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Review Article

GENE KICKED MOUSE: KNOCK OUT MOUSE AND ITS APPLICATION

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ABSTRACT

A knockout mouse is a laboratory mouse in which genes are inactivated, or "knocked out," an existing gene by replacing it or disrupting it with an artificial piece of DNA. The 2007 Nobel Prize in physiology or medicine is awarded to Drs Mario R. Capecchi, Martin J. Evans and Oliver Smithies for their discoveries of principles for introducing specific gene modifications in mice by using embryonic stem cells. Progress to gene targeting using embryonic cell was developed by Evans and his co-workers. Ingenious development of gene targeting has been made by introducing recognition sites for the enzyme Cre recombinase, called loxP sites, into existing genes. When mice carrying such "floxed" genes are mated with transgenic mice expressing Cre recombinase, the target gene of the offspring is modified through Cre action. Gene targeting has transformed scientific medicine by permitting experimental testing of hypotheses regarding the function of specific genes. The first area to which experimental geneticists turned their attention after the birth of gene targeting in mammals was monogenic diseases. Gene targeting has been exceptionally useful in cancer research. A large number of protooncogenes, tumor suppressor genes, angiogenetic factors etc have been targeted in different tissues in mice to shed light on the induction and spreading of tumours. Gene-targeted mouse models have also become increasingly important in studies of host defense against pathogens. Gene targeted mice have become indispensable in virtually all aspects of medical research.

Keywords: Knock out mouse, embryonic stem cell, Gene targeting.

INTRODUCTION

The 2007 Nobel Prize in physiology or medicine is awarded to Drs Mario R. Capecchi, Martin J. Evans and Oliver Smithies for their discoveries of principles for introducing specific gene modifications in mice by using embryonic stem cells. Their research has made it possible to modify specific genes in the germline of mammals and to raise offspring that carries and expresses the modified gene. The experimental genetic methods developed by Capecchi, Evans and Smithies, commonly called the knockout technology, have permitted scientists to determine the role of specific genes in development, physiology and pathology. It has revolutionized life science and plays a key role in the development of medical therapy.

What Is A Knockout Mouse?

A knockout mouse is a laboratory mouse in which genes are inactivated, or "knocked out," an existing gene by replacing it or disrupting it with an artificial piece of DNA. The gene loss often causes changes in a mouse's phenotype, which includes appearance, behavior and other observable physical and biochemical characteristics.

Discovery

Martin Evans identified embryonic stem cell and isolated it from the early embryo, the cell from which all cells of the adult organism are derived. It was established in cell culture, modified it genetically and reintroduced it into foster mothers in order to generate a genetically modified offspring. Mario Capecchi and Oliver Smithies, independently, discovered how homologous recombination between segments of DNA molecules can be used to target genes in the mammalian genome and developed methods to generate genetically modified mice. Such animals have become useful in medical research. Furthermore, the knowledge concerning stem cell biology and gene technology obtained during the research that led to the "knockout mouse" has changed our understanding of normal development and disease processes and identified new avenues for medical therapy.

General Strategy for Gene Targeting

In blastocyst the embryonic stem (ES) cells was isolated and cultured, then targeting vector was introduced by electroporation, the ES cells containing the targeted gene was selected by positive-negative selection is used to enrich for ES cells containing the modified genes. These ES cells are injected into blastocysts, which are injected into foster mothers to generate chimeric animal able to transmit the mutant gene to their progeny. To facilitate isolation of the desired progeny, the ES cells and recipient blastocysts are derived from mice with different coat colour alleles.

Progress to Gene Targeting

Embryonic stem cells

A stem cell is a cell that is capable of extensive proliferation, creating more stem cells (self-renewal) as well as more differentiated cellular progeny. Stem cells of somatic origin are necessary for renewal of the tissues of the adult organism. Hematopoietic stem cells in the bone marrow differentiate into blood cells, i.e. erythrocytes, megakaryocytes / platelets and the different types of leukocytes. While each somatic stem cell of the adult organism is committed to a certain line of differentiation, the early embryo contains stem cells that are totipotent, i.e. they give rise to all cell types in the developing organism. Therefore, the embryonic stem cells from the blastocyst could be used to create to a living mammalian organism has fascinated scientists for many years. The concept that differentiated cells and tissues are derived from undifferentiated stem cells ("Stammzellen") was already proposed a hundred years ago[1]. However, the precise properties remained elusive for many decades. In the 1950s, L Stevens found that mice of the 129Sv strain have a high frequency of such tumours. He also showed that their cells could develop into embryoid bodies, i.e. aggregates of

embryonic cells. When cells were transplanted, such aggregates could induce solid tumours with many different cell types[2,3]. A few years later, Kleinsmith and Pierce demonstrated that such tumours were derived from undifferentiated embryonal carcinoma cells[4]. The development of cell culture techniques permitted investigators to establish cultures of embryonal carcinoma cells (EC cells) from murine testicular teratocarcinomas. Several scientists including Martin Evans reported on such cultures in the early 70s[5-7]. Evans obtained 129Sv mice from Stevens, established a colony of mice and characterized the teratoma derived cells in culture[8]. These embryonal carcinoma (EC) cells could be grown on feeder layers of irradiated fibroblasts. When the latter were withdrawn, extensive *in vitro* differentiation occurred. It proceeded through a primitive embryonic endoderm, which clumped into embryoid bodies. Attachment on a solid surface gave rise to all kinds of cell types, including skin, nerve, beating cardiac muscle, etc. This showed that the EC cells differentiated in the same way as the inner cell mass of the mouse embryo[9]. Evans saw the potential in using these EC cells not only for cell culture studies but also for creating chimeric mice. In order to realise this, he made injections of EC cells into blastocysts and re implanted them into foster mice. The offspring was chimeric, with contributions from EC cells in nearly every tissue[10]. Similar findings were made by several other groups at about the same time[11]. However, chimeric mice carrying EC derived cells developed multiple tumours and could not contribute to the germ line due to karyotypic abnormalities. Evans found alternative strategy to obtain germ line transmission derived from cultured embryonic stem cells. By using monoclonal antibodies, he characterised cell surface macromolecules of EC cells and their counterparts, thus identifying molecular markers of early differentiation[12]. The results suggested that normal cells with a similar phenotype as EC cells could be found and used for experiments. In 1980, Evans teamed up with the embryologist Matt Kaufman to combine cell culture and embryo manipulation. Evans described in a later review[13], he had intended to use haploid embryos for cell culture but prepared some diploid ones as controls. These cells were the embryonic stem cells (ES cells) that became critical for the success of gene targeting. Evans' team set up blastocyst injection techniques to test whether indeed ES cells could contribute to functional germ cells and thus be used to create a chimeric mouse. They reported successful germ line transmission in 1984, in another landmark paper in Nature[14]. The next step was to determine whether ES cells could be used to introduce genetic material into the germ line. Evans and his fellow workers infected ES cells with a recombinant retrovirus before injecting them into blastocysts[15]. Retroviral DNA was identified in the founders and transmitted to the F1 generation, demonstrating introduction of the foreign DNA into the mouse germ line[16]. Gossler *et al*., 1986 reported germ line transmission of a neomycin resistance gene that they had introduced into ES cells by retroviral infection[17].

Transgenesis in Mammals

Transgenic Mice

The mouse has been a favourite animal for genetic studies for many decades and was an obvious choice for the first attempts to introduce new genes into the mammalian genome. Work in several other laboratories had defined conditions for manipulating fertilized mouse eggs and blastocysts in culture. Using the culture techniques, SV40 virus DNA was introduced into blastocysts, which was subsequently implanted into pseudopregnant foster mothers. SV40 DNA can be detected in the offspring but it was impossible to demonstrate with certainty whether the DNA was integrated into the host genome, or remained as episomes[18]. A few years later, the first transgenic mouse was created by infecting embryos with Moloney leukemia virus[19]. A DNA copy of the viral RNA was present in the genome of the transgenic mice and was transferred to the offspring in the Mendelian fashion, therefore virus DNA had been introduced into the mouse germ line. Subsequent development made it possible to introduce and over express a large number of transgenes in mice and also other mammals[20]. However, integration of the foreign DNA in the genome occurs at random and the numbers of copies vary. Although it is an important tool in life science, transgene technology lacks precision with regard to the inserted gene and cannot be used to manipulate endogenous genes in a predetermined manner. These inherent problems with transgenic over expression technique limit its usefulness.

Homologous Recombination

The principle of recombination between homologous genes has been known for half a century and was recognized by a Nobel Prize to Joshua Lederberg in 1958 for his studies in bacteria. In the 1970s, it became evident that eukaryotes employ a similar machinery to mediate exchange of genetic information between homologous chromosomes during meiosis. An early study in yeast was followed by experiments demonstrating recombination between retroviral DNA sequences in the mammalian genome and introduced oligomeric retroviral DNA. Pioneering work by Richard Axel showed that cultured mammalian cells defective in thymidine kinase could be rescued by introduction of the herpes virus thymidine kinase (tk) gene[21]. Mario Capecchi improved the method and used a fine glass pipette to inject DNA directly into the nucleus[22]. This improved the efficiency of gene transfer dramatically and Capecchi's method was rapidly adopted by other investigators to introduce new genes into fertilized mouse embryos and produce transgenic mice[23]. However, the transferred gene was still introduced at random in the host genome. Oliver Smithies had developed the concept that homologous recombination might be used to repair mutated genes. As early as the 60s he had already established that an allelic variant of haptoglobin had occurred through recombinatorial events[24]. Later on, he cloned human fetal globin genes and concluded that Gγ and Aγ had arisen through a process involving homologous recombination[25]. By 1985, Capecchi had shown that homologous recombination occurs with high frequency in mammalian cells and Smithies had used homologous recombination to insert a plasmid DNA sequence into a chromosomal gene of a human cell. However, all this work was carried out in cell culture. Smithies used homologous recombination to correct a mutant HPRT gene in cultured ES cells[26]. For this purpose, an ES cell line was used that carried a deletion mutation; this cell line had previously been used for production of mutant mice. The gene HPRT was repaired with a plasmid carrying the missing promoter and first 2 exons and Smithies showed that treated cells survived and grew in HAT selection medium, which requires HPRT enzyme activity. Smithies concluded that "This modification of a chosen gene in pluripotent ES cells demonstrates the feasibility of this route to manipulate mammalian genomes in predetermined ways". All the components were now in place for producing gene-targeted

mouse strains: the development of ES cell culture, gene modification in such cells can be transmitted to the germ line and registered in the offspring; the observation was that homologous recombination occurred with high frequency in the mammalian genome[27].

Development of Gene Targeting Technology

After the establishment of gene targeting technology, in several laboratories, they extended its use in significant ways. Ingenious development of gene targeting has been made by introducing recognition sites for the enzyme Cre recombinase, called loxP sites, into existing genes. When mice carrying such "floxed" genes are mated with transgenic mice expressing Cre recombinase, the target gene of the offspring is modified through Cre action[28,29]. Another site-specific recombinase, Flp, is also frequently used to construct conditional targeting of genes in mice[30]. The activity of the Cre, or Flp, gene can be controlled by placing it under a suitable promoter to achieve tissue-specific gene targeting[31]. Expression of Cre and hence targeting of the floxed gene can be restricted to e.g. T cells (lck promoter), cardiac muscle (cardiac myosin promoter), neurons (enolase promoter) or epithelia (cytokeratin promoter). Cre expression can also be controlled, by introducing an element into the promoter which requires a ligand such as a drug for induction[32]. Tetracyclin, type I-interferon and tamoxifen have been used to obtain drug-inducible promoters. A desired gene can be targeted by administrating the drug. By introducing a tamoxifen site into a tissue-specific promoter, gene targeting can be obtained selectively in a certain tissue when the mouse is treated with the drug. Cre-lox technology can also be used to replace an existing gene with another one[33]. Such "knock-in" has been used to replace murine immunoglobulin or MHC genes with human ones in order to "humanize" the mouse with regard to immune function. It has also been used to replace an allele with another one.

Application of Knock out Mouse

Gene targeting has transformed scientific medicine by permitting experimental testing of hypotheses regarding the function of specific genes. Prior to targeting of gene, our understanding of the role of genes in higher organisms was deduced from observations of spontaneous mutations in patients and experimental animals, linkage, administration of gene products to animals and to little extent, from cell culture experiments. However, cell culture is not helpful for understanding functions and diseases involving multicellular, integrative responses. Insight into organ systems such as the nervous system, the cardiovascular system and the immune system, were fragmentary at best, as was knowledge of mammalian development. As the cardiovascular physiologist Heimo Ehmke put it, ″cells don't have blood pressure"[34]. Cardiovascular physiologists switched from rats to mice as models, downscaling their instruments and techniques in order to study the genetic regulation of hemodynamics. A new era of genetic physiology was born. The genomes of man and mouse contain about 22,400 genes. Several thousand of them have already been investigated by targeting gene. These studies have provided a wealth of information about gene function in development and disease. They have helped fuse mechanistic molecular biology with integrative life sciences such as embryology, physiology and immunology and have prompted new technical developments in physiological sciences. For medicine, the modelling of diseases in human by gene targeting in mice has been particularly informative. Scientists use observations, hypotheses and deductions to propose explanations, theories, for natural phenomena. Predictions from theories are tested by experiment. Any theories which are cogent enough to make predictions can be tested reproducibly in this way. Therefore, scientific method is essentially a cautious means of building a supportable, evidence-based understanding of our natural world. Prior to gene targeting, genetic medicine lacked the means for experimental testing. If we make an analogy with Robert Koch's approach to infectious diseases, genetic medicine could apply the first of Koch's postulates (i.e. observe an association between microbe, or in this case, gene or allele and disease) and with the invent of gene cloning, the second one (isolate the microbe/gene from the diseased individual and establish it in culture), but applying Koch's third postulate (induce the disease by transferring the microbe/gene to a host organism) required gene targeting. By mutating a gene to destroy its function (knock-out) or switching it to a disease-associated allele (knock-in), disease is induced if the hypothesis is correct. Alternative approaches based on epidemiology are currently being developed but currently available methods do not have the precision of hypothesised experiments. This digression into theory may suffice to make the point that only by targeting candidate genes did it become possible to formally establish causality between gene and disease. Now look at some specific examples of the impact of gene targeting in medicine.

Monogenetic Diseases

The first area to which experimental geneticists turned their attention after the birth of gene targeting in mammals was monogenic diseases. The Lesch-Nyhan syndrome, a defective metabolism of nucleotide caused by a mutation in the HPRT gene, served as the model condition during development of the technology. One of the reasons for choosing this particular medical condition was because selection conditions for isolating transduced cells were available for HPRT. The initial examination of HPRT-/- mice was disappointing since neither neuropathological nor behavioural features of human disease could be observed[35,36]. This prompted analysis of purine salvage pathways in mice and led to the findings that mice depend largely on adenine phosphoribosyltransferase (APRT) for purine salvage and are therefore not as sensitive to HPRT deficiency as humans. Administration of an APRT inhibitor to HPRT-/- mice induced persistent self-injurious behaviour resembling the clinical features in human disease[37]. Cystic fibrosis is one of the most common monogenic diseases and was chosen for gene-targeting studies by Smithies and his co-workers[38,39]. The defective gene had been identified by linkage studies in patient families followed by molecular cloning. It turned out to be a cAMP-activated choride channel and was termed cystic fibrosis transmembrane conductance regulator (CFTR). By knocking out CFTR in mice, a condition was generated that reproduced many features of the human disease. Thus, CFTR-/- homozygotes displayed defective chloride transport in epithelia of airways and intestines, failure to thrive, meconium ileus and pathological alterations of gastrointestinal glands. These studies were among the first to create a model of a human disease by gene targeting in mice. They have been followed by an avalanche of such knock-out models.

Cardiomyopathy

The pathogenesis of inherited heart diseases have been explored successfully by gene targeting approaches[40,41]. For instance, targeting of genes encoding components of the contractile apparatus in cardiomyocytes leads to cardiomyopathy; targeted mutations in connexin proteins of gap junctions cause conduction defects; disrupted genes for transcription factors involved in heart development lead to congenital heart malformations; and targeting of genes controlling energy metabolism causes cardiomyopathy.

Complex Diseases

Complex diseases involving the action of more than one gene, and either, gene-environment interactions, represent a particular challenge for medical research. Inheritance and interactions are usually poorly understood, it has been difficult to dissect the contribution of an individual genetic factor and the distinction between causation and correlation has been problematic. To prove causation in such a complex system, experiments must permit detection of the effects of changing only a single variable at a time. Gene targeting made such experiments possible and has permitted proof of causation in complex diseases.

Cardiovascular Diseases

Oliver Smithies has been the leader in this development. Along with Nobuyo Maeda, he focused on two important, complex diseases, hypertension and atherosclerosis[42]. Twin studies suggest that genetic factors may account for approx 70 % of familial aggregation of essential hypertension. However, 10 genes have been shown to alter blood pressure and their gene products appear to interact in complex ways. In spite of discovery; that angiotensinogen (AGT) gene polymorphism is associated with essential hypertension; the genetics of disease has remained poorly understood. Little is known about the number of genes actually involved in human hypertension, their effect on blood pressure, their mode of transmission, or their interaction with other genes and environmental components. Smithies suspected that gene dose effects would impact on blood pressure levels and designed a new method for titrating gene dosage by producing mice with one, two or three functional copies of the AGT gene[43]. "Conventional" gene targeting was used to produce the one- and two-copy mice and gap-repair gene targeting to produce mice with three copies of the AGT gene. It resulted in proportionally higher levels of gene products (i.e. plasma angiotensinogen protein) and proportionally higher blood pressure with increasing gene copy number. When Smithies and co-workers targeted another important gene for blood pressure regulation, which codes for the angiotensin-converting enzyme (ACE), no linear relationship was observed, in spite of the effectiveness of ACE inhibitors in reducing blood pressure. The data was submitted to a computer simulation for complex interacting systems and could propose a model for blood pressure control through the renin-angiotensin system, which was proven to be useful for understanding essential hypertension[44]. It shows that gene dosage, gene expression and gene product clearance/catabolism must all be considered when evaluating the genetic regulation of blood pressure. In 1992, Nobuyo Maeda, working in Smithies' developed a mouse model of atherosclerosis by targeting the gene for apolipoprotein E (*Apoe*). The same gene was targeted independently by investigators[45]. The *Apoe*$^{-/-}$ mouse develops spontaneous atherosclerosis which is remarkably similar to human disease. Michael Brown and Joseph Goldstein (1985 Nobel Prize for discoveries concerning cholesterol metabolism) and their co-workers targeted the gene for the low density lipoprotein (LDL) receptor (*Ldlr*) and obtained a mouse that develops atherosclerosis when fed a cholesterol-rich diet[46]. The introduction of the two mouse models with defective *Apoe* and *Ldlr* genes have completely changed atherosclerosis research. By crossbreeding them with other gene-targeted mice, it has been possible to deduce the importance of genes regulating inflammation, lipid metabolism, blood pressure and other factors proposed to be involved in atherosclerotic cardiovascular disease[47]. They are also used abundantly in the pharmaceutical industry for development and testing of new drugs against coronary artery disease.

Cancer

Gene targeting has been exceptionally useful in cancer research. A large number of protooncogenes, tumor suppressor genes, angiogenetic factors etc have been targeted in different tissues in mice to shed light on the induction and spreading of tumours[48]. Gene targeting of tumour suppressor genes have helped clarify their role in the formation of tumours. Mice carrying a targeted p53 gene were predisposed to tumour development[49]. Conditional targeting (using Cre-lox technology) of the adenomatous polyposis coli (APC) gene induces colorectal tumours in mice and APC-targeted mice have become useful models for research on solid tumours[50]. Targeting of genes for endothelial growth factors and proteolytic enzymes have been essential for understanding mechanisms of neoangiogenesis and metastasis of solid tumours and are also used for developing therapeutic strategies to prevent spreading[51].

Other Diseases

Contemporary research into most if not all major human diseases involves gene targeting in mice and there are "knockout models" for endocrine, metabolic, neurological, inflammatory and other disorders. Gene-targeted mouse models have also become increasingly important in studies of host defense against pathogens. Gene targeted mice have become indispensable in virtually all aspects of medical research.

CONCLUSIONS

Development of knock out mouse has become wonderful tool to study the function of specific gene in the system. Its ability to generate predictable designer mutation in mouse gene has led to penetrating new insights into development, immunology, neurobiology, physiology and metabolism. The development of a gene knockout mouse has been a massive advance to the biomedical and pharmaceutical field presenting researchers with a very powerful tool for analyzing gene function during development, as well as in disease. Development of a knockout mouse has advanced the biomedical and pharmaceutical field presenting the researchers with a powerful tool for analysing gene function during development, as well as in disease process. Gene knockout has become invaluable experimental tools for modelling genetic disorders, assigning functions to genes, evaluating drugs and toxins and for helping to answer fundamental questions in basic and applied research.

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