Patología molecular

Transgenic mice and gene targeting in pathology

M. Gaboli, E. de Álava* and P.P. Pandolfi

Department of Human Genetics and Molecular Biology Program, Memorial Sloan-Kettering Cancer Center, Sloan-Kettering Division, Graduate School of Medical Sciences, Cornell University, New York, Estados Unidos.
**Departamento de Anatomía Patológica, Clínica Universitaria de Navarra, Pamplona, España.*

INTRODUCTION

In the last decade, a powerful tool for studying the molecular pathogenesis of human diseases, both congenital and acquired, has involved the insertion of human DNA into the germ line of mice, allowing the creation of animals known as "transgenic". Subsequently, refinements in transgenic technology have also made it possible to disrupt the function of specific mouse genes, therefore generating animals known as "knock out" mice. These murine models are being extensively utilized in basic research as well as in experimental pathology, and not only have they allowed for a better understanding of the molecular basis of diseases, but they have provided a unique system for studying the effects of altered gene expression or gene inactivation in a mammalian organism.

In this article, after presenting the above mentioned technology, we will review why and how transgenic and knock out mice have recently become interesting for pathologists.

TRANSGENIC MICE

The technology of introducing foreign genes into the mammalian germ line arose from the knowledge, obtained through gene transfer experiments in tissue culture, that new DNA could be inserted into the genome of a host cell and be efficiently expressed.

A transgenic mouse (or, more in general, a transgenic animal) is an organism that develops from an embryo at the one-cell stage after it has been injected with foreign DNA into one of its pronuclei. Therefore, all the cells of such an animal will contain the new DNA and may be affected in their functions by the information coded by the foreign DNA (Fig. 1). It becomes evident that a specific transgenic mouse can be created by choosing the DNA sequences to be introduced into the murine zygote and the modalities by which these sequences will be expressed. Moreover, specific biological questions, such as those regarding the pathogenesis of diseases, can be answered by creating adequate animal models.

The pathogenesis of cancer is a multi-step process characterized by the accumulation of critical genetic lesions within the cell. Sometimes, very well defined genetic lesions are associated with a specific tumor type. This is the case of the translocations which occur in leukemia, lymphomas, and in pediatric sarcomas, leading to the formation of fusion genes coding for novel proteins normally not present in the cells. In order to assess

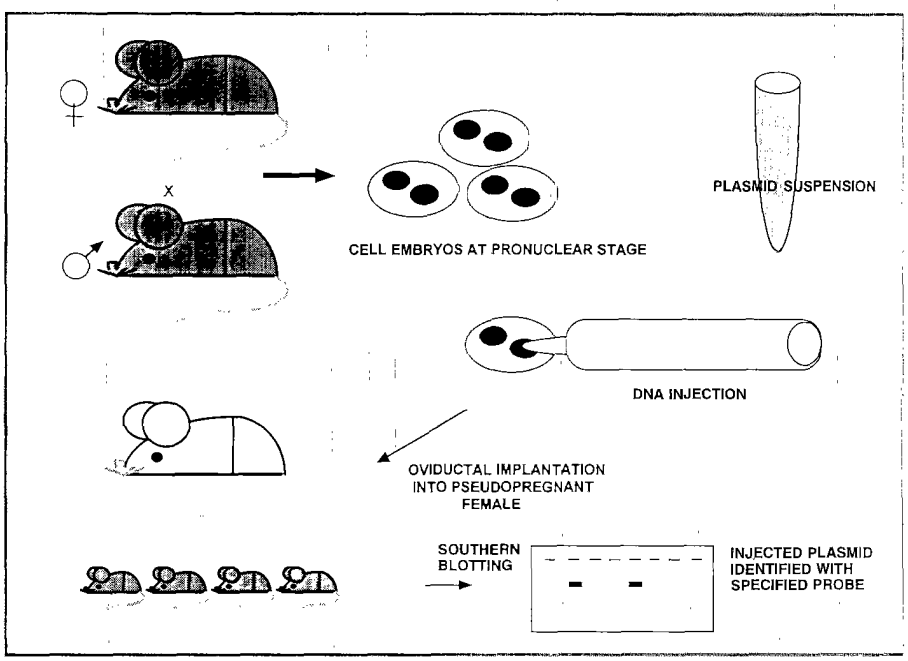

Figure 1. Production of transgenic mice. Females are superovulated, mated to fertile males, and sacrificed the next day (top left). Zygotes with pronuclei are recovered and one of the pronuclei is injected with DNA (center). Surviving embryos are reimplanted into pseudopregnant foster females, and DNA samples from newborns are evaluated for the presence of foreign genes.

the role played by any of these molecules in the pathogenesis of a disease, the aberrant gene can be inserted into the murine germ line and observe whether its expression is sufficient to reproduce the disease in the mouse.

The first step will be to prepare a DNA construct containing the fusion gene, or the corresponding cDNA, under the control of a promoter which will direct the expression of the protein of interest. The choice of the promoter represents a very important step in the experimental planning. There are promoters which are known to drive expression in all tissues, the so-called housekeeping promoters, such as the mammalian promoters of β-actin, ubiquitin C and glucose-6-phosphate dehydrogenase enzyme, or the viral promoters of cytomegalovirus immediate early (IE) genes. Nevertheless, there are also promoters that are known to drive expression only in specific organs or tissues. These are known as tissue specific promoters, such as the keratin 5 promoter, which is restricted to the skin; the cathepsin G promoter, which is restricted to cells of myeloid origin; the lymphocyte-specific kinase *lck* promoter, which is specific for T-cells at early stage of development; or the α1 antitrypsin promoter, which is liver specific. In the first case, with a housekeeping promoter, the expression of the gene of interest can be achieved in all the organs of the transgenic mouse, while in the second case only one or a few restricted organs will have the transgene expressed.

Each of the different promoters mentioned above can best serve to address specific scientific questions. For example, a tissue specific promoter makes it possible to achieve a closer reproduction of human pathologies that imply somatic genetic alterations, such as those occurring in most tumors, while a housekeeping promoter can better mimic the situation observed in a congenital disease. In addition, by using a tissue specific promoter it is possible to overcome the problem of toxicity, which sometimes arises when proteins are expressed in tissues where they are not normally found. This is the case of several fusion proteins, which result from chromosomal translocations associated to leukemias and sarcomas, whose expression in the embryo impairs its complete development, thus not allowing the transgenic mouse to be born. Finally, it should be mentioned that there are promoters whose activity is restricted to a certain time of the embryogenesis or tissue differentiation. For example, the adenovirus eIIa promoter targets the expression to a very early stage of preimplantation embryogenesis, while the promoters of molecules which are lineage

markers of commitment and stage differentiation in bone marrow cells, such as CD34, myeloperoxidase, or CD11b, can be utilized to restrict the expression of a fusion protein associated to a leukemia only to the bone marrow cells of a specific lineage and stage of differentiation. Thus by choosing the promoter to be used in the experiment the investigator is actually deciding the pattern of expression, and the expected phenotype of the transgenic mouse.

For years, the experience acquired in making transgenic mice suggested that additional sequences, such as introns or polyadenylation sites, may be added to the promoter and the gene of interest in order to make the construct sequences as similar as possible to the genomic DNA. In this way, the transgene may be recognized as "endogenous DNA" in the cells in which it is integrated, therefore undergoing correct and efficient transcription and translation. Several expression cassette for transgene, i.e., vector containing a promoter region, a cloning site for the gene of interest, an intervening sequence (usually an intronic sequence) and a polyadenylation site have been generated. Few of them have allowed for the fulfillment of one of the most ambitious goals in transgenic technology, i.e., achieving a position-independent, copy number-dependent expression of the gene of interest. It means that the transgene is expressed regardless of the site of the murine genoma in which it is integrated, and the amount of foreign protein synthesized depends only on the number of DNA molecules integrated.

Once the construct has been prepared, the cassette containing the information to be injected into eggs is released from the plasmid in which it was assembled and purified. The fertilized eggs are obtained from superovulated females that have been mated to fertile males the day before collection. After microinjection, the zygotes can be kept for a few hours in culture in an adequate incubator and then the surviving embryos are implanted into the oviducts of pseudopregnant foster mice (Fig. 1). Pups are born 19 to 21 days after transfer; after a couple of weeks DNA samples are collected from them (normally DNA is extracted from their tails) and are to be analyzed for the presence of the transgene by Southern blotting or PCR. The rate of integration is very variable (from 15% to 90% of the newborns), and the positive animals are indicated as founders. The mice are now ready to be studied according to the expected phenotype or, maybe, to an unpredicted one which can surprise the investigator. It is important to stress that any phenotypic feature observed can be ascribed to the transgene only when it is reproduced in more then one founder and in their offspring.

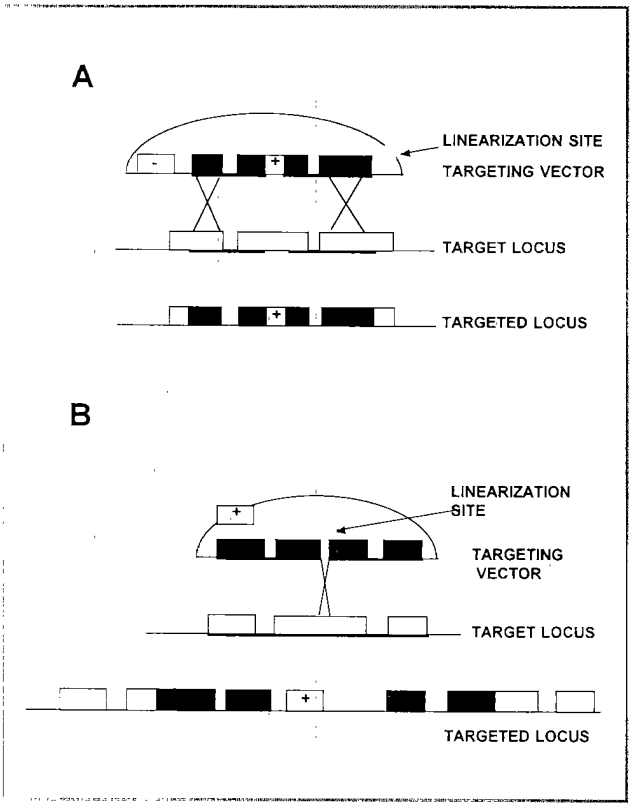

Figure 2. Gene targeting in embryonic stem (ES) cells. The thick lines represent regions of homology between the vector and the target locus; the thin lines represent the plasmids. The stippled and the white rectangles represent exons, in the vector, in the wild type locus and in the recombined one. The positive selection marker is shown as a rectangle that contains a +, while the negative selection marker is shown as a rectangle that contains a –. A) Replacement vector: upon recombination with the chromosomal DNA the positive selectable marker interrupts the target homology, therefore ablating the function of the gene, while the negative selectable marker is lost. The linearization site is outside the regions of homology. B) Insertion vector: upon recombination all the vector, including plasmid sequences is integrated into the target locus. The vector is linearized inside the region of homology.

GENE TARGETING IN MICE

Analysis of classical mouse mutants has been a useful tool for discovering the functions of gene products. Cloning of some of the involved loci has provided dramatic insight into mammalian development. However, there is only a limited number of mutant mice in which

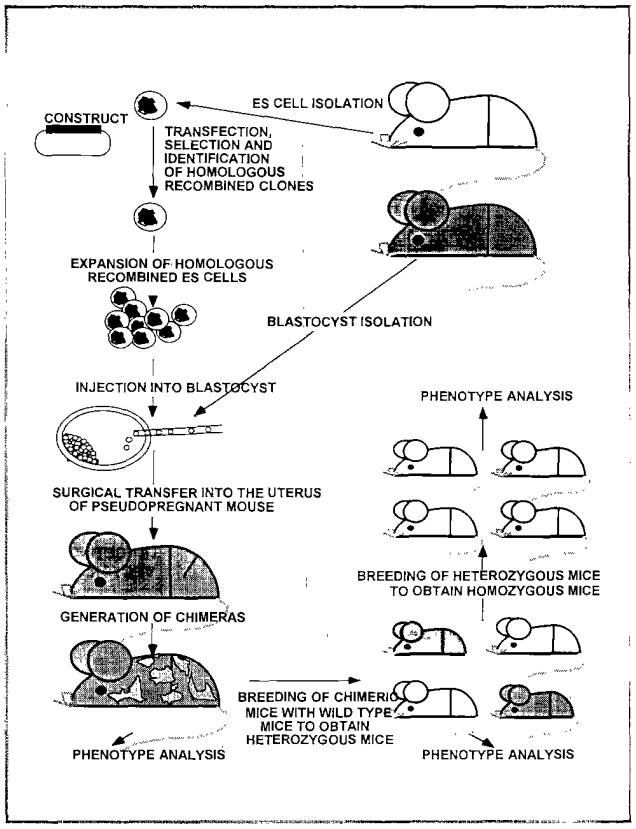

Figure 3. Production of gene targeted mice. Schematic representation of the steps required to generate gene targeted mice by homologous recombination.

the involved gene has been identified and cloned. In contrast, there is a large number of cloned genes whose position in the murine genoma does not correlate with any of the loci alterated in spontaneous mouse mutants. It is now possible to introduce mutations in virtually any locus and then generate "laboratory mutant mice" to study, *in vivo,* the function of the involved genes. When the introduced mutation causes complete disruption of the gene and no protein is coded by such a modified gene, the resulting animal is referred to as "knock out mouse" or "null mutant" for that specific gene. On the contrary, when the introduced mutation causes a misfunction, or a gain of function, and an aberrant protein is coded by the gene in question, the resulting animal is indicated as a "knock in mouse". In the last few years investigators have refined their skills at a level that is now possible to generate mutants which contain the same gene alteration found in human pathologies, for example ablation of a specific domain of a protein, introduction of aminoacid mutation and chimeric proteins such as those found in translocations.

Two key technologies have made this experiment system possible: the isolation of embryonic stem cells as permanent *in vitro* cell lines, and the discovery that mammalian cells could recombine introduced vector DNA with a homologous chromosomal target, a process known as gene targeting. We will analyze how the generation of a laboratory mutant mouse is achieved *via* these two technologies.

Embryonic stem (ES) cells are derived from 3.5 days postcoitum (dpc) mouse embryos and arise from the inner cell mass (ICM) of the blastocyst. ES cells can grow *in vitro* and retain the potential to contribute extensively to all the tissues of an animal when injected back into a host blastocyst, which is allowed to develop in a foster mother (ES cells are totipotent cells). The animal formed from the injected blastocyst is called "chimera" since it is formed by cells from two different individuals. The injected cells can form all or part of the functional germ cells of the chimera and in doing so establish themselves in the mouse germ line. This can occur even after the ES cells have been modified by the introduction of exogenous DNA (Fig. 3). In addition, it should be mentioned that because of their totipotency, it has been possible, under specific conditions, to obtain differentiation of ES cells to form cystic embryoid bodies capable of generating erythroid and myeloid cells in suspension culture. This possibility is of great interest not only for the study of normal and aberrant hemopoiesis (the second when the ES cells contain a specific gene alteration), but also for the development of gene transfer protocols, similarly to what is done with bone marrow hemopoietic stem cells, which are very difficult to identify, to obtain and to culture.

As said above, when a fragment of genomic DNA is introduced into a mammalian cell it can locate and recombine with the endogenous homologous sequences. This results in an exchange of DNA between the chromosome and the inserted vector, such that it is possible to substitute a normal endogenous sequence with any mutated or unrelated one (Fig. 2). This type of homologous recombination, which is known as gene targeting, has been widely used, particularly in mouse ES cells, to make a variety of mutations in many different loci, so that the phenotypic consequences of those specific genetic modifications could be assessed in the organism. The

design of a targeting vector is a very important step in the gene targeting experiment and it has to be considered as the moment in which the researcher, by choosing which kind of mutation will be introduced into the gene of interest, determines the expected phenotype.

The two most common types of vectors currently utilized for gene targeting, replacement vectors and insertion vectors, and the results of their homologous recombinations with chromosomal DNA are presented in Fig. 2. The fundamental elements of any targeting vector are regions of homology to the target locus (Fig. 2). These are fragments of genomic DNA corresponding to the sequences flanking the region that will be mutated. The second important element of a targeting vector is a gene encoding a selectable marker, the most common being the neomycin resistance gene which confers neomycin resistance to the cells in which is introduced. In what are known as replacement vectors for knock out, the neomycin resistance gene cassette replaces the sequences that have to be removed so that, upon homologous recombination, once integrated into the chromosome, this gene will cause the inactivation of the target gene (Fig. 2). Alternatively, the selectable marker can be simply introduced into the target locus without replacing any sequence and without causing its disruption (for example by being positioned inside an intron). In this case, selection for correctly recombined ES cells can still be achieved, but the target gene is preserved. A different kind of mutation can then be introduced into the coding region contained in the targeting vector, so that a misfunction will be caused upon homologous recombination event (knock in). Finally, a targeting vector can also contain a negative selectable marker, the most common of which being the *Herpes simplex* virus thymidine kinase, a viral enzyme which can phosphorylate the drug gancyclovir, making it active. This marker is located outside the region of homology and will be lost if correct recombination occurs; however in the case recombination does not take place, gancyclovir will kill the cell harboring it when exposed to this drug (Fig. 2).

Once the targeting vector has been constructed, it is transfected in its linearized form into ES cells, and positive and negative selection will be carried out with the proper drugs. The resistant clones, in which the homologous recombination has occurred correctly, will now be carrying the mutation in heterozygosity. After cariotype analysis, the ES cells of the euploid clones are injected into 3.5 dpc host blastocysts collected from a mouse strain different from the one which was utilized to isolate the parental ES cells (see above, Fig. 3). The injected blastocysts are then transferred into the uteri of pseudopregnant foster mice. Chimeric pups will be born after about 17 days and if the coat colors of the two strains of mice utilized to obtain the parental ES cells and the host blastocysts are different, these newborns will show chimerism on the coat by developing patches of different color (Fig. 3). As discussed above, the ES cells harboring the mutation can contribute to the generation of the germinal line of the chimeras. If this is the case, when chimeric mice are mated to wild type mice, their offspring will contain several animals heterozygous for the mutation.

Finally, by interbreeding the heterozygotes, all the possible genotypes for the mutation are obtained according to Mendelian frequencies, unless the introduced mutation has an effect on the development of the mouse and confers a disadvantage to the animals carrying it in homozygosity. It should be mentioned at this point that all the animals obtained in the different steps of this kind of experiment should be regarded as potential mutants and careful analysis of any abnormality in their phenotypes should be done.

APPLICATIONS TO PATHOLOGY

The above described technology makes it possible to either target normal genes or insert and express "engineered" genes in several organs, or in the whole organism. The development of lesions and of abnormal functions in reproducible animal models can be assessed and described. Let us give several examples of animal models already useful to study the pathogenesis of diseases.

Among **degenerative diseases,** mice with mutations in Collagen III gene develop rupture of the great vessels or intestines, as in Ehlers-Danlos syndrome. Those with mutated keratin 1 gene develop severe blistering as appears in epidermolytic hyperkeratosis, while severe chondrodysplasia, including alterations in the ossification plate, with loose and shortened cartilage, is seen in mice carrying collagen II mutations. Huntington's disease can be reproduced by inserting CAG trinucleotide repeats in HD gene, and similar animal models can be potentially used in other neurode-

generative diseases, many of them sharing this molecular feature.

Regarding inflammatory lesions, a severe chronic synovitis, with a heavy inflammatory infiltrate similar to the pannus observed in rheumatoid arthritis can be induced in transgenic mice with overexpression of HLA DR1. Mice overexpressing tumor necrosis factor or proteins of the lymphocytic choriomeningitis virus develop respectively demyelinating lesions and chronic inflammation in the central nervous system. Complement-dependent liver necrosis in animals "perfused" with human blood can be modulated by inducing the expression of decay acceleration factor; this is potentially interesting in xenogenic liver transplantation.

The field of **cardiovascular pathology** is one of intense research. For example, mice with overexpression of lecithin:cholesterol acyltransferase (an enzyme that favors esterification and storage of excess cholesterol) have not only higher levels of low density lipoprotein, but also less arteriosclerosis, assessed by image analysis and by immunohistochemical expression of proliferation antigens in the "tunica media" of the vessel wall. Several reports have been made on models involving overexpression of genes related to the renin-angiotensin-aldosterone system; high blood pressure, lesions such as myocardial hypertrophy, severe glomerulonephritis, and a higher endothelial proliferation in vascular branching sites have been described. An interesting topic related to infarction is the expression of nitric oxide synthase (NOS) isoforms. Mice in which this enzyme has been knocked out have smaller areas of ischemia around brain infarcts than normal mice; this control on local circulation can potentially be applied to other organs (i.e., liver, in cirrhosis with portal hypertension). A transgenic model for dilated cardiomyopathy is the knock out of LMP, a gene whose product links the actin cytoskeleton to the contractile apparatus in the myocardial cells, and whose inactivation leads to atrophy and disorganization of the cytoskeleton.

The ability to inactivate/activate gene function at a particular time in a particular tissue of adult mice provides an excellent model of somatic mutations, a characteristic feature of **human neoplasia.** Oncogenes can be placed under the control of their own regulatory sequences, or under the control of "ectopic" sequences, which are either widely active or tissue-specific. The affected tissues often suffer a developmental disturbance, and in most cases a tumor develops from the cells expressing the oncogene. In this way MYC, RAS, NEU, or FOS genes have been targeted to specific tissues, almost always leading to tumors in the expressing organs. Since not all cells proceeded to malignancy, it became clear that additional molecular events were needed to have a tumoral phenotype. For example, when the RET-PTC1 fusion gene, which is present in human papillary thyroid carcinoma, is inserted in transgenic mice, they develop thyroid tumors that reproduce the morphology of papillary thyroid carcinoma, but are indolent and unable to metastasize. Many of the secondary molecular events are related to inactivation of tumor suppressor genes, such as RB or p53. In the above mentioned example, when these mice express viral proteins that inactivate p53 or RB, such as HPV-16 E7, they develop undifferentiated, fully malignant thyroid carcinomas.

Another example in which several steps are required for full neoplastic transformation is given by the overexpression of cyclin D1, usually seen in squamous cell carcinomas of the aerodigestive tract. Transgenic mice engineered to have overexpression of cycline D1 develop dysplasia of squamous epithelium at this particular location. But, it is likely that additional events such as p53 inactivation or RAS mutations are needed for a malignant tumor to arise. Similarly, when transgenic mice having non-metastatic breast carcinoma are crossed with transgenic mice harboring mutations in p16 (MTS1), a tumor suppressor gene that inhibits cycline D1, develop metastasis. This example illustrates an additional advantage of transgenic animals: it is possible to create hybrid lines by cross-breeding mice with specific alterations making it possible to study simultaneously more than one lesion in their pathogenetic pathways.

The above animal models are particularly useful to study the influence of exogen factors on a genetic predisposition, for example cooperative carcinogenesis including external stimuli (low iodine diet, UV radiation, tobacco, etc.) on a stable, mutated background, (i.e. effect of a fat-rich diet on an obesity prone genetic background), as well as the interaction/cooperation between different genetic alterations in causing an overt disease, achieved by crossing different mice with specific molecular alterations.

LIMITATIONS OF TRANSGENIC/ KNOCKOUT MODELS

A limitation of transgenic/knockout models is that significant differences in the phenotype are sometimes observed between humans and mice carrying analogous gene-

tic mutations. For example, p53 -/- mice develop a tumor spectrum just partly similar to that of patients with Li-Fraumeni cancer syndrome. In general this may be due to the two following reasons: different gene function among species; and different functional redundancy among species. In other words, a particular function can be carried out by the product of only one gene in a given species, while products of several genes may be required in another species to produce the same function. Inactivation/mutation of one gene in the latter species will probably not lead to an abnormal phenotype. Complete functional redundancy can result in knock out mice without any obvious phenotype. Finally, a disadvantage more than a real limitation is the high cost of these kinds of experiments, in addition to investments required to build and maintain the appropriate laboratories and animal houses.

CONCLUSIONS

For pathologists, transgenic/knockout models are of interest because in general they contribute to the study of the pathologic basis of disease; they help us to confirm the pathogenetic meaning of many of the genetic alterations that we can find in clinical samples; they also help us to focus our research on the genetic alterations in which a pathogenetic role has been demonstrated. Pathological expertise is required to analyze the pathological features of a transgenic animal compared to a normal one, and to validate any transgenic model by "certifying" that the lesions found in the animals correspond to the expected ones, based on human pathology. Both our traditional tools (morphology) as well as our new ones (immunohistochemistry, electron microscopy, molecular pathology) can be used to serve these purposes.

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