From Genetically Altered Mice to Integrative Physiology

Max Gassmann and Thierry Hennet

Transgenic and gene-targeted mice permit the study of the function(s) of the single gene(s) in a whole organism, thereby relating molecular biology and integrative physiology. To demonstrate the potential of transgenic models, we present in this review some physiologically relevant information obtained from genetically engineered mice.

Animal models have in many instances contributed to the advancement of physiological knowledge. In contrast to cell and tissue culture, in vivo models allow the assessment of phenomena such as tolerances, complementation, and redundancy in biological pathways. In several cases, animal models have unexpectedly led to breakthroughs that paved the way to fundamental discoveries. For example, in 1889 Mering and Minkowski surgically removed the pancreas from dogs to study the function of this organ. Surprisingly, the animals showed increased levels of blood glucose, thereby pointing to the essential role of the pancreas in blood sugar regulation. The modern analogy to this experiment would consist in removing the insulin gene from the animal's genome by "molecular surgery," thereby coupling integrative physiology with genetically engineered animals. Procedures that allow the introduction or removal of genes have been developed and optimized during the last 20 years. This achievement now allows scientists to study the functions of genes in the context of physiological mechanisms.

The classical transgenic mice: pronuclear DNA microinjection

In most cases, a transgene can be defined as an expression cassette consisting of a gene driven by a promoter of choice. The classical transgenic procedure consists of randomly introducing such a transgene into the genome to generate a gain-of-function mutation (reviewed in Ref. 1). To this end, the DNA containing the transgene is microinjected into the male pronucleus of fertilized mouse oocytes (Fig. 1). Subsequently, viable embryos are implanted into pseudopregnant foster mothers. On the average, 10–30% of the resulting litters bear the transgene in their genome. In general, a transgenic mouse line is established when the transgene is effectively transmitted to the following generations in a Mendelian way. Although the transgenic DNA is present in all cells, transgene expression is dependent on many factors such as the chosen promoter and enhancer elements, the number of incorporated copies, and the locus of integration.

Basically, two kinds of pathophysiological situations can be simulated in transgenic mice, namely, the systemic effects at an altered protein level and the consequences of ectopic gene expression. For example, because the role of angiotensinogen and renin in hypertension obviously cannot be studied in cell culture models, different transgenic mouse lines were generated that produced high serum levels of angiotensinogen or renin or both. Analyses of single and double transgenic mouse lines clearly established the requirement of both components to reach high plasma angiotensin II, thereby causing arterial hypertension (12). In addition, transgenic mice expressing angiotensinogen and renin provide physiological models to study disorders linked to high blood pressure, such as pregnancy-associated hypertension.

Transgenic mice can reproduce diseases, provided these are due to gain-of-function mutations. This is the case in amyotrophic lateral sclerosis (ALS), an autosomal dominant neurodegenerative human disease. Because some ALS patients carried point mutations in the Cu/Zn-superoxide dismutase (SOD) gene, it has been postulated that altered SOD levels are related to...
the development of ALS. Indeed, transgenic mice expressing the mutant SOD gene develop an age-related neurodegenerescence reminiscent of the human disorder (11). Thus the transgenic model clearly established the role of the mutated SOD gene in the etiology of ALS.

The main limitation of the classical transgenic approach is linked to the uncontrolled integration of the transgene into the host genome. This random integration may influence the expression of genes situated close to the transgene, and the locus of integration may affect the expression of the transgene itself. Therefore, it is mandatory to generate several transgenic mouse lines with comparable transgene expression patterns that show identical phenotypes. This cumbersome drawback became obsolete with the emergence of targeted mutagenesis techniques.

**Targeted mutagenesis in mice**

Most targeting experiments performed to date were aimed at the generation of null-mutant animals, commonly referred to as “knock-out” mice (reviewed in Ref. 13). A critical aspect of targeted mutagenesis was to define the conditions required to achieve specific genetic alterations at high yield in mice. However, this breakthrough would have remained useless if the mutation could not be introduced into the mouse germline. This critical step was made possible by the establishment of embryonic stem (ES) cell lines. Pluripotent ES cells are derived from the inner cell mass (ICM) of blastocysts. When leukemia inhibitory factor is omitted, wild-type and mutant ES cells spontaneously differentiate into embryoid bodies (right).

**FIGURE 1.** Generation of transgenic and gene-targeted mice and embryoid bodies. The transgene is microinjected into the male pronucleus because it is larger in size and has a better position compared with female counterpart. An unpredictable copy number of injected DNA integrate randomly into the mouse genome (left). Several pluripotent embryonic stem (ES) cell lines have been established from the inner cell mass (ICM) of blastocysts. On transfection with the targeting construct, homologous recombinant ES cells are microinjected into recipient blastocysts. Use of ES cells isolated from an agouti mouse strain (e.g., 129/Sv) and recipient blastocysts from a black strain (e.g., C57BL/6) gives rise to chimeric animals with 2 coat colors (middle). When leukemia inhibitory factor is omitted, wild-type and mutant ES cells spontaneously differentiate into embryoid bodies (right).

"Pluripotent ES cells are derived from the inner cell mass of mouse embryos..."
severe kidney disease and occasionally to peri-trast, the disruption of the COX-II gene led to a
ng functions. The inactivation of the COX-I gene
ates the prostaglandin pathway, is encoded by
of genes encoding related proteins. For example,
ry, 1 2 3 4
well as size of the homologous arms flanking the mutation in the vector. Usually between 1 and 75% of the ES clones resis-
tant to neomycin will harbor an homologously recombined
targeted allele.

birth to chimeric mice. When the injected ES
cells contribute to the formation of germ cells in
chimeric mice, the ES cell genotype can be prop-
agated to the next generations.

Since the first mouse with a targeted gene dis-
ruption was presented in 1989 (14), more than
1,000 targeted mutations have been described.
Most targeted mice have been generated to char-
acterize developmental and physiological func-
tions of genes in vivo. The approach is espe-
cially powerful in revealing the distinctive functions
of genes encoding related proteins. For example,
the cyclooxygenase (COX) activity, which initi-
ates the prostaglandin pathway, is encoded by
the two genes, COX-I and COX-II. Whereas
COX-I is constitutively expressed, COX-II expres-
sion is inducible among others by inflammatory
mediators such as tumor necrosis factor or
lipopolysaccharide. Both isoforms are the targets
of nonsteroidal anti-inflammatory drugs (NSAID).
It was believed that the negative side
effects of NSAID were due to the inhibition of
COX-I, which was assumed to fulfill housekeep-
ing functions. The inactivation of the COX-I gene
showed no effect on the viability, health, and fer-
tility of the corresponding null-mutant mice (8).
Also, the gastric ulceration associated to NSAID
was missing in COX-I null-mutant mice. By con-
trast, the disruption of the COX-II gene led to a
severe kidney disease and occasionally to peri-
tonitis (9). In addition, it was shown that the
inflammatory response to arachidonic acid
remained unaltered in COX-II-deficient mice.
These targeted mutagenesis experiments have
permitted revision of many conclusions about
the supposed distinctive roles of COX isoforms
and the distinction between cellular and physio-
logical effects.

Sometimes the study of knockout mice unveils
unexpected phenotypes and thereby highlights
unsuspected functions of the disrupted gene.
Although such discoveries are serendipitous,
they should not be regarded as marginal. The tar-
ged mutagenesis strategy has boosted the pace
of knowledge in many fields by providing new
insights into the functions of various genes. It is
known, for example, that mammals express a
dioxin receptor, although no endogenous physi-
ological ligand has been detected. Binding of
dioxin or other aryl hydrocarbons induces het-
erodimerization of the dioxin receptor with the
aryl hydrocarbon receptor nuclear translocator
(Arnt). Interestingly, null-mutant mice lacking the
dioxin receptor showed a spectrum of hepatic
defects and in one case a depressed immune sys-
tem. Thus it appears that the dioxin receptor is
required, at least for normal liver function. On
the other hand, the disruption of the het-
erodimerization partner Arnt resulted in embry-
onic lethality due to the lack of blood vessel for-
mation (reviewed in Ref. 15).

Another example is the inactivation of the neu-
ronal nitric oxide synthase (NOS), which revealed
an unexpected physiological role for this gene
product in the normal function of the gastroin-
testinal tract (5). Indeed, mice without neuronal
NOS have dilated stomachs and hypertrophied
intestinal tract (5). Indeed, mice without neuronal
NOS have dilated stomachs and hypertrophied
circular muscles of the stomach and pylorus.
These features seem to be secondary to alterations
in pyloric relaxation or motility, which obviously
depends on NOS-containing neurons. Surprising-
ly, the lack of the neuronal NOS gene has no
effect on the architecture of the central nervous
system, although this gene is widely expressed in
this tissue. The dilatation and circular muscle
hypertrophy observed in the stomach of mice
deficient in neuronal NOS recall the human dis-
ease infantile pyloric stenosis. In addition to this
gastric phenotype, it was observed that male null-
mutant mice are hyperaggressive while females
showed no such deviant behavior (10). These two
unexpected phenotypes detected in neuronal
NOS-null mice illustrate the usefulness of target-
ed gene disruption in mice to relate a gene with
human diseases.

Although the targeted mutagenesis approach
has become an invaluable resource, it is not
devoid of constraints. Sometimes a gene disrup-
tion results in embryonic death of the null-

FIGURE 2. Gene targeting by homologous recombination.
To replace a gene of interest (exons shown as black boxes) by
a mutated copy, a targeting vector is constructed containing
the mutated gene flanked by 2 arms of homologous genomic
sequence. Here, the reading frame of exon 2 is interrupted by
insertion of an exogenous gene (neo). When this targeting
vector is introduced into embryonic stem (ES) cells, it can
locate and recombine with the homologous wild-type allele.
A selection marker, like the neomycin resistance gene (neo),
is normally added to targeting vector to allow selection of ES
cells that have integrated the vector into their genome.
Although not fully understood, molecular mechanisms of
homologous recombination are believed to rely on a double-
reciprocal exchange surrounding the mutation. Efficiency of
homologous recombination depends on factors like the
degree of identity between the target allele and the vector, as
well as size of the homologous arms flanking the mutation in
the vector. Usually between 1 and 75% of the ES clones resis-
tant to neomycin will harbor an homologously recombined
targeted allele.

...mice without neuronal NOS have dilated stomachs....
mutant animal. In such cases, one might exploit the capability of wild-type and targeted ES cells to differentiate in vitro, thereby forming a variety of cell types. On withdrawal of the leukemia inhibitory factor, ES cells differentiate into three-dimensional structures termed embryoid bodies (Fig. 1). These embryo-like structures are viable and recapitulate several aspects of early mouse embryogenesis (2). For in vivo studies of lethal phenotypes, however, approaches have been developed that restrict the inactivation of the target gene to specific cell types or to defined developmental stages. This approach, also called “conditional” gene disruption, relies on the application of site-specific recombination systems.

**Conditional mutagenesis in gene-targeted mice**

The site-specific recombinase Cre (causes recombination) derived from the bacteriophage P1 cleaves and ligates DNA at a specific recognition site termed loxP (locus of x-over), which is an asymmetric 34 base pair (bp) DNA sequence (6). A loop is formed when four Cre proteins bind to two loxP sites (Fig. 3). When the two loxP sites involved in the reaction are in the same orientation, the recombination results in the excision of the DNA region flanked by the two loxP sites. This simplicity is the key to the versatility of the site-specific recombinase and ensures its successful adaptation to any species and any cell type. Because the recombination depends solely on the presence of the recognition sequence and of the recombinase itself, conditional recombination can be achieved by controlling the expression of the Cre protein.

Conditional gene targeting relies on the insertion of loxP sites around a specific gene into the mouse genome by homologous recombination, a procedure that is now known as “floxing.” Mice bearing a “floxed” gene are then bred with mice expressing a Cre transgene in a specific tissue (Fig. 3). Some of the resulting progeny will harbor both the floxed region and express the Cre recombinase in specific cell types. In those cells the floxed DNA region will undergo recombination and be excised from the genome (4). Such an approach has been applied to inactivate the DNA polymerase β (polβ) gene specifically in T-lymphocytes (3). Whereas the systemic null mutation was lethal to embryos, mice that lost the polβ gene in T-cells showed no impaired survival. In another case, the polβ null mutation was achieved postnatally by inducing the expression of Cre using the interferon-inducible Mx promoter (7). These very recent examples clearly demonstrate the potential of the site-specific recombination system in the mouse. Various lines of Cre transgenic mice have been produced and additional lines are currently being characterized, thus continuously extending the sites where a gene of interest can be conditionally inactivated.

**Conclusion**

The establishment of techniques that modify the genome has provided ways to reproduce genetic alterations in the mouse. In view of
issues concerning species variability, it remains important to define whether the mouse represents an adequate model for the human physiological or pathological condition to be investigated. Globally, genetically altered mice have already provided a great deal of knowledge in assessing the roles of specific proteins in numerous different pathways. The rapidly growing number of genetically engineered mice makes it difficult to keep abreast with the whole area. We encourage the curious reader to consult the helpful Transgenic/Targeted Mutation database Tbase (http://www.gdb.org/Dan/tbase/tbase.html) and further Internet resources mentioned therein. We anticipate that physiologists will be challenged with an overwhelming number of genetically altered mice representing an enormous source of information. The future of physiology appears to be firmly linked to the study of genetically engineered animal models.

We thank C. Bauer, E. Berger, T. Rülicke and R. H. Wenger for helpful discussions and critically reading the manuscript and C. Gasser for doing the artwork. We apologize that many important contributions could not be cited due to space limitation.

Our work has been supported in part by the Swiss National Science Foundation.

References