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Tumorigenesis in Transgenic Mice: Identification and Characterization of Synergizing Oncogenes

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Abstract Transgenic mice carrying oncogenes present a useful model with which to assess the tissue-specific action of oncogenes. These mice are usually predisposed to a specific type of neoplastic growth. The tumors that arise are usually monoclonal in origin and become only apparent after a variable latency period, suggesting that additional events are required for tumor formation. Identification of these additional events is highly relevant: it might give access to the genes that can synergize with a preselected oncogene in tumorigenesis and could facilitate the identification of the biochemical pathways in which these genes act. Retroviruses can be instrumental in identifying cooperating oncogenes. Proto-oncogene activation or tumor suppressor gene inactivation by insertional mutagenesis is an important mechanism by which the non-acute transforming retroviruses can induce tumors in several species. Owing to the sequence tag provided by the provirus, the relevant proto-oncogene can be directly identified by cloning of the DNA flanking the proviral insertion site. We have exploited this potential of retroviruses by infecting Eμ-*pim*-1 and Eμ-*myc* transgenic mice, which are predisposed to lymphomagenesis, with Moloney murine leukemia virus (MuLV). A strong acceleration of tumor induction ensued upon infection of these mice with MuLV. More importantly, it allowed us to identify a number of additional common insertion sites marking both previously known as well as new (putative) oncogenes. In a significant portion of the tumors more than one oncogene was found to be activated, indicating that within this system the synergistic effect of at least three genes can be established. Furthermore, the distribution of proviruses over these common insertion sites suggests that this methodology enables us to assign each of these genes to distinct complementation groups in tumorigenesis. To get insight into the function of these oncogenes, we have inactivated one of them, the *pim*-1 oncogene, via homologous recombination in ES cells. We have shown that by using a *pim*-1 targeting vector in which transcription and translation of the resistance marker was made dependent on the acquisition of both a promoter and an in-frame translational initiation codon, the gene could be inactivated with high efficiency. Subsequently, we inactivated the second allele by using a different selectable marker. The absence of the *pim*-1 gene product does not appear to have any detrimental effect on the growth of ES cells and hematopoietic cells derived from these ES cells in vitro, despite the fact that *pim*-1 is highly expressed in wild-type ES cells. More detailed analysis of *pim*-1-less cells and mice will be necessary to gain insight into the relevance of the highly conserved *pim*-1 proto-oncogene in development and maintenance of mammalian organisms.

Key words: oncogene, transgenic mice, homologous recombination, lymphomagenesis

Cancer is a multi-step process of mostly genetic and epigenetic alterations that accumulate during the lifetime of an organism [1,2]. These alterations might be caused by exposure to carcinogenic agents or simply result from mistakes, made in processes such as DNA repair and DNA replication. Some cell types appear more vulnerable to transformation than others. This might be explained by the notion that cells at particular developmental stages exhibit a pattern of gene expression which closely resembles that of transformed cells. The mutation of only one or two genes might then be sufficient to cause transformation. Retinoblastoma in man might exemplify this [3]. However, in most forms of cancer many events are required to yield a highly malignant tumor cell. Many of the genetic lesions have been shown to cause the activation of oncogenes or inactivation of tumor suppressor genes. In colon tumors the co-incidence of mutations in p53, DCC, *ras*, and other, unknown, loci are an illustrative example of this multi-step model [4]. Specific genetic lesions are often associated with specific tumors, suggesting that the "wiring" of regulatory circuits differs from one cell type to the other and, accordingly, different sets of oncogenes and tumor suppressor genes

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must be genetically altered in these cells to cause malignant transformation [5].

How can we find synergizing sets of cancer genes and how can we determine their individual contribution to oncogenesis? Mice with specific gain-of-function and loss-of-function mutations in oncogenes and tumor suppressor genes can be instrumental in this endeavor. It is difficult if not impossible to extract this information from the genetic alterations found in outgrown tumors: not only is it difficult to identify the genes that are mutated or deleted but one might assume that, as the number of proliferating cells in a tumor increases, genetic alterations will further accumulate, some of which may confer only minor selective advantages to the cell. Nevertheless, these relatively insignificant mutations might be scored as prevalent events in the outgrown tumor. Therefore, there is need for model systems in which this process can be mimicked and experimentally controlled.

Recently, all the genetic tools to introduce mutations as found in tumors and in inherited diseases have become available: transgenesis [for review, see 6] to introduce dominant acting genes in all cells of an organism and embryonic stem (ES) cell manipulation [for review, see 7] to introduce both gain-of-function as well as loss-of-function mutations in either some (chimeric mice) or all (offspring of germ-line chimeric mice) cells of the organism. Mice carrying the mutations can provide insight into the transforming potential of putative oncogenes and the tumor-preventing activity of tumor suppressor genes. They also allow analysis of the immediate effects of mutations on proliferation and differentiation of somatic cells before additional mutations have occurred. Furthermore, cross-breeding between transgenic mice carrying different mutations, or the generation of chimeric mice carrying a combination of these mutations in some of their cells can reveal the synergistic effects of these lesions in transformation. Finally, transgenic mice bearing a particular oncogene or defective tumor suppressor gene can be used to search for (new) oncogenes or tumor suppressor genes that synergize with the mutant gene by using retroviruses as an insertional mutagen [8].

C-*MYC* AND *PIM*-1 ONCOGENES

The c-*myc* oncogene plays a predominant role in many tumors. Chromosomal rearrangements, proviral insertions, and gene amplification are mechanisms frequently observed in c-*myc* activations. Recently, it has been shown that c-*myc* encodes a DNA binding protein, which, in concert with other nuclear factors, can bind to specific DNA motifs, thereby regulating transcription of genes that are probably of pivotal importance for cell proliferation and differentiation [9,10].

The *pim*-1 oncogene encodes two serine/threonine-protein kinases with cytoplasmic localization [11]. The *pim*-1 gene was identified as frequently activated by proviral insertion in murine leukemia virus-induced lymphomas [12,13]. The gene is expressed in cells of the hematopoietic system, in gonads, and in ES cells. The *pim*-1 mRNA can be induced in lymphoid cells by a variety of growth factors and mitogens; the protein is extremely short-lived [11 and references therein]. This suggests a role for *pim*-1 in cell growth or differentiation. However, at present it is not known in which signal transduction pathway *pim*-1 functions. Oncogenicity of *pim*-1 is mediated by overexpression of the normal *pim*-1 proteins rather than a *pim*-1 mutant protein, this in contrast to most other oncogenes derived from protein kinase genes. Transforming activity of *pim*-1 is not observed in vitro in fibroblasts or REF cells. However, the oncogenicity of *pim*-1 has become apparent in transgenic mice [14; see below].

Eμ-*PIM*-1 AND Eμ-*MYC* TRANSGENIC MICE ARE PREDISPOSED TO LYMPHOMAGENESIS

To get insight into the transforming capacity of *pim*-1, we have produced transgenic mice expressing the *pim*-1 gene in both the B- and T-cell compartment at levels comparable to those found in MuLV-induced tumors [14]. This was achieved by inserting the immunoglobulin enhancer upstream of the *pim*-1 promoter and a MuLV LTR in the 3′ untranslated region of the gene. The lymphoid organs in the Eμ-*pim*-1 transgenic mice do not show imbalances in the different subpopulations of cells as analyzed by FACS, although an enlargement of the spleen is reproducibly observed. The tumor incidence of the Eμ-*pim*-1 lines 64 and 66 is low. Only after monitoring relatively large numbers of mice did a significant difference in tumor incidence become apparent [14].

The tumors that appear after a long latency period are monoclonal T-cell lymphomas. In the majority of those tumors a very high expression of the c-*myc* oncogene is observed in accordance with the notion that *pim*-1 and c-*myc* synergize very efficiently in lymphomagenesis (see follow-

ing text). However, no gross rearrangements in the c-*myc* locus were found in these tumors. Although the *pim*-1 transgene is also expressed in pre–B cells, only T-cell lymphomas are found. This might be due to the genetic background of mice that were used in these experiments (BL/6xCBA).

Eμ-*myc* transgenic mice, overexpressing the c-*myc* gene in B-cells by virtue of the presence of an immunoglobulin heavy chain enhancer (Eμ) in the promoter region, have been instrumental in determining the role of c-*myc* in B-cell lymphomagenesis [15,16]. Eμ-*myc* transgenic mice show an expansion of the pre–B-cell compartment from early on in life [17,18]. It has been suggested that cells in this compartment are particularly vulnerable to oncogenic transformation [19]. Pre–B-cell lymphomas occur with a significant frequency in Eμ-*myc* transgenic mice: 50% of the mice develop tumors before 5 months of age. The tumors that arise are monoclonal in origin, indicating that additional events are required. Direct analysis of these tumors has given little information on these additional events. In a few cases, a mutant *ras* gene was found [20]. However, it is unclear what genetic changes are responsible for the other tumors that were found in Eμ-*myc* transgenic mice.

MULV INFECTION OF Eμ-*PIM* AND Eμ-*MYC* TRANSGENIC MICE ALLOWS THE IDENTIFICATION OF SYNERGIZING ONCOGENES

One approach to identify the additional events necessary for malignant transformation in Eμ-*myc* or Eμ-*pim*-1 transgenic mice takes advantage of the capacity of retroviruses to act as insertional mutagens, thereby activating proto-oncogenes or inactivating tumor suppressor genes, while at the same time tagging these genes to allow subsequent identification [8]. With the assumption that proviral integrations are relatively random, flanking regions of integrated proviruses are cloned and used to screen independently induced tumors for the presence of proviruses in the corresponding region of DNA. If an integration in that region is found more than once, it is assumed that there is selective growth advantage associated with proviral insertion in that locus. In the past, a number of oncogenes have been identified by this approach [for review, see 8].

This methodology has proven to be extremely powerful when used in combination with Eμ-*pim*-1 and Eμ-*myc* transgenic mice. MuLV infection results in a much faster induction of T-cell lymphomas in Eμ-*pim*-1 transgenic mice than in their non-transgenic littermates [14]. In nearly all of these tumors in Eμ-*pim*-1 mice, either c-*myc* or N-*myc* appeared to be activated by proviral insertion, whereas in nontransgenic littermates insertion in these loci was found in only 30% of the cases. Importantly, in a significant fraction of these tumors integration in another common insertion site has been found, indicating that the provirus tagging technique can easily identify two additional steps in these tumor-prone mice [Van Lohuizen, unpublished results].

Complementary observations have been made in Eμ-*myc* transgenic mice, which overexpress the c-*myc* oncogene in their B-cell compartment, thereby predisposing the mice to pre–B-cell lymphomagenesis [21,22]. In these mice MuLV causes acceleration of pre–B-cell lymphomagenesis (see Fig. 1). Here, activation of the *pim*-1 gene was found as a frequently occurring event. We and others have identified additional common insertion sites in these pre–B-cell tumors [21,22]. One of these common insertion sites harbors a highly conserved, not previously identified gene, *bmi*-1, encoding a 46 kD protein with nuclear localization. It has a cystein-rich motif, also found in a number of other proteins such as Rad-18, RAG-1, and HSV-IE110, genes with functions in DNA repair, DNA rearrangement, and transcriptional regulation, respectively. Other novel common insertion sites found in these tumors are *pal*-1 and *bla*-1. The genes residing in these loci still have to be identified. An important aspect transpiring from this analysis is the potential functional relationship between these genes. As can be seen in Figure 2, tumors induced by MuLV in Eμ-*myc* mice show the presence of proviruses in four common insertion sites. In a significant portion of these tumors, which are mostly of monoclonal origin as judged from the immunoglobulin heavy chain gene rearrangement patterns, proviral insertion in two different common sites is found, indicating a synergistic interaction between the Eμ-*myc* transgene and the genes affected by these insertions. This brings the number of traceable synergizing genes in this experimental system to three. It is not surprising that more than two genetic events are required for tumor formation in this system. Crossbreeding between Eμ-*myc* and Eμ-*pim*-1 transgenic mice also points to the necessity of additional events for tumor growth (see below). Besides evidence for synergism between oncogenes, the data might suggest that

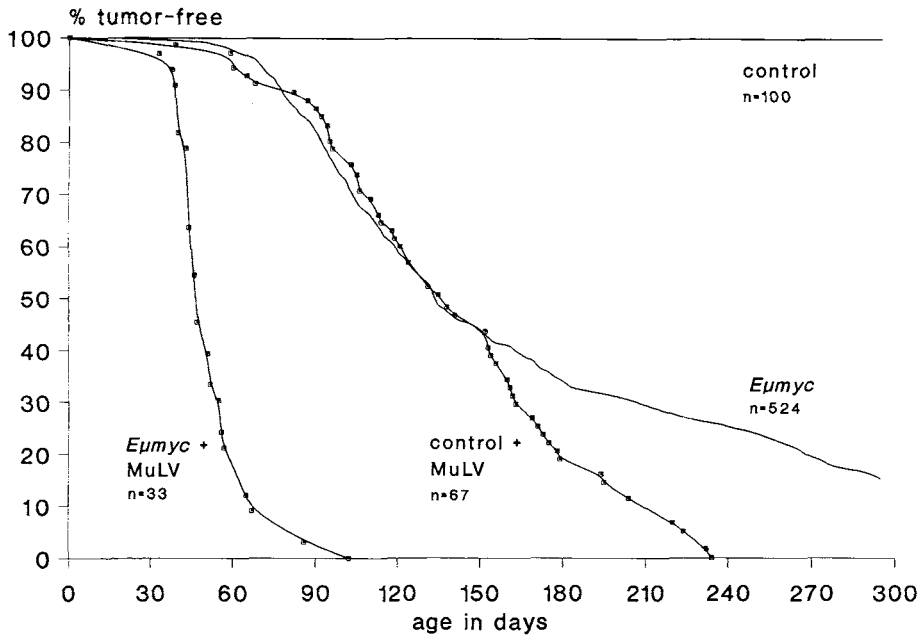

Fig. 1. Acceleration of B-cell lymphomagenesis in Eμ-*myc* mice by MuLV infection. Vertical axis: percentage of tumor free mice; horizontal axis: latency period of tumor development in days. Note the rapid onset of B-cell lymphomas in Eμ-*myc* transgenic mice infected with MuLV as compared to spontaneous B-cell lymphomagenesis in Eμ-*myc* mice. Curve "control + MuLV" represents T-cell lymphomas that develop in non-transgenic littermates infected with MuLV. (Reproduced from Van Lohuizen et al., 1989, with permission of Cell Press.)

some of these genes act in a similar pathway and therefore might substitute for each other in transformation. The most unequivocal example in this respect is provided in T-cell lymphomagenesis induced by MuLV in Eμ-*pim*-1 transgenic mice. In nearly all tumors, activation of either c-*myc* or N-*myc* is observed. However, activation of both genes in one cell is never seen. Apparently, when either one of the two is activated by proviral insertion, activation of the other by proviral insertion does not mediate sufficient additional advantage, and, therefore, cells in which this has occurred will not grow out selectively. Although one might have predicted this for genes such as c-*myc* and N-*myc*, which are highly related, the observation is possibly quite informative for, for example, *bmi*-1 and *pal*-1, the activation of which seems mutually exclusive in pre–B-cell lymphomas; these genes might directly or indirectly act on the same target(s).

POTENCY OF SYNERGISM BETWEEN *PIM*-1 AND *MYC*

The MuLV-tumor-acceleration studies with Eμ-*pim*-1 and Eμ-*myc* transgenic mice suggest that crossbreeding of Eμ-*pim*-1 and Eμ-*myc* mice in the absence of exogenous tumor-accelerating agents should uncover the potency of the synergistic interaction between *pim*-1 and c-*myc*. Indeed, a dramatic synergism was seen in crossings between Eμ-*pim*-1 and Eμ-*myc* mice. Fetuses that co-express both transgenes succumb perinatally from lymphoblastic leukemia [23]. However, even in this rapidly occurring leukemogenic process, additional events appear to occur already early on, resulting in differences in tumorigenicity of the leukemic cells from these fetuses when transplanted into histocompatible recipients; approximately 60% of the leukemic cells of these fetuses gave rise to monoclonal tumors in the recipient mice. If the cells from one fetus were transplanted into a series of recipient mice and the resulting tumors were analyzed with respect to IgH chain rearrangements, it appeared that each tumor showed a different pattern of rearrangement, suggesting that clonal selections occurred upon transplantation of the leukemic cells. Our data are best compatible with a model of tumorigenesis in which a polyclonal pre–B-cell proliferation, induced by the Eμ-*myc* and Eμ-*pim*-1 genes in the early embryo, is frequently followed by additional (epi)genetic events, leading to the formation of malignant cells. The stochastic nature of these events could explain the variation in the

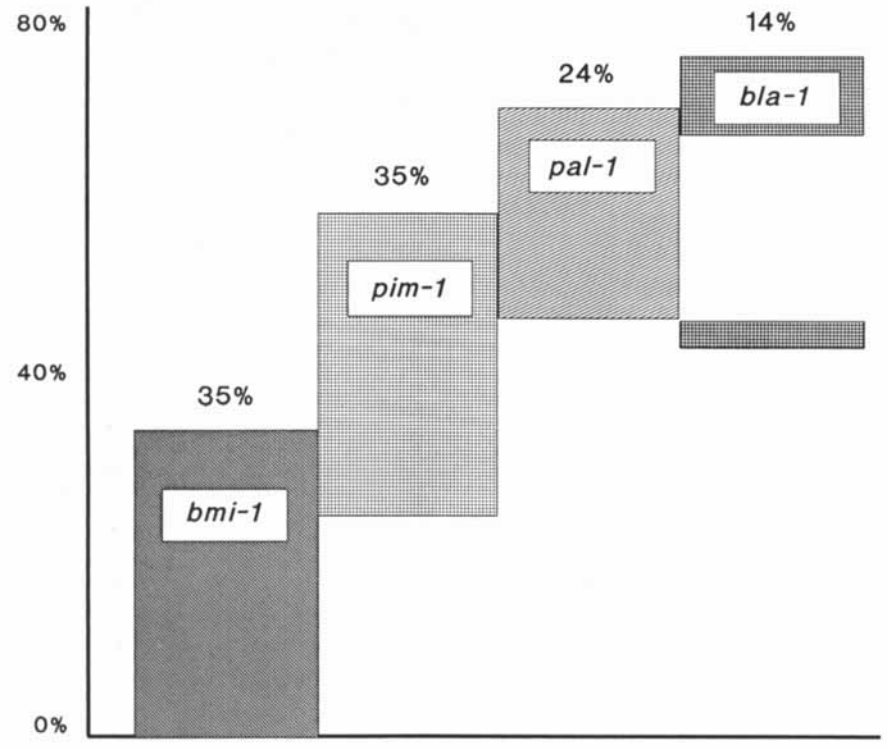

Fig. 2. Coincidence of common proviral insertion sites in MuLV-induced tumors in Eμ-*myc* transgenic mice. Tumors carrying proviral insertions in more than one common insertion site are indicated by overlapping bars. Percentages of occupation of the individual sites is denoted over each bar. Approximately 20% of tumors do not have a proviral insertion in any of these sites. (Reproduced from Van Lohuizen et al., 1989, with permission of Cell Press.)

severity of the disease we observed in these fetuses and might also account for the lack of transplantability of some of the fetal blood samples. Upon transplantation, additional selective forces appear to act on these cells, resulting in the outgrowth of further adapted subclones of tumor cells that are marked by different D-J joins. These observations are in accordance with the notion that in MuLV-induced pre–B-cell lymphomas in Eμ-*myc* transgenic mice activation of more than one putative oncogene is frequently found [22,23].

FUNCTIONAL ANALYSIS OF ONCOGENES BY TARGETED INACTIVATION IN ES CELLS AND MICE

Identification of novel genes synergizing in transformation leads to a number of questions, such as what the normal function of these genes is, how aberrant expression results in transformation, and what the molecular basis of the cooperation with a particular oncogene in transformation is. Loss-of-function mutations might give insight into some of these issues and provide the reagents to more fruitfully pursue biochemical and cellular biological analyses on the involvement of the gene product in signal transduction. We have followed this approach for the *pim*-1 oncogene. The *pim*-1 proto-oncogene was inactivated in ES cells by homologous recombination using a targeting construct in which G418 resistance was made dependent on the acquisition of both a promoter and an in-frame AUG initiation codon upon integration of the DNA construct into chromosomal DNA [24]. This should provide a strong selection for obtaining clones that have undergone homologous recombination as opposed to random integration. And indeed it appeared to be the case. Eighty-five percent of the G418-resistant colonies obtained with the above mentioned targeting construct carried a disrupted *pim*-1 allele. When a hygromycin construct was used a similarly high frequency was observed.

This allowed us to consecutively inactivate both *pim*-1 alleles in ES cells. When ES cells in which one *pim*-1 allele was disrupted by the insertion of a Neo construct were targeted with a hygromycin-containing construct, a large fraction of the hygromycin-resistant clones showed

disruption of both *pim*-1 alleles. This indicates that *pim*-1, which is highly expressed in ES cells, is not required for normal growth of ES cells in vitro. Upon in vitro differentiation of these ES cells, no differences were noted between the cells lacking *pim*-1 and normal ES cells. Also hematopoietic differentiation in vitro into hemoglobin-producing cells seemed unaffected [van Roon et al., unpublished results]. In the meantime, we have shown that these ES cells can also contribute to chimeric mice and to the mouse germ line [van Roon et al., unpublished results]. Mice lacking a functional *pim*-1 allele generated by using a different targeting strategy (collaboration with the group of M. Hooper, Edinburgh), appear viable, and show no obvious phenotype [Laird et al., in preparation]. A detailed analysis of the hematopoietic system of these mice is in progress in order to assess the function of *pim*-1 in this system.

CONCLUDING REMARKS

Retrovirus-induced acceleration of tumorigenesis in transgenic mice carrying oncogenes appears to be a very efficient method to identify oncogenes that synergize with the transgene. The data indicate that, once an inventory of all the common insertion sites in these lymphomas has been made, it will be possible to assign the relevant genes, present in these loci, to distinct complementation groups in transformation. By generating transgenic mice with these newly found genes and repeating the retroviral infection, we not only can firmly establish and possibly expand these complementation groups, but we can also assess the relative contribution of these genes to early and/or late steps in the tumorigenic process.

The approaches I have sketched here might not only greatly facilitate the identification of signal transduction pathways in which many of these genes act, but also help to define genetic alterations that are associated with non-proliferative aspects of cancer cells, such as invasiveness and metastatic potential. The use of retroviruses for this purpose does not have to be restricted to tumors of the hematopoietic system. MMTV can be used in the mammary system, and infection of cells with different MuLVs in vitro or even of embryos in utero might permit access to a variety of other cell types. Subsequently, the inactivation of the genes identified in this way in the mouse germ line can provide insight into the normal function of these genes in growth and development. In case the gene is expressed in cultured cells, there is now also the option to study the effects of loss-of-function mutations directly in vitro, without the necessity to generate germ-line chimeric mice. This should expedite the composing of the network of genes involved in tumorigenesis and help to define their function in both normal and malignant cell growth.

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