely as a mitogenic stimulus in the CNS, rather than a patterning signal in this model, since developmental abnormalities were not evident.

A further gene belonging to the *dpp*/tgf-β pathway was isolated by positional cloning upon the observation that extensive deletions of chromosome 18 are present in human pancreatic carcinomas. This led to the final identification of DPC4, which is now believed to be involved in more than 50% of pancreatic carcinomas. DPC4 is highly homologous to *mad*, and displays frequent mutations in its carboxy terminus which may induce transcriptional activtion (Chu, 1997; Frank *et al*, 1997; Lagna *et al*, 1996; Moskaluk and Kern, 1996).

Transgenic mouse models have provided considerable insights into the *hedgehog* pathways. The group of M Scott, who initially discovered mutations of the *patched* gene in nevoid basal cell carcinoma syndrome, linked the *shh* gene to a keratin promoter in order to obtain overexpression in the skin (Johnson *et al*, 1996; Oro *et al*, 1997). Transgenic mice showed alterations similar to those seen in BCNS (such as polydactily and spina bifida (McMahon and Chuang, 1996)) as well as alterations

involving skin structure and epidermal cell proliferation (Fan *et al*, 1997; Oro *et al*, 1997). These findings prompted a search for *shh* mutations in human cancers, but only somatic mutations were detected in approximately 10% and 30% of the total and desmoplastic cases, respectively (Pietsch *et al*, 1997; Raffel *et al*, 1997; Vorechovsky *et al*, 1997; Xie *et al*, 1997).

A further model is based upon alteration of *patched*, whose mutations are linked to BCNS in humans. The neurooncological relevance of this gene derives from the occurrence of aggressive medullobalstomas in a small percentage of BCNS patients. Mice with a homozygous ablation of *ptc* died early *in utero* E9.0–E10.5) with several congenital abnormalities, confirming the essential role of *ptc* as an inducer of dorsalization (Goodrich, 1997). Most interestingly, a high incidence of medulloblastomas was documented in the cerebellum of hemizygous mice. Predictably, *gli* was highly expressed in these tumors. The incidence of medulloblastoma increased with age (8.3% at 5 weeks and 9–10 weeks, up to 30% in animals at 12–25 weeks), similarly to what is observed in other models. This suggests that additional changes, such as loss of the second allele of *ptc* and possibly secondary genetic lesions, are required for formation of medulloblastomas.

Choroid plexus tumors

It has been long known that transgenic expression of the SV40 viral enhancer and large-T antigen efficiently induces tumors of the choroid plexus (Palmiter *et al*, 1985). More recently, mutant of the IgH intronic enhancer ENHiH coupled to the SV40 large-T antigen was shown to induce choroid plexus tumors (Enjoji *et al*, 1995). This enhancer contains motifs which direct expression to B-cells, whose removal (Enjoji, 1994) renders the construct permissible for expression in brain cells. In a cell line established from such choroid plexus tumors (which expresses typical markers such as stransthyretin and alpha2-macroglobulin), an *ets*-like transcriptional regulator was shown to bind to the transgenic enhancer and to be probably important for directing expression to the choroid plexus (Enjoji, 1994). The choroid plexus tumor model proved extremely useful also for studying the interactions of SV40 large T antigen with p53, RB, and apoptogenic proteins such as *bax*, as discussed in detail in a former section of this review [Saenz Robles, 1994#1331; (Yin *et al*, 1997).

Multiple endocrine neoplasia

The hallmark of multiple endocrine neoplasia type 2 (MEN 2) syndrome is the development of medullary thyroid carcinomas (MTC), which are responsible for the high lethality of this syndrome (DeLellis *et al*, 1986). MEN 2 is one of the very few hereditary cancer syndromes with a dominant pattern of inheritance. The gene responsible is the RET transmembrane receptor (Myers *et al*, 1995), and mutations responsible for MEN 2 appear to activate the RET kinase (Eng *et al*, 1994, 1995; Mulligan *et al*, 1994a,b). A different type of mutation is responsible for a certain percentage of familial Hirschsprung's disease: these are inactivating, loss-of-function mutations (Pasini *et al*, 1995). Accordingly, in RET deficient mice there is not development of enteric nervous system, kidney agenesis, and lack of intestinal autonomic ganglia (Schuchardt *et al*, 1994, 1995). Several studies have shown that RET is the receptor for the glial cell derived neurotropic factor (GDNF) (Durbec *et al*, 1996; Treanor *et al*, 1996; Trupp *et al*, 1996). Three additional animal studies confirmed these results by showing tht mice lacking the GDNF gene display both kidney agenesis and abnormalities of the enteric nervous system (Moore *et al*, 1996; Pichel *et al*, 1996; Sanchez *et al*, 1996).

While MEN2B seems to be due to a unique mutation in the active site of the tyrosine kinase domain (Carlson *et al*, 1994; Donis Keller, 1995; Pandit *et al*, 1996), in MEN2A mutations affect one of five cysteine residues clustered in the extracellular domain of the receptor (Chi *et al*, 1994; Donis Keller *et al*, 1993; Mulligan *et al*, 1994a,b). When a ret construct with a typical MEN2A mutation was linked to the calcitonin gene related peptide promoter in order to target its expresion to thyroid C cells, hyperplasia of thyroid C cells occurred and was followed by multifocal and bilateral medullary thyroid carcinomas in three independent transgenic lines of mice (Michiels *et al*, 1997). These intriguing results confirm the role of RET as a dominant oncogene responsible for thyroid tumorigenesis.

Pituitary tumors

Due to its interest for molecular endocrinology, mammalian reproduction, and development, carcinogenesis in the pituitary gland has been addressed by a wealth of transgenic models over the past few years. In several instances, tumor growth or carcinogenesis were dependent on the hormonal status. A transgene encoding the polyoma early region promoter linked to a cDNA encoding polyome large T antigen induced pituitary tumors (Helseth *et al*, 1995) which produced adrenocorticotropic hormone (ACTH). At 13–16 months of age, such animals develop pituitary macroadenomas with elevated ACTH plasma levels. Transplantation of such tumors into nude mice led to a considerable increase in ACTH levels and the weight of the

Table 1 Transgenic models of neurocarcinogenesis

Tumor type	*Transgene or ablated gene*	*Phenotype*
Pineoblastoma	Tph–LacZ	Expression in pineal gland (Huh *et al*., 1994)
	Tph–T-ag SV40	Aggressive pineal tumors (Son *et al*., 1996)
Neuroblastoma	Tyrosine hydroxylase N-myc	Neuroblastomas: (Weiss *et al*., 1997)
Astrocytic tumors	GFAP (2.3 kb)–T-ag SV40	Choroid plexus hyperpl. +undifferentiated tumors (Danks *et al*., 1995)
	GFAP (whole gene) v-src	Astrocytomas (Weissenberger *et al*., 1997)
Olfactory neuroblastoma (ONB)	Omp T-ag sv40	Neuroblastomas (ectopic) (Servenius *et al*., 1994)
	Renin/angiotensin E1 adeno-12	Olfactory neuroectodermal tumors (Sugiyama *et al*., 1995)
Primitive neuroectodermal tumors (PNET)	Tyrosine hydroxylase T-ag sv40	PNET (Fung *et al*., 1994; Fung and Trojanowski, 1995)
	JCV–T-ag	PNET (Franks *et al*., 1996)
	Neuron specific enolase — *Dbl* oncogene	PNET (only in $p53^{+/-}$) (Colucci *et al*., 1995)
Gatekeeper genes ablation	$p53^{-/-}$	Tumor predisposition, occasional embryo lethality (Norimura *et al*., 1996)
	T-ag SV40 mutant	Slowly growing tumors P53 bind./bax apoptosis (Bowman *et al*., 1996; McCarthy *et al*., 1994) [Yin, 1997#1313]
	$Atm^{-/-}$	Extremely sensitive to •-rays (Barlow *et al*., 1996)
	$Bcl\text{-}2^{-/-}$	Axonal repair impeded (Cen *et al*., 1997)
	$P107^{-/-}$	Embryo lethality (e14.5) Affected: cns and liver (Jiang *et al*., 1997; Zacksenhaus *et al*., 1996)
Caretaker genes ablation	$Brca1^{-/-}$	Embryonic lethality (e5–6) (Hakem *et al*., 1996)
	$Brca2^{-/-}$	Embryonic lethality (e7.5–8.5) Sharan, 1997#1910)
Neurofibromatosis (Nf-1)	$Nf\text{-}1^{-/-}$	Lethality heart+nervous system (Brannan *et al*., 1994)
	$Nf\text{-}1^{-/+}$	Phaechromocytomas and myeloid leukemias (Jacks *et al*., 1994b)
	$Reconst.Nf^{-/-}$	Myeloprolif. (JCML) (Largaespada *et al*., 1996)
	$Nf\text{-}1^{-/-}$	Hypersens to GM-CSF (Bollag *et al*., 1996)
	$NF\text{-}1^{-/-}$Schwann	Similar to v-ras (Kim *et al*., 1995)
	$Ras\text{-}gap^{-/-}$Neurofibromin inact.	Die E10.5 : cns+endoth (Henkemeyer *et al*., 1995)
	By Htlv-tax	Localized neurofibromas: Additional mechanisms (Feigenbaum *et al*., 1996; Hinrichs *et al*., 1987)
		Size reduct.-PK-A involv.
Hedgehog Signaling	Hoxb-4a enhancer-Wnt½ and $p53^{-/-}$	Cooperation in carcinog. between wnt and p53. Mitogenicity of wnt
	Keratin-14-Shh	Basal cell nevus syndrome (Fan *et al*., 1997; Oro *et al*., 1997) (Dickinson *et al*., 1994; Donohower *et al*., 1995; Gunther *et al*., 1994; Lee *et al*., 1995)
	$Ptc^{-/-}$	Lethal e9-e10.5
	$Ptc^{+/-}$	Large size, syndactily, cerebellar medulloblastoma (30% at 5–6 months) (Goodrich, 1997)
Multiple Endocrine Neoplasia (MEN)	$Ret^{-/-}$	Renal agenesis/Hirschsprung's disease (Schuchardt *et al*., 1994, 1995, 1996)
	$GDNF^{-/-}$	Comparable to $ret^{-/-}$: both show same phenotype
	CGRP promoter (2 kb) $Ret^{cys634\rightarrow arg}$	Bilateral C cell hyperplasia multifocal MTC (3 weeks–2 years) (Michiels *et al*., 1997)
Choroid plexus tumors	SV40/IgH enhancer T-ag SV40	Tumors: ets-like trascription factor activation (Enjoji, 1994, 1995) (Saenz Robles *et al*., 1994; Symonds *et al*., 1994; Yin *et al*., 1997)
Pituitary Tumors	PyLT	Micro and macroadenomas Cushing's syndrome (Helseth *et al*., 1992, 1995; Holm *et al*., 1993)
	LH/FSH promoters-Tag SV40	Pituitary tumors/immortal cell lines (Alarid *et al*., 1996; Turgeon *et al*., 1996)

animals. Therefore, this transgene model provides a realistic model of human Cushing's disease (Helseth *et al*, 1992; Holm *et al*, 1993).

In a very interesting model developed by the group of P Mellon, the SV40 large T antigen was expressed under the regulatory control of various promoters, in order to test whether different target cells may be immortalized in specific stages of differentiation (Alarid *et al*, 1996). Cells from an immature gonadotropic cell line are less differentiated and express the alpha subunit as well as the gonadotropin releasing hormone receptor, while more differentiated cell lines were obtained by oncogenesis with SV40 large T linked to promoters of LH/FSH (Alarid *et al*, 1996). A L β T2 gonadotropic cell line, also obtained from a transgenic mouse containing the SV40 large T antigen linked to the rat LH β-subunit regulatory region, was shown to respond to steroid hormone regulation and to pulses of gonadotropin releasing hormone (Turgeon *et al*, 1996).

Ependymomas

As discussed in a previous review article (Aguzzi *et al*, 1995), it has been amply demonstrated in the past that transgenes encompassing the early region of the SV40 virus display a tropism for ependymal cells and choroid plexus, and SV40 large T antigen is capable of inducing both ependymomas and choroid plexus tumors when driven by its homologous promoter. Carcinogenesis of these target cells could be induced also by the oncogenes of the human papillomavirus type 16, E6 and E7. Therefore, some type of specificity for viral sequence expression or viral promoters seems to be present in ependymal cells. Intriguingly, also the major immediate early promoter of human cytomegalovirus (HCMV), when introduced into transgenic mice, (Fritschy *et al*, 1996), targeted expression of a reporter gene to ependymal cells and choroid plexus epithelia (among other, predominantly neural crest-derived targets). Since HCMV can cause severe and devastating congenital disorders in human embryos, as well as ependymitis in immunocompromised adults, such studies are of potentially great interest in understanding whether pathology correlates with specific gene expression. These data are consistent with the presence of specific, probably transacting, regulatory mechanisms which permit expression of viral genes in these cells.

Concluding remarks

The increasing number of transgenic animals utilized for studies on neurocarcinogenesis is documented by the plethora of new articles published only two years after our initial review. Certainly, the most recent transgenic systems aim at increased specificity and reproducibility of the natural situations which they attempt to model. The new tools for specific targeting of genomic loci have now made such goals attainable.

The wealth of information now available on the biochemical pathways of signal transduction, allows for more meaningful experiments. Moreover, transgenic studies have become a natural complement to reverse genetics, in which susceptibility genes are being detected by linkage analysis and positional cloning. On the basis of the discoveries of the last few years, we predict that transgenic mice will not only be instrumental for understanding the function of newly discovered oncogenes and tumor suppressor genes, but also for uncovering unexpected functions of genes long known.

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