Mouse models for human diseases

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Mice are increasingly being used as models for the study of various human diseases. This is primarily because among mammalian models, they are most amenable to genetic manipulations. As we attempt to understand the molecular mechanism of diseases, it is imperative that the genes involved in the disease process be identified. One approach is to study mouse mutants with symptoms analogous to human diseases, and try to identify the genes responsible. Another approach is to manipulate the expression of genes suspected to be involved and see how they affect the disease development. This review briefly discusses the concept of manipulating gene expression by transgenic and gene knockout technology and illustrates this with how these techniques are used to study the mechanism of diabetic complications.

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Introduction

Many animals, including primates, dogs, cats, pigs, rabbits, rats, and mice have been used extensively as model systems to study various human diseases to circumvent the need for experimentation on human subjects. In recent years, mice have become the model of choice for several reasons: they have a relatively short life cycle; they become sexually mature about forty days after birth; and each litter usually has from eight to 15 pups. Thus, large numbers can be available for experimentation in a relatively short period of time. But more importantly, it is the possibility of obtaining mutant mice that mimic certain human disease conditions, and the possibility of manipulating their genes that make them such an attractive model system. Some of the mutants arose spontaneously from breeding stocks; some were obtained by mutagenesis; and recently, many mouse mutants have been developed by recombinant DNA technology, i.e. genetically-engineered mice. These mutant mice are invaluable models for studying how abnormalities in the function of genes lead to various diseases.

In animal experiments, there is always the question of how relevant the findings are to human diseases. It is well known that there are differences in the metabolism and physiology of different species leading to differences in their susceptibility to diseases. For example, mice and rats are resistant to atherosclerosis, mutagens induce a different spectrum of tumours in different animals, and different species develop different complications associated with diabetes. In most cases, these species differences are primarily due to differences in the levels of participating enzymes rather than to the involvement of different sets of enzymes. Herein lies the great advantage of using mouse models to study human diseases. In the situation where the mouse is resistant to developing a certain disease because the level of the key enzyme responsible is low, the level of that enzyme can be increased by introducing extra copies of the gene into transgenic mice. Alternatively, when mice do not develop a certain disease because of a high level of certain enzymes, that enzyme activity can be eliminated by "knocking out" the gene coding for the enzyme. Such mice that have been

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engineered to be more human-like in certain aspects of their metabolism or physiology are likely to provide valuable insights as to how the human body functions. While the transgenic technique can be used in other animals, the gene knockout experiments can currently only be done in mice.

Researchers in the early days of experimental research preferred to use large animals because it is difficult to perform physiological experiments on mice, and sometimes it is difficult to obtain enough mouse tissue for enzyme and other biochemical analyses. But these disadvantages are less of a concern now because most physiological techniques such as measurement of conductivity, or ion channel functions etc. can be done at the cellular level, and similarly, biochemical analytical techniques such as the assay of various metabolites requires only several microlitres of sample. Furthermore, large amounts of enzymes or proteins can be produced in *Escherichia coli* or in cell culture systems for enzymatic or structural analysis once the gene for the protein has been cloned.

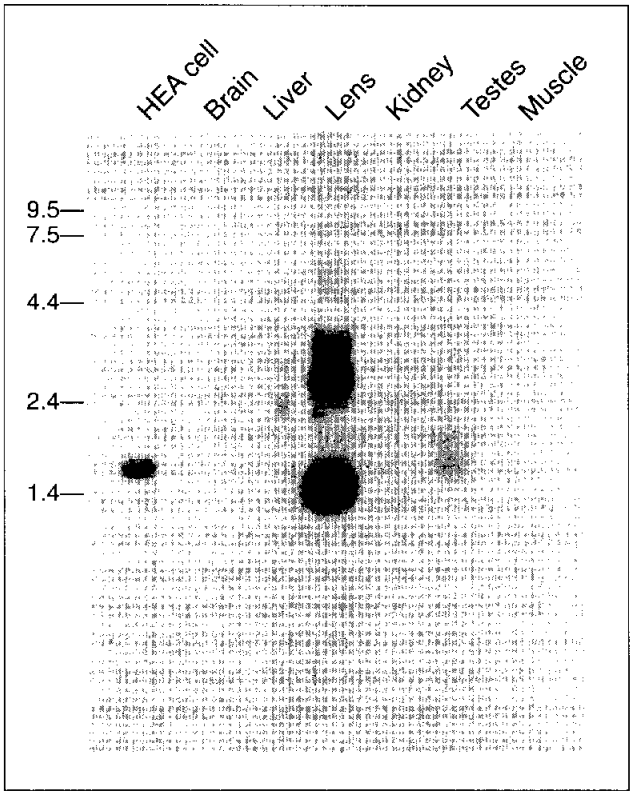

Fig 1. Analysis of transgene expression in the CAR 222 by Northern blot hybridization. Total RNA was extracted from tissues of CAR222 heterozygous transgenic mice at the age of 6 weeks and subjected to Northern blot analysis by using a 32P-labeled transgene construct as the probe. The unit of RNA size marker is kb. HEA=human endothelial cell line.

Spontaneous mutants and mutants resulting from non-targeted mutagenesis

Several large mouse breeding facilities such as the Jackson Laboratory in Bar Harbour, Maine, USA, that house tens of thousands of mice, routinely find mice with unusual behaviour or an unusual appearance in their colonies. Mutant mice with abnormal gait, size, eye development, etc. are readily identifiable. Mutants with subtle changes in enzyme activity and immune response can be identified through screening programmes. Through the efforts of these breeding facilities and many research laboratories, several hundred mutant mouse strains have been identified. Some of these mutants have been derived from parents given radiation or chemical mutagens to increase the chance of obtaining a mutant strain. Many of the mutant phenotypes have a human counterpart. Examples include: obesity (ob/ob), diabetes (db/db), congenital cataract, dwarfism, spinal muscular atrophy (sma), and neuron degeneration (mnd/mnd) [similar to the Batten Disease]. There are many other examples. Unravelling the mechanisms that lead to the mutant phenotypes in these mice will no doubt help us to understand the cause of analogous human diseases. The gene for obesity in mice has been cloned, and genes responsible for other mouse mutant phenotypes should be identified in the near future by the positional cloning approach that has identified the genes for cystic fibrosis, Duchenne's muscular dystrophy, and Huntington's disease. The advantage of the mouse system is that there is no shortage of family members for linkage analysis.

Mutants engineered by recombinant DNA techniques

The spontaneous mutants or mutants induced by mutagens that are selected for study are based on their phenotypes and the task is to find the genes responsible for these mutant phenotypes. Sometimes the situation is reversed. There are a number of cloned genes whose functions are not yet determined. Examples include some oncogenes that have been cloned based on their association with the disease state but their physiological functions are unknown. Some genes have been cloned because the proteins they encode are thought to have certain functions in vitro and it is important to confirm their functions in vivo. One could over-express these genes in transgenic mice or mutate them in gene knockout mice and see what the resulting phenotypes are.

Transgenic mice

Transgenic mice are simply mice that have foreign genes incorporated into their genome.[1-3] This can be the entire

Fig 2. In situ localization of transgene mRNA expression in lens of CAR222 mice.
(a) The left hand panel shows the photomicrograph of the lens treated with the sense control probe.
(b) The right hand panel shows the photomicrograph of the lens treated with the antisense probe.

gene including its own promoter, exons, and introns. More often, however, just the exons (cDNA) are fused to an appropriate promoter and injected into the pronucleus of the fertilized egg where the injected DNA or the transgene can integrate into any of a number of chromosomes. The injected eggs are then transferred to the oviducts of the foster mothers that have been prepared by mating with a vasectomized male. The eggs are allowed to develop to term, and after weaning, DNA samples are extracted from a small section of the tails and the presence of the transgene can be determined by PCR or Southern blot analysis. If performed by skilled staff, 10% to 30% of the pups will have the transgene integrated into their genome. The transgenic founders are then mated with non-transgenic partners to see if the transgenes can be transmitted to the next generation.

The expression of the transgene can be tested in the second generation transgenic mice, usually by determining the presence of mRNA and protein in appropriate tissues. The expression of transgenes is unpredictable as it depends on the promoter and the number of copies of transgene integrated, which ranges from a few to about one hundred. But most importantly, it depends on the site of integration. Transgenic mice are usually designed to see what happens when a function is gained. One can also design the transgene, however, to express an antisense RNA to block the translation of a message by binding to the complementary sequence of the target mRNA.[4] Alternatively, the transgene can express a ribozyme,[5] which is an RNA molecule that can bind to the complementary sequence of the target mRNA and cleave it.

Gene knockout mice

Although one can suppress the expression of a gene by expressing antisense transcript or ribozyme in transgenic mice, it is not always effective. To ensure complete elimination of the gene function, one can inactivate the endogenous gene by replacing it with a mutated copy of the gene by a homologous recombination technique.[6-8] Usually, the neomycin-resistant gene is first inserted into one of the exons of the genomic DNA clone of the gene to be replaced. The insertion of the neomycin-resistant gene inactivates the replacement gene and also serves as a positive selection marker. The herpes virus thymidine kinase (TK) gene, which renders cells sensitive to the antiviral drug gancyclovir is also inserted at one end of the replacement gene to serve as a negative selection marker. Since homologous recombination is a rare event, the gene replacement step is done in embryonic stem (ES) cells instead of in fertilized eggs. The replacement gene is introduced into approximately one million ES cells by electroporation, and the transfected cells are then cultured in growth medium containing the drugs neomycin and gancyclovir. When the replacement gene integrates into the chromosome at random sites it usually carries with it both the neomycin-resistant gene and the TK gene. These cells will be resistant to neomycin but sensitive to gancyclovir. The process of integration by homologous recombination with the endogenous gene usually eliminates the TK gene, and these cells are thus resistant to gancyclovir as well as to neomycin.

Table 1. Lens aldose reductase enzyme level and rate of galactose cataract development

Transgenic mouse line no.	Lens AR* enzyme level (nmol/min/mg)	Time to reach stages of galactosaemic cataract† Stage I	Stage II	Stage III
CAR222	37.9±3.57	2 days	14 days	21 days
CAR223	11.1±1.20	N§	N	N
CAR435	48.9±3.75	1 day	14 days	21 days
CAR440	11.1±0.63	N	N	N
CAR648	133.7±8.50	1 day	10 days	14 days
CAR222+ ARI‡		N	N	N
Normal mouse	1.4±0.09	N	N	N
Normal rate	37.4±1.30	7 days	14 days	21 days

*AR aldose reductase
† after 50% galactose diet
‡ aldose reductase inhibitor
§N no observable cataract

The neomycin- and gancyclovir-resistant ES cells are first tested to see if the replacement gene indeed replaced one copy of the endogenous gene. This is usually done by Southern blot analysis of the DNA extracted from the cells. ES cells that are confirmed to have one copy of the target gene knocked out are then injected into blastocysts. Since ES cells are totipotent, they will contribute to the formation of various tissues in the developing embryo. This is evident in the pups born because the ES cells are usually derived from mice with one fur colour (agouti), while the blastocysts are from mice with another colour (black). As a result, the chimeric mice will have patches of agouti and patches of black fur. Sometimes the ES cells will contribute to germ cell lineage such that the mutated gene is transmitted to subsequent generations. This again can be tested by Southern blot analysis of DNA from these mice. Since usually only one copy of the targeted gene is replaced in the ES cells, the mice derived from these cells will be heterozygous for the mutation. One can perform experiments with these mice to see the effect of reducing the level of gene function by half. To completely eliminate the gene's activity, breeding between siblings generates mice homozygous for the mutation.

One limitation in transgenic and gene knockout experiments is that many genes that are involved in disease development in adults are also essential for embryonic development. Thus, over-expressing these genes in transgenic mice or eliminating their functions in gene knockout mice sometimes results in abortive development at the embryonic stage. These problems can be circumvented by putting the transgene under the regulation of an inducible promoter such that its transcription is repressed at all times until the inducing agent is administered to the mice.[9] One can also design the gene to be knocked out only in certain tissues or only after a certain stage of development to avoid the lethal effect of the mutation during embryogenesis.[10]

In the following section, an example of using a transgenic mouse model[11] to test a specific disease theory is presented. The genetic approach circumvents the need to use chemical inhibitors whose specificity is always questionable. Thus, unambiguous conclusions can be drawn from these experiments.

The genetic analysis of diabetic complications

It is well known that a large proportion of both insulin-dependent diabetes mellitus (IDDM) and non-insulin–dependent diabetes mellitus (NIDDM) patients develop secondary diseases as a result of chronic diabetes. These diabetic complications include the development of cataracts, retinopathy, neuropathy, and nephropathy. High blood glucose had long been thought to be the primary cause, and this was confirmed by the results of the Diabetic Control and Complications Trial.[12] It is not clear, however, how hyperglycaemia leads to these complications.

There are two models that have gained strong support from animal experiments. One is that glucose can covalently attach itself to proteins without the aid of

enzymes. The attached glucose slowly changes to advanced glycosylation end-products that induce cross-linking between proteins, leading to diabetic lesions.[13] This model is supported by the fact that aminoguanidine, a drug that inhibits protein cross-linking, suppresses some symptoms of diabetic complications in diabetic animals.[14] However, this drug also inhibits other enzymes, including nitric oxide synthetase,[15] which is involved in many cellular processes.

The other is a much older model and involves the enzyme aldose reductase (AR). This enzyme is able to reduce glucose to sorbitol with the aid of the co-factor nicotinamide-adenine dinucleotide phosphate (reduced) [NADPH]. It is thought that under hyperglycaemic conditions, the accumulation of sorbitol somehow causes diabetic lesions.[16] Sorbitol accumulates because it does not readily diffuse out of the cell. Although the enzyme sorbitol dehydrogenase (SDH), together with the co-factor NAD^+, can convert it to fructose, this process is presumably too slow to cope with the increase in sorbitol that occurs in the hyperglycaemic state. This model was first proposed to explain diabetic cataract because of the large amount of sorbitol found in cataractous lenses,[17] and also because of the presence of AR in lens cells.[18,19]

Other supporting evidence for this model is that animals such as dogs, rabbits, degu, rats, and humans that have high levels of AR in their lenses are susceptible to developing diabetic cataract, while mice that have low levels of AR in their lenses are not susceptible to this disease.[20] The strongest evidence comes from the use of AR inhibitors, which can suppress cataract and other diabetic complications in experimental animals.[21-23] Clinical trials of these drugs were unsuccessful, however, leading to the speculation that the beneficial effect of AR inhibitors may be the result of the inhibition of other enzymes. Kinetic[24,25] and X-ray crystallographic studies[26] also support the view that AR is unlikely to be able to reduce glucose under physiological conditions. To resolve these controversies we used genetic manipulations to test the role of AR in diabetic cataractogenesis, avoiding the use of chemical inhibitors.

Since mice have low levels of AR in their lenses and are not susceptible to developing diabetic cataract, the experiment was designed to increase the AR level in the lenses of transgenic mice to see if they then developed cataracts. The human AR cDNA was cloned, fused to the mouse alpha A-crystallin promoter and injected into mouse oocytes. This promoter had been shown to express heterologous genes specifically in the lens epithelial cells in transgenic mice.[27] This is an important concern because cataract is one of the symptoms for a number of unrelated syndromes. Thus, it is possible that over-expression of AR in other tissues may indirectly contribute to cataract development, complicating the interpretation of the results.

Several lines of transgenic mice were identified by polymerase chain reaction (PCR) amplification of the

Table 2. Rate of diabetic cataract formation and lens sorbitol level in heterozygous and homozygous transgenic mice

Transgenic mouse line no.	Transgene genotype	Lens AR* enzyme level (nmol/min/mg)	Time to reach stages of diabetic cataract			Lens sorbitol level† after having diabetes for		
			Stage I	Stage II	StageIII	1 week	2 weeks	3 weeks
CAR222	heterozygous	37.9±3.57	N‡	N	N	1.3±0.11	1.9±0.40	3.0±0.69
	homozygous	75.4±4.29	17 days	28 days	ND§	5.2±0.14	6.0±0.48	7.4±0.67
CAR648	heterozygous	133.7±8.50	14 days	25 days	31 days	15.8±1.31	17.5±1.23	19.1±1.86
	homozygous	270.3±15.26	7 days	12 days	20 days	20.5±1.08	25.4±1.54	18.9±1.79
Normal mouse		1.4±0.09	N	N	N	0.3±0.12	0.3±0.11	0.3±0.02

*AR aldose reductase

† amount of sorbitol is expressed in μmol/g wet weight of lens and the values are mean ± S.D. from four mice

‡N no observable cataract during course of experiment

§ND not determined

transgene, and five were chosen for detailed analysis. The AR activity in the lens of these transgenic mice was measured and the result is shown in Table 1. The AR activities in non-transgenic mouse lens and normal rat lens are also shown. In non-transgenic mice, the lens AR activity is only approximately 4% of that of rat lens. Transgenic line CAR648 has the highest lens AR activity, having 3.5 times that of rat lens, while the lens AR activity in CAR222 and CAR435 are similar to that of rat. Lines CAR223 and CAR440 have only one-third of the AR activity seen in rat lens. Interestingly, the level of transgene expression is not proportional to the copy number of the transgene (data not shown), indicating that expression of the transgene is strongly affected by the neighbouring sequences in the chromosome.

The expression of human AR from the transgene in brain, liver, lens, kidney, testis, and muscle was examined by Northern blot hybridization of RNA extracted from these tissues, and the result is shown in Fig 1. Only lens contained the human AR mRNA, confirming the specificity of the alpha A-crystallin promoter. The types of cells in the lens expressing the AR transgene were determined by in situ hybridization. As shown in Figures 2a and b, AR transgene is expressed in the epithelial cell layer and in the bow region of the lens. This is similar to the site of expression of the endogenous AR gene, indicating that the transgene is expressed in cells that normally contain AR, and its expression merely increased the level of AR in these cells.

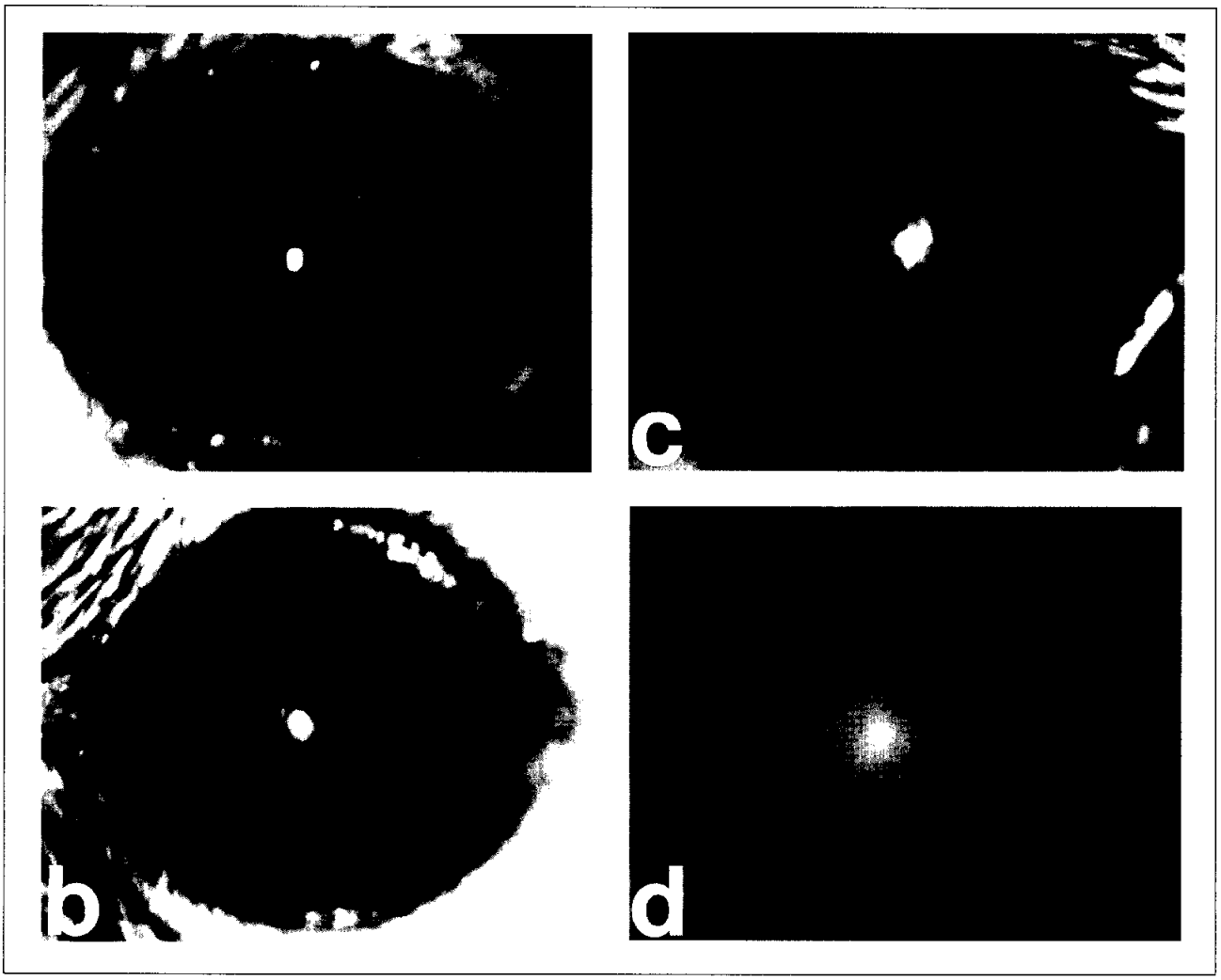

Fig 3. Three stages of cataract development in transgenic mice.
(a) Normal lens: no sign of cataract.
(b) Stage I: small vacuoles appear in the peripheral region of the lens.
(c) Stage II: vacuoles cover the entire lens and fuse together so that individual vacuoles disappear.
(d) Stage III: complete opacification occurs through the entire lens.

Under normal rearing conditions, none of the transgenic mice developed cataract, indicating that over-expression of AR in the lens did not by itself cause cataract (Fig 3a). These mice were induced to become diabetic by a single injection of streptozotocin (200 mg/kg body weight, Sigma, US), and their blood glucose monitored periodically by blood glucose test strip (Line 4-Blood glucose strip, Haemo-Glukotest, Boehringer Mannheim, UK). Only those with blood glucose greater than 500 mg/dL throughout the experimental period were included in the study.

When the heterozygous transgenic mice were induced to become diabetic, only CAR648, the line with the highest lens AR activity, developed cataract (data not shown). Since only one line developed cataract, the result was suggestive, but did not prove that a high level of AR made the mice susceptible to developing diabetic cataract. Lines CAR222 and CAR648 were mated with their siblings to develop homozygous lines to double the AR activity. Enzyme assay showed that the homozygous mice had twice as much AR activity as their heterozygous siblings (Table 2). When the homozygous and heterozygous CAR222 and CAR648 mice were induced to become diabetic, homozygous, but not heterozygous CAR222 developed cataract, and homozygous CAR648 developed cataract more rapidly than did their heterozygous siblings. Furthermore, the rate of cataract development, as judged by the time it took to reach the three stages of cataract defined in Figure 3, was proportional to the lens AR level. These results are summarized in Table 2. These experiments demonstrate unambiguously that AR is the key enzyme in the development of diabetic cataract, and that it is able to reduce substantial amounts of glucose to sorbitol in vivo (Table 2), contrary to the predictions of enzyme kinetic studies and X-ray crystallographic analysis.[24-26]

Although we firmly established that AR is the key enzyme in the aetiology of diabetic cataract, the mechanism is still not clear. One model suggests that the accumulation of sorbitol causes osmotic stress, leading to an influx of water and the swelling of lens cells, which is observable under a microscope.[28] Another model points to the reduced level of NADPH, the co-factor for AR, during the reduction of glucose to sorbitol or the reduced level of NAD^+, the co-factor for SDH, during the conversion of sorbitol to fructose as the major cause.[29] A third model suggests that it is the fructose[30] formed from the oxidation of sorbitol and its derivative fructose-3-phosphate (both potent non-enzymatic glycosylation agents) that are the main culprits.[31] To see which model is correct, one can block the conversion of sorbitol to fructose by knocking out the gene for SDH and see whether cataract development is accelerated or delayed. If cataract development is due to sorbitol accumulation, this will accelerate cataract formation. If fructose or fructose-3-phosphate is the main cause, this will prevent cataract formation. If the reduction of NADPH level is the culprit, cataract development should not be affected, and if the reduction of NAD^+ level is to be blamed, blocking the conversion of sorbitol to fructose should also prevent cataract formation.

Fortunately, there is a naturally occurring SDH-deficient mutant called C57LiA,[32] which made knocking out this gene unnecessary. Homozygous SDH-deficient mice were mated with homozygous CAR222 and CAR648. All the progeny of these matings (F1) were heterozygous for SDH deficiency and AR transgene. The siblings of F1 mice were mated with each other, and their offspring (F2), with 9 possible genotypes, were induced to become diabetic and their rate of cataract development monitored. The results, shown in Table 3, clearly show that mice deficient in SDH accumulate higher levels of sorbitol and develop cataracts at a faster rate. This experiment convincingly shows that the accumulation of sorbitol is the major cause of diabetic cataract development, and effectively rules out other models.

Although mice are not susceptible to developing diabetic cataract, they are prone to developing diabetic neuropathy and nephropathy. We are employing a different strategy to study the role of AR in these two diseases. Since there are substantial amounts of AR in the nervous tissues and kidneys of the mouse, we are trying to knock out the AR gene to see if the resultant mutant mice become less susceptible to developing these complications.

Future prospects

The experiments described in the previous section illustrate the power of genetic manipulation in the analysis of a disease process. It is evident that the mouse model is an indispensable tool, and it is gaining popularity rapidly. Although the development of transgenic and gene knockout mice remains technically challenging and requires substantial investment in equipment and facilities, many research centres and academic institutions provide them as a service to their research communities. Several companies also offer these services for a fee. These services should greatly facilitate the application of mouse genetics in biomedical research.

Table 3. The effects of sorbitol dehydrogenase deficiency on the rate of diabetic cataract development and lens sorbitol level in the F2 mice generation

Transgenic line no.	Genotypes of F2 mice	Time to reach stages of diabetic cataract Stage I	Stage II	Stage III	Amount of lens sorbitol* 1 week after diabetes
CAR222	$hAR^{-/-}$ $mSD^{+/+}$	N†	N	N	0.24±0.01
	$hAR^{-/-}$ $mSD^{+/-}$	N	N	N	0.39±0.05
	$hAR^{-/-}$ $mSD^{-/-}$	N	N	N	0.57±0.04
	$hAR^{+/-}$ $mSD^{+/+}$	N	N	N	1.38±0.11
	$hAR^{+/-}$ $mSD^{+/-}$	N	N	N	1.81±0.09
	$hAR^{+/-}$ $mSD^{-/-}$	N	N	N	2.74±0.27
	$hAR^{+/+}$ $mSD^{+/+}$	17 days	28 days	ND‡	5.00±0.13
	$hAR^{+/+}$ $mSD^{+/-}$	15 days	25 days	ND	5.43±0.41
	$hAR^{+/+}$ $mSD^{-/-}$	13 days	21 days	ND	6.82±0.16
CAR648	$hAR^{-/-}$ $mSD^{+/+}$	N†	N	N	0.25±0.06
	$hAR^{-/-}$ $mSD^{+/-}$	N	N	N	0.40±0.03
	$hAR^{-/-}$ $mSD^{-/-}$	N	N	N	0.57±0.07
	$hAR^{+/-}$ $mSD^{+/+}$	14 days	25 days	ND	16.07±1.42
	$hAR^{+/-}$ $mSD^{+/-}$	14 days	21 days	ND	16.96±0.92
	$hAR^{+/-}$ $mSD^{-/-}$	12 days	20 days	ND	17.35±2.16
	$hAR^{+/+}$ $mSD^{+/+}$	7 days	12 days	20 days	20.79±0.66
	$hAR^{+/+}$ $mSD^{+/-}$	4 days	7 days	14 days	22.56±1.17
	$hAR^{+/+}$ $mSD^{-/-}$	2 days	6 days	10 days	26.51±1.98

* amount of sorbitol is expressed in μmol/g wet weight of lens and the values are mean ± S.D. from three to six mice
†N no observable cataract during course of experiment
‡ND not determined

As we are gaining a better understanding of the regulation of gene expression, we are also acquiring more tricks and techniques to target the expression of transgenes in the tissues that we want to investigate and have them expressed when we want. The gene knockout technology is also becoming more and more sophisticated. Genes can be mutated only in the tissues desired, and the mutation event can be induced to occur at any stage of development or at any time during adulthood. It is likely that some of these techniques can be applied to gene therapy.

The human genome project will undoubtedly contribute to the increased use of mouse models. This project will identify more than 100 000 new genes based on DNA sequence information without any knowledge of their functions. The vast majority of these genes will have mouse counterparts and their functions can more easily be identified in mice.

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