 0014-4754/95/111028-12$1.50 + 0.20

Genetic knockouts in mice: an update

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Received 20 February 1995; received after revision 12 April 1995; accepted 2 May 1995

Abstract. Gene disruption technology in mammals, by homologous recombination in embryonic stem cells, is a powerful method to manipulate the mouse germ line. In the past decade it has produced a wealth of knowledge concerning neuronal development, neurodegenerative disorders and the roles of oncogenes, Hox genes and growth factors during development. A surprising variety of genes, however, have given unexpected and disappointing results. A gene/function redundancy theory proposed by many investigators to explain the unexpected results has been supported in certain cases by the generation of double knockout mice. Modification of the basic technology now allows the investigators to carry out a variety of manipulations including conditional or tissue-specific knockouts. This may provide a better opportunity in the future for the gene therapy approach to correct the genetic disorder.
Key words. Gene disruption; gene targeting; homologous recombination; phenotype; redundancy; stem cells.

Introduction

Developmental biologists are always interested in knowing the function of a gene during development. To address this question, the mouse has become a favorite experimental organism of choice, mainly due to the availability of improved technologies and genetics. Rapid progress in molecular biology techniques has made it possible to develop transgenic mouse models which have become extremely valuable for use in biomedical research (developmental biology, growth factor, biology etc.) and the treatment of many devastating human disorders (cancer, cardiovascular disease, etc.). Added to this advancement is the development of a knockout technique[13,14] which can be used to introduce germ line mutations in a predetermined way in virtually any gene of interest. In this article, an attempt is made to summarize the results of this rapidly evolving technique and to address how much information we have been able to gain from gene knockouts. No attempts have been made to discuss the entire literature exhaustively. For more detailed treatment of the subject, readers are requested to consult several other recent reviews[12–14,39,45,101,112,126].

Gene disruption technology

The terminology 'gene disruption', 'gene knock-out', 'gene targeting' and 'targeted gene replacement' refers to the process by which a predetermined specific alteration in a chosen cellular gene is introduced in mouse embryonic stem (ES) cells by homologous recombination[12,13]. This results in inactivation of a specific gene in the mouse genome. It is a powerful technique which not only provides opportunities to alter and understand the functions of a gene in the context of the whole animal during development, but also enables the investigator to produce animal models of human diseases.

Briefly, the basic principle of the technology (fig.) consists of constructing a targeting vector by inserting a positive marker sequence (usually a neomycin resistance gene) within a coding sequence of the test gene. This vector also contains another marker (usually the herpes thymidine kinase gene) which in the later stages of the procedure helps to avoid selecting cells which contain a randomly inserted gene. When this vector is introduced into mouse embryonic stem cells, a homologous recombination takes place (identical sequences between the targeting vector and the normal gene on a chromosome will be aligned) and as a result, the preexisting normal gene on a chromosome is replaced by the altered gene. By growing the cells in medium containing the drugs G418 (which kills those cells not containing the targeting vector) and ganciclovir (which kills the cells having randomly inserted targeting vector) one can select only those cells harboring the modified gene. The ES cells containing the targeted mutation in one of the chromosomes are then inserted into recipient mouse embryos (blastocyst stage) and the chimeric mice are screened using coat color as selection marker. The chimeric males are then mated and the resulting offspring harboring the mutation (heterozygotes) are selected and mated once again to each other. The resulting mice (one in four) now carry the mutated gene in both alleles (homozygotes) and hence are deficient in normal functional genes.

This basic method has been modified numerous times to increase the efficiency of targeting frequency[12,136], to replace both alleles of an endogenous gene[24,135] with different selectable markers, to repeat targeting at a

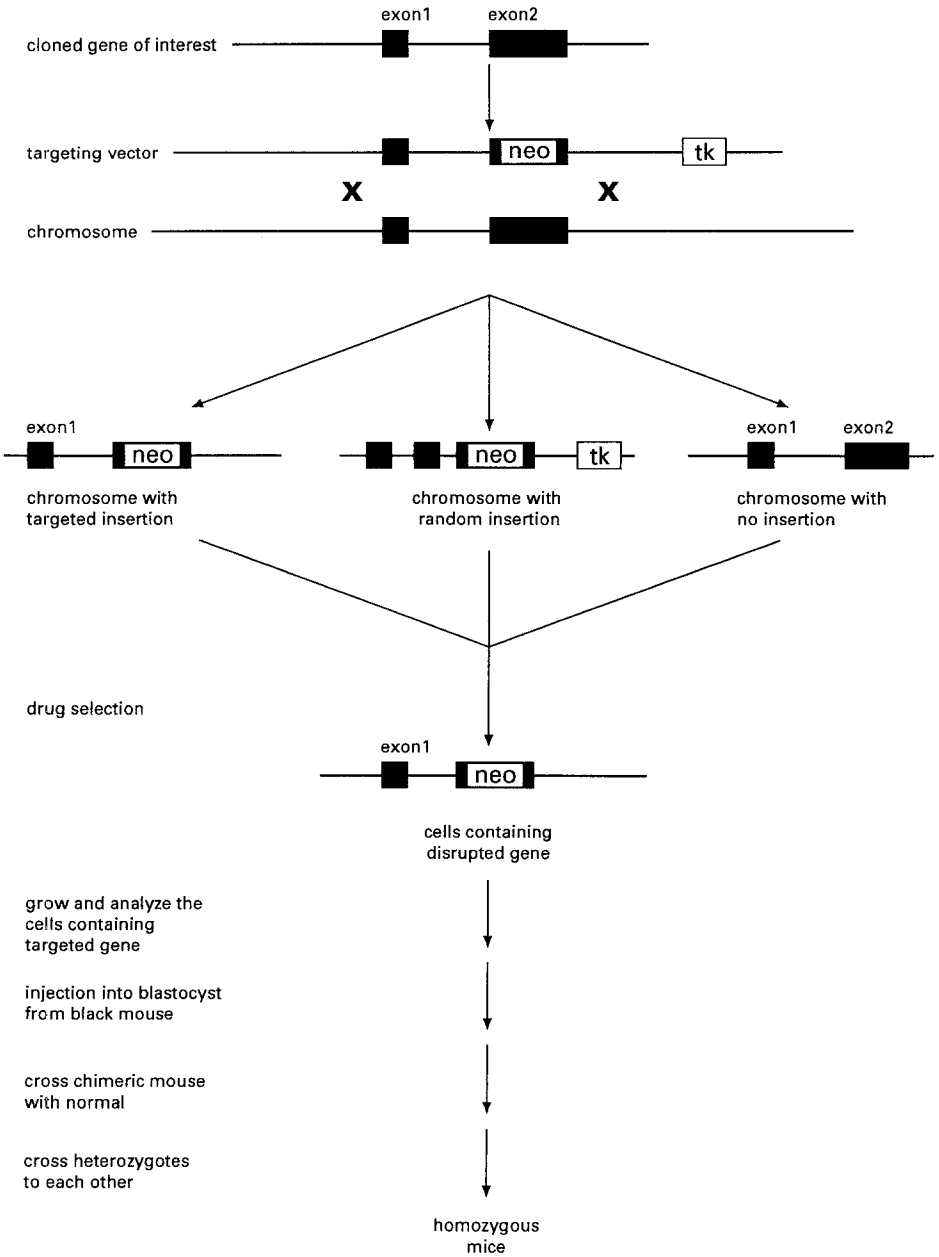

Figure. A schematic illustration of the basic strategy of gene targeting by homologous recombination in embryonic stem cells[12–14]. The figure shows the homologous recombination at the hypothetical locus. The position of neomycin (neo) resistance gene (positive selection) replacing part of the exon2 (black box) and herpes thymidine kinase (tk) gene (negative selection) (white box) are shown. The targeting vector carrying the disrupted gene is incorporated into the genome through homologous recombination. The selection of stem cell colonies containing targeted insertion is achieved by using two different drug systems. These embryonic stem cells contain one copy of disrupted gene in their chromosome.

locus[7,121,151], to generate a wide range of mutations[51,144], to duplicate a locus[117], and to carry out a conditional gene targeting or tissue-specific knockout[46,107]. The technique makes use of the Cre-lox P recombination system from bacteriophage P1 and the Cre mediated excision which is a powerful method specifically to remove unwanted DNA. Technically two mice strains, one carrying a cell-type specific Cre transgene and the second carrying the target gene but also containing the three lox P sites, in addition to the markers, are crossed. A site-specific recombination occurs in only those cells in which the Cre gene is expressed. On the other hand the target gene will remain functional in all other cells which lack Cre transgene. Using this tissue-specific knockout method, it is now feasible to address other functions of a gene during later stages of life. In addition, with a replacement vector it is also possible to introduce a point mutation into a targeted gene in the genome to understand the phenotype of the mutated gene[102]. More interestingly, the

Table. Additional genes whose functions are disrupted.

Disrupted gene	Tissue distribution	Mutant phenotype	Reference
mGluR1	central nervous system	reduced LTP deficient in LTD	1, 2, 22
Dopamine D1 receptor	brain	reduced striatal expression of dystrophin	27, 154
Vimentin	mesenchymal cells	normal	20
MAG	peripheral nervous system	subtle change in myelin	84
En-1	mid-hindbrain	multiple developmental abnormalities	153
Type X collagen	chondrocytes	normal	100
Mash-2	extraembryonictrophoblast	essential for development	47
IKaros	T cells	necessary for the development of all lymphoid lineages	40
RARβ2	all cell types	normal	82
Perforin	cytotoxic T lymphocyte and natural killer cells	mice are unable to clear lymphocytic choriomeningitis virus infection	63, 78, 146
IRF-1	ubiquitous	not essential for type I interferon gene expression	97
5-lipoxygenase gene	granulocytes, mast cells	resistant to lethal effect of shock	18
Hoxa-4	hindbrain, spinal cord	homeotic transformation	54
β2 subunit of $Na,^{+}$-K^{+} ATPase	central nervous system	degeneration of neural and photoreceptor cells	80
PLP	oligodendrocytes	behavioral changes and impairment in neuromotor coordination	11
IRS-1	pancreas	growth retardation and insulin resistance	6, 131
IPF-1	β-cells	mice lack pancreas	60
c-mpl	hematopoietic tissue	less number of platelets and megakaryocytes	48
Apolipoprotein C-III	liver	hypotriglyceridemia	79
GATA-2	blood cells	haemotopoietic defect	140
Srm	ubiquitous	normal	66
Ryanodine-receptor	skeletal muscle	abnormalities of the skeletal muscle	130
Neurotrophin-3	neurons	sensory and sympathetic deficiency, severe movement defects	32, 34, 118, 137
Fmr1	brain	hyperactivity, macroorchidism and learning deficiency	10
IgE	mast cell	active anaphylaxis	91
E-cadherin	oocytes and all cells of early embryos	fail to form a trophectodermal epithelium	72, 98
LMP-7	all cell types	reduced levels of MHC class I cell-surface expression	37
vav	hematopoietic lineages	erythroid and myeloid developments are normal	158
HNF-3β	node, floorplate, notochord, gut	essential for notochord and a gut tube formation	4, 150
HNF-4	visceral endoderm, liver	embryonic lethality	17
RXRα	ubiquitous	myocardial and ocular malformations	64
β-casein	mammary gland	not needed for milk production	68
Acrosin	sperm	not essential for sperm penetration	8
Angiotensinogen	liver	hypotension	133
*α i2		normal	85
evx1	embryonic ectoderm	abnormal phenotype	120
NF1	widespread	abnormal cardiac development	57
GM-CSF	myeloid cells	pulmonary pathology	28, 122
α-lactalbumin	mammary epithelial cells	highly viscous milk	127
TAP1	widespread	lack $CD4^{-}$ 8^{+} T cells deficient in antigen presentation	62
Leukotriene	granulocytes, macrophages and mast cells	alteration in in flammatory response	43
CD40	B lymphocytes	failure of β cell proliferation	16
CD23	B cells	normal B- and T-cell development	157

Table (continued)

Disrupted gene	Tissue distribution	Mutant phenotype	Reference
c-mos	widespread	females have reduced fertility	19, 50
CREB/ATF	widespread	normal	56
EDNRB	endothelins	produces megacolon	55
Pax5/BSAP	B cell	involved in midbrain patterning	143
E2A	widespread	required for β cell development	9, 159
βAPP	brain	spatial learning impaired	88
Wnt-4	kidney	required for pretubular cell aggregation	123
Igf2/Mpr	widespread	lethal at birth	147
TNF-R2	widespread	normal T-cell development	30
α2(v) collagen	connective tissue	spinal deformities and skin and eye abnormalities	3
Hoxd-11	forelimbs and hindlimbs	homeosis in the skeleton, forelimb malformations	36
Adenylyl cylcase type I	brain	changes in behavior and long-term potentiation	152
FGF-4	widespread	essential for development	38
NMDA receptor ϵ1 subunit	brain	reduction in long-term potentiation and spatial learning	104
IL-2R gamma	lymphoid cells	essential for natural killer cell development	26
fgfr-1	mesoderm	mesodermal patterning is defective	25, 155
NF-kappa B (50)	widespread	multiple defects in immune response	111
NF-kappa B/rel B	thymus and spleen	hematopoietic abnormalities	149
NF-IL6	macrophages	mice are susceptible to infection	132
CRH	hypothalamus and cerebral cortex	glucocorticoid is required for fetal lung maturation	87
tal-1/SCL	widespread	essential for embryonic blood formation	114
ANP	cardiac atria	salt sensitive hypertension	58
GFAP	central nervous system	normal but susceptible to scrapie prions	42
$Gp91^{phox}$	widespread	chronic granulomatous	95
MARCKS	widespread	perinatal death and abnormal brain development	129
Thrombomodulin	vascular cells, platelets	embryonic lethality	52
ICE	monocytic lineage	IL-1β production is defective	74
GAP-43	growth cones	abnormal neuronal pathfinding	128
HL	hepatocytes	mild dyslipidemia	53
VCAM-1	vascular and non-vascular cells	necessary for the formation of umbilical cord and placenta	49, 70
hoxb-5 and hoxb-6	mesoderm	affects brachiocervicothoracic structure	96
CRABPII	widespread	normal	71
Hepatocyte growth factor	widespread	impaired placenta and embryonic lethality	109, 141
CBS	widespread	severe growth retardation	148
N-ras	widespread	normal	142
apolipoprotein B	intestine and liver	embryonic lethality	33
Tcf-1	lymphocytes	thymocyte development is blocked	145
β2 nAchR	brain	normal behavior	93
Bcl-x	widespread	massive apoptotic cell death	86
mGluR6	retina	necessary in synaptic transmission to the ON bipolar cell	81
laminin β2	muscle cells	essential for the motor nerve terminals formation	89
Msx1	epithelial or mesenchymal	cleft palate and craniofacial abnormalities	105
BDNF	brain	excessive degeneration in ganglia ganglia	31, 59
neurotrophin-3 receptor gene trkC	brain	abnormal movements	65
nerve growth factor	brain	sensory and sympathetic ganglion cell loss	23
Trk/NGF receptor	neurons	extensive neuronal cell loss	115

Table (continued)

Disrupted gene	Tissue distribution	Mutant phenotype	Reference
IL-6	T cells	impaired immune response	67, 94
CD-3 Eta	T cells	lethal	92
t-PA/u-PA	blood cells	extensive fibrin deposition	15
endothelin-1	vascular endothelial cell	craniofacial abnormalities	69
Hox 11	brain	normal but lack spleen	99
lymphotoxin	lymphocytes	lack lymph nodes	139
mdr1a	renal tubules, hepatocyte, brain, testes and intestine	deficiency in blood-brain barrier	108
α1 (IX) collagen	hyaline cartilage	degenerative joint disease	35

mGluR1 = metabotropic glutamate receptor 1; LTP = long-term potentiation; LTD = long-term depression; MAG = myelin associated glycoprotein; En-1 = homeobox containing engrailed gene 1; Ikaros = genes encoding a family of zinc finger DNA binding proteins; RARβ2 = retinoic acid receptor β2; IRF1 = interferon regulatory factor 1; PLP = proteolipid protein; IRS1 = insulin-receptor substrate-1; IPF-1 = insulin promoter-factor-1; c-mpl = the receptor for thrombopoietin; GATA-2 = a transcription factor; Srm = a non receptor tyrosine kinase; Fmr1 = the gene involved in the fragile X syndrome; LMP-1 = one of the proteasome subunits; HNF-3β = a member of the family of transcription factors; HNF-4 = a transcription factor of the steroid hormone receptor super family; Mash-2 = a mammalian member of the achaete-scute family; RXRα = retinoic acid receptors (α, β, γ) activated by 9-cis retinoic acid; αi2 = inhibitory G protein subunit; evx1 = murine gene homologous to the drosophila even-skipped (eve) gene. GM-CSF = Granulocyte/macrophage colony-stimulating factor; TAP1 = transporter associated with antigen processing 1 gene; vav = oncogene; CD23 = the low affinity receptor for IgE; c-mos = a protooncogene; CREB = the cAMP response element binding protein; ATF = activating transcription factor; EDNRB = endothelin-β receptor gene; Pax5/BSAP = B cell specific transcription factor encoded by the Pax5 gene (paired box containing gene); E2A = helix-loop-helix transcription factors gene; βAPP = β-amyloid precursor protein gene; Wnt-4 = encodes a secreted glycoprotein. Igf2/Mpr = insulin like growth factor receptor type 2 also known as mannose-6-phosphate receptor; TNF-R2 = tumor necrosis factor receptor type 2; FGF = fibroblast growth factor; NMDA = N-methyl-D-aspartate; IL-2R gamma = interleukin 2 receptor gamma chain; fgfr-1 = fibroblast growth factor receptor-1; NF-kappa B = a heterodimeric transcription factor; NF-IL6 = nuclear factor IL-6; CRH = corticotrophin-releasing hormone; tal-1/SCL = T-cell leukaemia oncoprotein; ANP = atrial natruretic peptide; GFAP = glial fibrillary acidic protein; $gp91^{phox}$ = a gene involved in X-linked chronic granulomatous disease, phox stands for phagocyte oxidase; MARCKS = myristoylated alanine-rich C kinase substrate; ICE = interleukin-1β converting enzyme; GAP = growth-associated protein; HL = hepatic lipase; VCAM = vascular cell adhesion molecule; hox = homeotic genes; CRABP = cellular retinoic acid binding protein; CBS = cystathionine β-synthase; Tcf-1 = T-cell factor-1; β2 nAchR = nicotinic acetylcholine receptor β2 subunit; Bcl-x = a member of the protooncogene bcl-2; mGluR6 = metabotropic glutamate receptor subtype 6; Msx1 = homeobox gene; BDNF = brain derived neurotrophic factor; Trk = a receptor tyrosine kinase; NGF = nerve growth factor; IL-6 = interleukin-6; t-PA/u-PA = tissue-type and urokinase type plasminogen activator; Hox 11 = homeobox gene; mdr1a = gene encoding a drug transporting p-glycoprotein.
*null mutant ES cells and not null mutant mice.

technology is also used to correct the singly mutated gene into the normal allele[113] in the mouse human hybrid cell line which may provide a better handle in the future for gene therapy to correct a single gene defect in certain human disorders.

Single gene disruption

In general, mice with null mutations at a single locus show a wide range of phenotypes (depending on the gene). Some of the phenotypes are normal or less severe than anticipated based on their mode of expression, while others are lethal for embryonic development (table). Many of these genes are discussed in the previous article[112]. It is once again surprising that mice deficient for platelet-derived growth factor B (PDGFB) and its receptor (PDGFβ) show defects only in development of kidney and being severely anemic. No other tissues, such as heart, central nervous system and major blood vessels, in which PDGFβ receptor is also expressed are found to be affected in these mice[73,119]. Since PDGFα receptor can bind both PDGF A and B[110], it is possible that the α-receptor may be able to compensate for the loss of a functional β-subunit in certain cell types and that the β-receptor has only a restricted function. It is interesting to see what kind of phenotype results in mice lacking PDGFα receptor or double mutant mice. Similarly, minimal phenotypes have also been observed in mice lacking type X collagen[100] and vimentin[20] although these genes are believed to be important for skeletal development and cytoskeletal structure respectively. In order to explain the apparent lack of phenotypes in several cases, many investigators have advanced a gene-function redundancy or superfluous and non-functional theory[29,134] which appears to be a fact of life as evidenced by the following double knockout mice.

Paralogous genes knockouts

There are many examples to support the theory of gene/function redundancy. For instance, previously it has been shown that independent disruption of hoxa-3 and hoxd-3 produces minimal effects. However, when both genes are simultaneously disrupted a more extensive

homeotic transformation is observed indicating that synergistic interactions between these two genes are essential to change the phenotypes[21]. Another notable example is retinoic acid receptors. Although retinoic acid has been shown to produce a wide spectrum of malformation when it is exposed to embryos, the disruption of individual retinoic acid receptors (RARs) did not produce expected results. However, RAR double mutant mice have shown multiple abnormalities such as malformation of the head, eye, vertebrae limb, heart, neck respiratory tract, thyroid gland and genito-urinary system[76,83]. These studies demonstrate not only the essential roles of retinoic acid receptors during development, but also their functional redundancy. The Src family of tyrosine kinases also belongs to this category. These kinases (Src, Fyn and Yes) are expressed in a variety of cell types. When these genes are independently disrupted (reviewed in ref. 112), they produce minimal phenotypic changes and often these cannot be correlated with their levels of expression[5,44,77,124]. However, increased severity has been observed in double mutant mice (src/fyn and src/yes) which die prematurely shortly after birth; fyn/yes mice develop a renal disorder. These results once again support the notion that the Src family kinases are functionally interchangeable and the absence of one member may signal other members to substitute for the function in some cells[125]. This recruitment effect could be at the level of 1) a functional redundancy of the other member, 2) an increase in expression of a related member and 3) a change in catalytic activity. The net result is to nullify the blocking effect. For instance, the myogenic transcription factor myo D when inactivated produced no morphological abnormalities in skeletal muscle, but the Myf-5 mRNA level was elevated significantly (3.5-fold). However, when myf-5 and myo D were simultaneously disrupted a more severe phenotype was observed[103]. Hence, it is likely that the increased expression of myf-5 in myo D knockout mice has compensated for the loss of myo D function. Thus, there appears to be increasing evidence for the gene/function redundancy theory which has long been proposed and discussed in detail by others[134,138].

Neuronal development

Gene targeting technology has already demonstrated its usefulness in understanding the role of several genes in neuronal development[45,61]. Knockout mice lacking NMDA1 receptor which is involved in the long-term potentiation (LTP) in the CA1 region of the hippocampus died soon after birth[75] suggesting a critical role in development. Similarly, mice lacking a non-receptor tyrosine kinase Fyn have demonstrated its involvement in the induction of LTP[44]. Surprisingly, however, when a nitric oxide synthetase (NOS) gene was disrupted, mice showed normal LTP implying that either NOS is not required for LTP or its function is compensated by a related molecule, once again supporting the redundancy theory in the neuronal system[90]. Similarly, Rab3A, the small GTP binding protein does not seem to be involved in neurotransmitter release but in 'efficient docking'[41].

Knockout mice can also be useful models for understanding neurodegenerative disorders and the behavioral role of genes. For instance, mice lacking serotonin 5 HT_{1B} receptor are found to be normal but show aggressive behavior suggesting its role in controlling behavior which has long been suspected[106]. Similarly, a mouse model of Tay-Sachs disease was produced by the targeted disruption of the Hexa gene[156]. These mice are deficient in β-hexosaminidase A, a key enzyme, the mutation of which is responsible for the neurodegenerative disorder. The findings that these mice exhibit similar biochemical and pathological abnormalities of the human Tay-Sachs disease may provide further opportunities to understand the molecular basis of the disorder in detail and possibly to derive a method to correct it by placing the functional gene into the nervous system.

Conclusions

Although there are many questions such as the genetic background of mouse strains used in the studies and the maintenance and stability of redundant genes during the process of natural selection, the homologous recombination technique has made a significant contribution to the field of developmental biology. The question of genetic control of development has not been answered, but accumulated data concerning the roles of Hox genes, growth factors and oncogenes in development and the redundant nature of our genetic make-up have provided some answers. Refinements in the technique allowed investigators to introduce and/or correct a mutation in a targeted gene in the genome. These kinds of mice are suitable for understanding the effects of mutated genes. The technique also helped to produce animal models for human genetic disorders[116] and it is now feasible to address other functions of a gene during later stages of life using the tissue-specific knockout method. In the future, the generation of double mutant mice may aid in understanding many biological processes such as protein:protein interactions and their roles in normal and disease states. The genetic redundancy of several genes may provide a simple means of gene therapy for many human disorders. The ability to interfere with the function of a specific gene in a spatio-temporal manner using site-specific recombinase may provide a method to inactivate the disease-causing gene in certain stages of life. This may give another simple way to approach gene therapy. Although many ethical and feasibility questions remain to be addressed, the

ability to introduce germ line mutations may some day become useful for correcting inherited genetic disorders. The combination of biochemical and genetic approaches may also help to understand psychiatric disorders and the complex central nervous system.

Note added in proof

While this article is in press, studies on mice lacking factor VIII gene[1], Gelsolin[2], tyrosine hydroxylase[3], dopamine β-hydroxylase[4], 5-HT_{2c} serotonin receptors[5], ADP-ribosyltransferase[6], Lim 1 (a homeobox gene)[7], Hoxa 10[8], Vav[9], P-selectin/ICAM-1[10], interleukin-1β converting enzyme[11], connexin 43[12], Wnt-7a[13], activins or activin receptor type II[14,15] and follistatin[16] have been reported. The results once again show mild and severe phenotypes.

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Acknowledgements. I would like to thank Drs M. K. Hartzer and F. J. Giblin of Oakland University for their valuable corrections and suggestions. My sincere regards to the anonymous reviewers whose constructive suggestions and comments immensely helped to improve the quality of the manuscript. I would also like to thank Alice M. Carleton for her expertise in typing the manuscript. This work was supported in part by a grant from the Retinopathy of Prematurity Foundation (ROPARD) and William Beaumont Hospital Research Institute (RI-95-01).

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