ed in relation to another major national concern: the life expectancy of the Dutch population is increasing more slowly than the European average. Although this trend is not fully understood, health-related behavior seems to play a role. Accordingly, Dutch Health Minister Ab Klink has prioritized health promotion and the integration of preventive care into the health insurance package. Much is expected from better collaboration between public health workers and general practitioners, who have specific responsibility for their registered populations.

In the Netherlands, patients and doctors generally seem willing to accept the regulated market orientation, provided that competition leads to better health care for all. It is also increasingly recognized that optimal care and prevention, apart from improving health, are important for the market itself, since they stimulate employment, societal participation, and economic development.[5]

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Knock Out, Knock In, Knock Down — Genetically Manipulated Mice and the Nobel Prize

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In Stockholm this fall, the Nobel Prize in Medicine or Physiology was awarded to Martin Evans, Oliver Smithies, and Mario Capecchi for their discoveries of "principles for introducing specific gene modifications in mice by the use of embryonic stem cells." The methods they developed make possible exquisitely detailed studies of the function of almost any gene in a living animal. Given the high degree of similarity between the mouse and human genomes, this technology of gene manipulation has important clinical implications.

The concept of genetically engineering a mouse is straightforward: devise a specific genetic modification in a chromosome of embryonic stem cells and use these modified cells to generate mice that can transmit the new

trait to their offspring. The method's simplicity rests on two principles: the ability to exchange specific chromosomal DNA sequences in mammalian cells by means of homologous recombination and the manipulation of embryonic stem cells in a way that allows inheritance of the genetic modification.

During sexual reproduction, meiosis halves the chromosomal content of a diploid germ cell, yielding a haploid gamete. The gamete fuses with another haploid gamete to become a diploid zygote, which has a new pair of chromosomes — one from the egg, one from the sperm. As it develops, the zygote recombines chromosomes at sites of homologous genes derived from the two parents (homologous recombination), creating a unique combina-

tion of genes (and ensuring genetic variation within a population). Homologous recombination also occurs in somatic cells during the repair of a damaged DNA strand, with the intact copy on the partner chromosome serving as a template.

In the 1960s, Oliver Smithies found experimental evidence that homologous recombination generated allelic variation in human haptoglobin genes, a large family containing multiple copies of functional and inoperative genes. In 1985, Smithies and colleagues introduced a short DNA sequence from the human beta-globin locus into an erythroleukemia cell line and were able to detect a specific exchange of the beta-globin gene with the homologous sequence in about 1 in every 1000 cells.[1] Since this frequency was much

higher than would have been expected if the introduced DNA had integrated randomly into the cells' genome, the experiment demonstrated the feasibility of targeted recombination of genetic material.

While Smithies was conducting this work, Mario Capecchi was devising a method for introducing DNA directly into the nucleus of a cell, using a tiny glass pipette. This technique allowed the efficient transfer of genetic material into random chromosomal locations, creating the possibility of producing transgenic organisms. Capecchi noted that multiple copies of the introduced gene were positioned in specific configurations that resulted from homologous recombination. These studies established that homologous recombination can occur in somatic cells and revealed its potential for use in genetic engineering. By generating cell lines that harbored an inoperative mutant copy of a drug-selection gene, Capecchi built an elegant system for testing cells' ability to undergo homologous recombination. He was able to rescue the genetically defective mutant cells by introducing a functional copy of the gene into their DNA.[2]

Smithies' and Capecchi's work on cultured somatic cells fueled a race to introduce genetic changes into an animal's germ line. Correcting a genetic defect in a way that ensured heritability of the correction would, however, require cell lines that contribute to the formation of germ cells. Both teams turned to the work of Martin Evans, who had characterized embryonal carcinoma cell lines that had originated from mouse testicular teratocarcinomas. These cell lines could

be induced to differentiate into multiple tissue types, indicating their potential for stem-cell–like behavior. Evans injected cultured embryonal carcinoma cells into mouse blastocysts, which were then implanted into a foster mother. The result was a line of chimeric mice containing tissue derived from the cultured carcinoma cells. But those cells had been derived from a genomically unstable tumor, so Evans and his colleagues next developed a pluripotent embryonic-stem-cell line from mouse blastocysts.[3] By injecting blastocysts with cultured embryonic stem cells that were infected with a retrovirus, they generated chimeric mice in which retroviral DNA was detectable in both somatic and germline cells. Subsequently, Evans used genetic engineering to create a mouse model of human disease: the molecular phenotype of the Lesch–Nyhan syndrome was recapitulated by injecting blastocysts with embryonic stem cells bearing a retrovirus that inactivated the mouse hypoxanthine phosphoribosyltransferase gene (*hprt*).

Evans, Smithies, and Capecchi quickly sought to repair mutated genes in embryonic stem cells. Smithies and Capecchi focused on correcting defects of the *hprt* gene in such cells by identifying and selecting cells that had undergone homologous recombination, thereby eliminating the mutant gene.[4,5] This work, in which gene targeting was accomplished by homologous recombination, led to the development of a general method by which a specific gene in an embryonic stem cell can be inactivated; the genetically altered cell, after implantation into a surro-

gate mother, ultimately gives rise to a strain of mice that is homozygous for the inert gene — the "knockout mouse." The technique has been used to generate thousands of different kinds of knockout mice with features of particular human diseases. More remarkable is the transformation of our understanding of gene function: rather than relying on spontaneous mutations to deduce gene function, we can now use experimentally targeted mutations to test a gene's functional role prospectively.

Initially, knockout mice were produced by replacing or disrupting the coding exons of a gene with a drug-selection marker. Such mice could be used to study only the effects of the loss of a gene, not a specific mutation. For the latter purpose, a "knock-in" method was developed, in which a mutated DNA sequence is exchanged for the endogenous sequence without any other disruption of the gene. Some knock-in strategies rely on the use of gene vectors with flanking sequences, termed *loxP*, that on exposure to an enzyme called Cre recombinase undergo reciprocal recombination, leading to the deletion of the intervening DNA. With this method, it is possible to replace a gene sequence with a sequence of the investigator's choice and to delete unnecessary sequences (see diagram). The gene for Cre recombinase has been knocked into targeted loci in a way that brings its expression under the direction of the endogenous gene promoter, thus allowing tissue-specific or temporal-specific expression of the Cre enzyme and hence recombination of *loxP* sites that flank the gene of interest. Applications of this
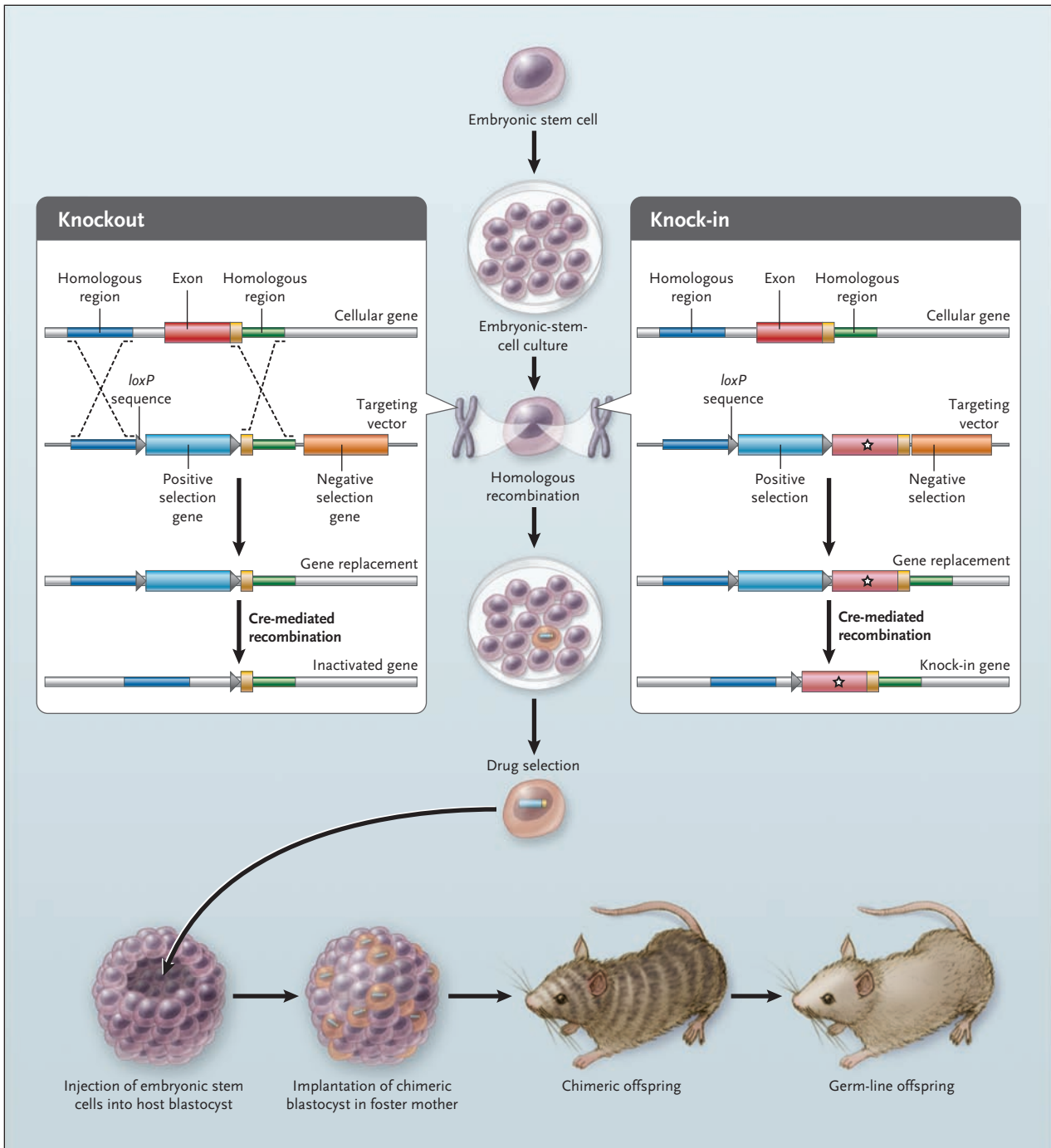
Knockout and Knock-in Mice.

A gene-targeting vector (left panel) is constructed to delete a specific exon of a gene in embryonic stem cells. Several kilobases of DNA on either side of the target gene are cloned around a drug-selection marker. After the cloned DNA (targeting vector) is introduced into the stem cells, positive and negative drug selection occurs in culture. The left panel shows a targeting vector that was constructed with *loxP* sequences flanking the positive drug-selection gene. Cre recombinase can delete the DNA sequence between the *loxP* sites, thereby deleting a specific gene in the embryonic stem cells. Knock-in mice (right panel) are generated by replacement of an endogenous exon with one harboring a mutation of interest. The gene-targeting strategy is similar to that used for knockout mice, except that a replacement exon (indicated by a star) is exchanged with the endogenous exon. Cre–*loxP* strategies can delete most traces of the targeting vector. Once the desired stem-cell clone is selected, it is injected into a blastocyst, which is implanted into the uterus of a foster mother. If the gene-targeted stem cells contribute to germ cells in the chimeric mice, subsequent offspring will harbor the gene-targeted mutation (germ-line transmission).

method are numerous, and some are already clinically useful. For example, knock-in of segments of the human immunoglobulin gene into the mouse genome enables mice to produce therapeutically useful humanized antibodies. As gene-targeting technologies and strategies evolve, it may become possible to create mouse models of polygenic human diseases such as diabetes and hypertension.

Given the success of gene targeting in mice, it is reasonable to envision clinical applications of a similar strategy. In principle, it should be possible to genetically modify stem cells to restore the function of a disabled gene in specific tissues. There is potential, for example, for correcting the mutant common gamma-chain gene in hematopoietic stem cells of patients with X-linked severe combined immunodeficiency to restore the development of lymphocytes.

Can other gene-modification techniques be used in stem cells? Last year's Nobel Prize was awarded for the discovery of RNA interference, in which genes are silenced or "knocked down" by short pieces of double-stranded RNA. This discovery has expanded our concept of heritable regulators of gene expression to include an RNA molecule. It is now possible to use viral vectors to insert interfering RNA into stem cells to reconstitute or otherwise modify the activity of genes in selected tissues. These and other methods are quickening the pace of development of clinical applications of targeted gene therapy, whose potential has been revealed by this year's Nobel Prize winners.

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