The Pluripotential Embryonic Stem Cell

Origin and Application in Medicine

JUNE 8, 1995

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" If you here the word, "Impossible!" spoken as an expletive, followed by laughter, you will know that someone's orderly research plan is coming along nicely. "

"The Planning of Science" in
The Lives of a Cell, Lewis Thomas

MEDICAL GRAND ROUNDS
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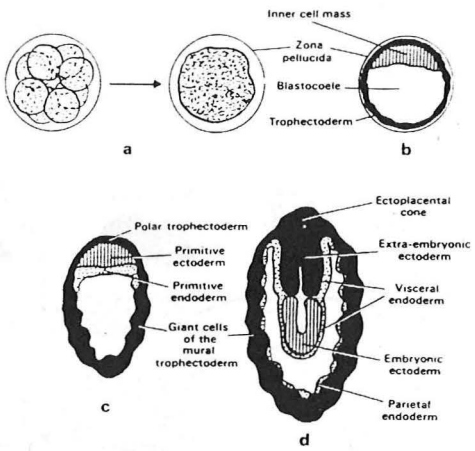
INTRODUCTION

The past two decades of research in the broad fields of molecular and cellular biology have witnessed the dramatic infusion of new techniques and approaches to scientific problems. Some of these have had profound impact on the practice of medicine; examples include PCR-based techniques for diagnosis of infectious diseases, the introduction and growing use of viral and nonviral delivery systems for human gene therapy, the ability to clone genes implicated in complex human diseases by expression and positional cloning strategies, etc. Occasionally, a new technique seems to suddenly appear in the repertoire of approaches that dramatically changes the way we think about scientific problems. While this often occurs by serendipity, it occasionally does so by logical progression of carefully conceived experiments in two disparate areas of biology that then converge at the right time and place. The subject of today's Medical Grand Rounds is an example of the latter.

My goal today is threefold : (1) to provide for you the historical background and demonstrate the techniques for one of the most powerful approaches in molecular genetics today (the "knock-out" mouse - homologous recombination in embryonic stem cells), (2) to provide for you examples of how this technology is changing the way we think about scientific and clinical problems, and (3) to introduce you to the prospects that we might one day use these cells in clinical medicine to approach problems ranging from heart failure to neurodegenerative diseases.

FIG. 1. Principal stages in early development of the mouse embryo



a: early embryo (at the 8-cell stage) progressing to compacted morula
b: blastocyst ($3\frac{1}{2}$ days after coitus) still inside the zona pellucida
c: zona-free implanting blastocyst $4\frac{1}{2}$ days after coitus
d: post-implantation embryo, at the egg-cylinder stage, showing derivatives of the trophectoderm (solid black), the primitive endoderm (stippled), and the primitive ectoderm (hatched area)

Figure 1. Mammalian developmental biology (all you need to know for now).

I. Historical Background - The pluripotent embryonic stem cell

The beginnings of this technology can be traced to a single, seminal paper by Beatrice Mintz published in 1975 (1). It had been known for at least two decades that explantation of normal day 6-7 mouse embryos (egg cylinder stage, see Figure 1) to extrauterine sites (most commonly the kidney capsule) led to the development of teratomas. These tumors frequently contained cell types representative of all three germ layers (ectoderm, mesoderm and endoderm) on initial passage. Cell lines, known as embryonal carcinomas (ECs), could be established from these tumors and these were a readily available source of tumor cells capable of limited differentiation in tissue culture (reviewed in 2). Mintz and her colleagues had established several of these, and in the course of their characterization, had adopted one of these to grow as an ascites. This particular cell line (OTT 6050) readily formed "embryoid bodies" - aggregated masses of differentiated and undifferentiated cells. Single cells from these EBs were shown to be capable of forming tumors when they were injected subcutaneously, implying that these undifferentiated tumor cells retained ***pluripotential*** characteristics (ability to differentiate into many different cell types). It was not clear whether these cell lines were capable of forming cell types representative of all germ layers *in vivo* (***totipotency***), and they deduced the most rigorous test of that would be to demonstrate contribution of the carcinoma cells to

the normal differentiation of all tissues in a mouse (Figure 2). "For this to occur, the initially malignant cells would presumably have to be brought into association with early embryo cells so that the latter could provide an organizational framework appropriate for normal development " (1).

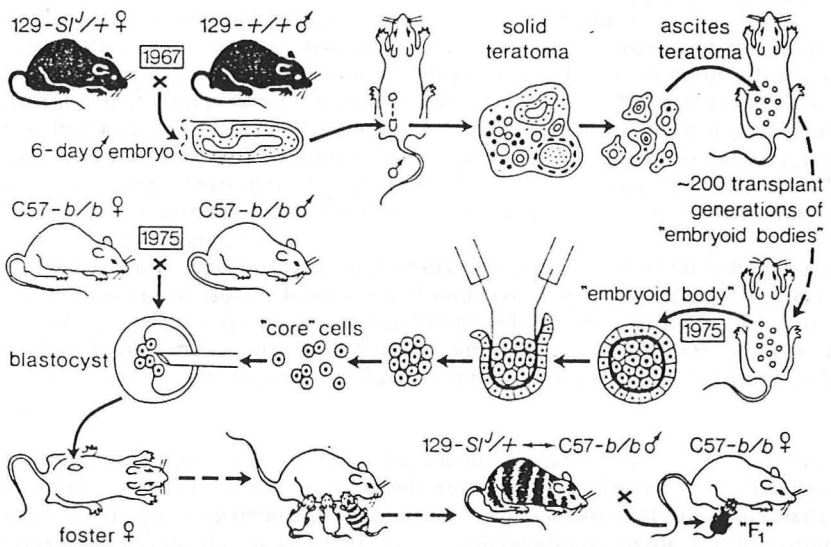


Figure 2. Pluripotency of malignant EC cells.

This was an astounding leap of logic at the time, since virtually everyone working on these cells "knew" that tumors would develop as a result of such an experiment. Even Mintz and her colleagues conceded this point for cells grown in culture, so they decided to use the core cells in the EBs grown as an ascites - reasoning that these were somehow less likely to have undergone dramatic shifts in chromosome complement (for which there was some data) since they were carried *in vivo* . She proposed these represented the most undifferentiated cells available, and subsequently injected these into normal blastocysts (remember - these had been carried in animals as an ascites tumor for nearly eight years !). To the astonishment of the scientific community, these "tumor" cells contributed not only to normal development, but contributed to the germline ! Chimeric (mosaic) mice made with these cells gave rise to essentially normal mice (1) !

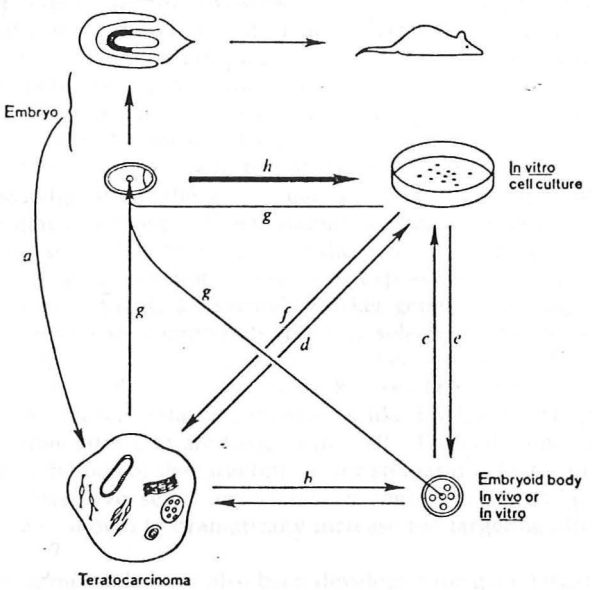
Two major scientific endeavors ensued as a result of this work, and cannot be stated any more succinctly than her original paper - "Thus, embryonal carcinoma cells from embryoid body cores offer new possibilities for studies of mammalian regulatory systems : the EC cells could first be experimentally mutagenized and selected during a brief *in vitro* sojourn and then "cycled" through mice via blastocyst injections. Participation in differentiation of a mosaic individual would permit developmental and biochemical analyses of the mutations; conversion of some cells to gametes would enable genetic analyses and mapping of the mutated regions through recombination and segregation during meiosis" (1).

Secondly, "these studies also offer a new means of analyzing the basis for malignancy. The capacity of EC cells to form normally functioning adult tissue demonstrates that conversion to neoplasia did not involve structural changes in the genome, but rather a change in gene expression. Maintenance of a proliferative stem-cell way of life, hence commitment to malignancy, appears to have been due solely to an organizational disturbance during development. Its complete reversibility in a normal environment is unequivocal, i.e., not attributable to selection for nonmalignant cells."

These results precipitated numerous attempts (and established several prominent careers) to isolate from normal embryos a growth factor(s) or cocktail that would prevent the formation of tumors or inhibit their growth. To date, no such factors have been identified, and it now appears that the growth of these "EC" cells as an ascites simply allowed for the growth of an undifferentiated clone of true ES cells.

Several attempts were made in the ensuing years to derive cell lines from normal embryos in culture but were largely unsuccessful. Then in 1981, Evans and Kaufman (3) in the Departments of Anatomy and Genetics at the University of Cambridge (and Gail Martin at UCSF, ref 4) independently made another seminal observation. Evans and Kaufman reasoned that success at establishment of such cell lines might depend on three critical factors : "(1) the exact stage at which pluripotential cells capable of growth in tissue culture exist in the embryo, (2) explantation of sufficiently large number of these precursor cells from each embryo, and (3) tissue culture conditions most conducive to multiplication rather than differentiation of these embryonic cells." They used a clever technique to enhance the proliferation of the inner cell mass (ovariectomy, which delays implantation by up to 2 days) - thus increasing their likelihood of maintaining the growth of undifferentiated inner cell mass cells. In addition, they used mitotically-inactivated fibroblasts as a "feeder" layer (these had previously been shown to aid in maintenance of the undifferentiated phenotype of some EC cell lines through continuous passage). They explanted these delayed-implantation blastocysts in tissue culture, and then "picked off" the developing egg cylinder like structures after a few days for subsequent passage. Proliferating colonies of EC-like cells were observed, and karyotype analysis revealed these to have a normal chromosome complement (both 40 XX and 40 XY). They designated the clones EK cells to indicate they had been derived directly from normal embryos (the "missing link" experiment, see Figure 3). When these cells were injected subcutaneously, they

formed tumors just as EC cells, with many different cell types. Furthermore, when they "relaxed" the culture conditions by growing the EK cells in the absence of feeders, they aggregated and formed embryoid bodies which subsequently differentiated into a host of cell types (pluripotent). Finally, they speculated these cells could be used as a vehicle for transfer of mutant alleles into the mouse genome, either selected in cell culture or inserted into the cells via transformation with specific DNA fragments.



Inter-relationships of cell lines, teratocarcinomas and embryoid bodies with normal mouse embryos. Arrows indicate routes of cell transfer: *a*, formation of teratocarcinoma by ectopic implantation of embryos; *b*, formation of embryoid bodies from teratocarcinoma and vice versa; *c*, derivation of cell culture from embryoid bodies; *d*, cell culture obtained directly from solid tumours; *e*, differentiation to embryoid bodies from culture; *f*, formation of solid tumours on reinjection of cells from culture; *g*, transfer of embryonal carcinoma cells either from cell culture or from the core of an embryoid body or from a solid tumour back to a blastocyst. All these procedures may result in chimaerism of the resulting mouse; *h*, the missing link supplied here.

Figure 3. The "missing link" experiment - ES cells from normal embryos.

Three years later this same group, now joined by two brilliant postdoctoral fellows (Allan Bradley and Elizabeth Robertson), demonstrated these cell lines would give rise to germline chimeras (5) and the stage was set for a revolution in mouse molecular genetics.

II. Homologous recombination - introduction of precise mutations into chromosomal loci

While the developmental biologists were busy developing ES cell lines, another independent line of investigation led to development of strategies for introduction of precise mutations into mammalian genes by using cloned copies of the genes (6-9). This technique, known as "gene-targeting" (homologous recombination between DNA sequences residing in the chromosome and newly introduced DNA sequences) allows for the specific alteration of genes in mammalian cell lines (Figure 4). To accomplish this task one must first clone the gene of interest and decide where to put the mutation (this usually requires some previous knowledge about the gene, such as where it is expressed, its presumed function, essential exons, etc). Most commonly, this is done by simply disrupting (or deleting) an essential coding region of the gene (typically early exons) such that you render the gene nonfunctional despite its expression. Disruption of the gene is usually done by introducing a selectable marker gene (neomycin, hygromycin) that confers resistance to toxic compounds (positive selection). Strategies to increase the frequency of targeted disruption have also been developed (10-13); the most common technique employs the thymidine kinase (TK) gene (8,9). In the presence of gancyclovir (or closely related compounds like FIAU) the TK protein generates nucleoside intermediates that are toxic to the cell. Typically, one introduces the TK gene at one or both ends of the targeting vector so that it is ***lost*** during homologous recombination (negative selection). This strategy, known as "positive-negative" selection, has been shown to dramatically increase the targeting efficiency in murine ES cells.

Additional methods have also been developed for gene-targeting (but will not be discussed today) that allow single point mutations to be introduced (by using an insertion type targeting vector (13) versus a replacement vector as demonstrated in Figure 3). Additionally, some recent techniques have shown promise for tissue-specific gene knock-outs employing binary systems (14).

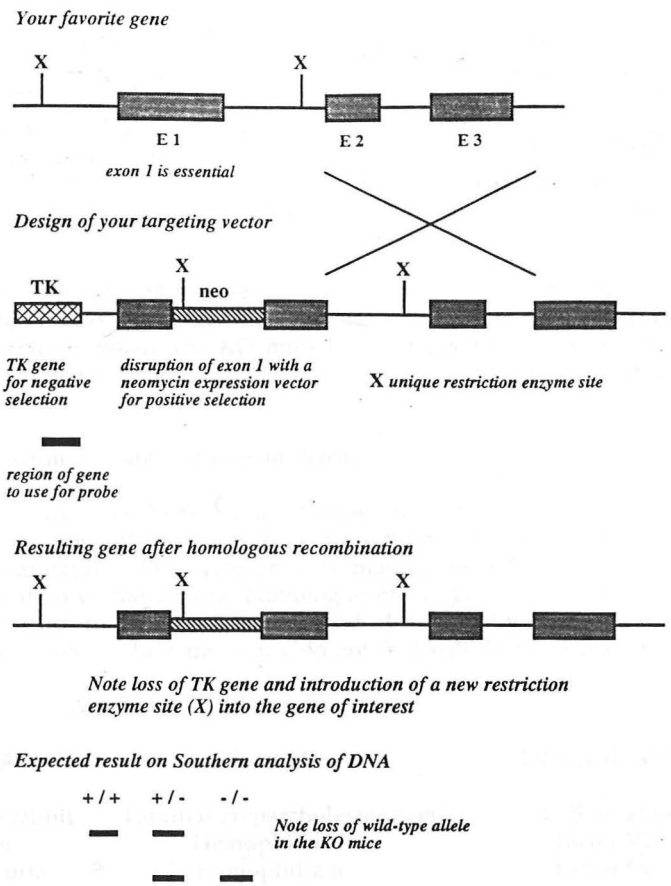


Figure 4. " Gene-targeting " via homologous recombination.

III. "The New Genetics"

It is an understatement that this technology has revolutionized mouse molecular genetics, and Mario Capecchi went so far as to entitle it "The New Genetics". Beginning with the first description of a targeted mutation in ES cells and creation of mutant mice (random proviral insertion into the HPRT locus on the X chromosome in XY ES cell lines, refs 15-17) in 1987 (18), there are now over 100 induced mutations in the mouse (obviously beyond the scope of this lecture). The technique has become one of the most powerful methods in molecular genetics today for the investigation of the functions of genes, since it allows one to assess the

consequences of loss of function throughout the life of the animal. It is almost impossible to pick up a recent issue of Science, Cell or Nature without several new "knock-out" mice described. As a result of its widespread application in developmental biology, some very interesting (and confusing !) mutations have been created. This has also led to the establishment of the Induced Mutant Resource Laboratory at the Jackson Laboratories in Bar Harbor, Maine, a facility dedicated to the maintenance of stocks of mutant mice for distribution to investigators worldwide.

I will spend some time reviewing some of these mutations, and will concentrate on two areas - (1) the application to clinical medicine (disease models) and (2) instances where the KO mice have changed the way we think about the function(s) of genes. It should be obvious that the technology can be applied to any cloned gene.

A. Animal models of human disease

There are over 2000 human diseases thought to be the result of recessive mutations; any one of these would be a candidate for creation of a mouse with the analogous mutation. The necessary information needed would be the cloning and characterization of the murine homologue of the gene. Several mouse models of human disease have been created, and dramatically recapitulate the disease phenotype present in humans (many do not !). I will discuss some of these in detail.

1. Are animal models useful ?

ANIMAL	DISEASE	DISEASE GENE
Watanabe rabbit	**Familial Hypercholesterolemia**	**LDL receptor**
Irish setter	**Hemophilia A**	**Factor VIII**
Beagle hybrid	**Hemophilia B**	**Factor IX**
Golden retriever	**Duchenne's Muscular Dystrophy**	**Dystrophin**
Cattle	**Urea cycle**	**Argininosuccinate synthase**
Plott hound/beagle	**Hurler's syndrome**	**alpha-L-iduronidase**

Table I. Animal models of human disease.

Although it may be obvious to some, the development of animal models of human disease and their utility is still debated. I reviewed the literature on many aspects of this and compiled a table of large animal (larger than a mouse) models of human diseases (Table I, all spontaneous mutations). Their utility in the development of strategies aimed at correction of human disorders is incontestable (in my view) since in every instance where a large mammal model of a human disease exists, gene therapy strategies (and other approaches as well) have been developed based upon preliminary data in these systems. Thus, the ability to experimentally create large animal models of human disease through ES cell

technology (instead of waiting for nature to provide them) represents an exciting new area of biomedical research.

2. Apolipoprotein E deficient mice ("furry balls of cholesterol")

Apolipoprotein E (apoE) is one of ten apolipoproteins that are found on the surface of lipoprotein particles (19). Its synthesis, principally by the liver (> 90% - but by almost every tissue in the mammal as well), results in a 34 kd glycoprotein found primarily on VLDL, IDL and HDL particles. It functions primarily as a ligand for receptor-mediated removal of lipoproteins from the plasma via the LDL receptor, but it has been postulated to play roles in immunoregulation, cell proliferation and differentiation (20). There are three common structural alleles in humans (ref 19 ; E4, E3. and E2) and population studies suggest these may play a role in susceptibility to coronary disease (21) and perhaps late onset Alzheimer's (22). Although extremely rare, there are reports of complete deficiency of apoE (23), and these individuals are very susceptible to atherosclerosis.

In order to investigate the role of apoE in cholesterol transport, and its possible role in other cellular processes, the gene was targeted in murine ES cells and mice were created which lacked apoE (refs 24, 25; Figure 5). These mice had markedly elevated levels of serum cholesterol on either a Chow diet (4.5% fat (w/w), low cholesterol) or a typical " Western diet " (21% fat, 0.15% cholesterol), as shown in Table 2. Furthermore, their lipoprotein profiles were markedly abnormal as well (Figure 6). The normal mouse has primarily HDL cholesterol - apoE-deficient mice had elevated levels of all lipoproteins but most dramatically VLDL.

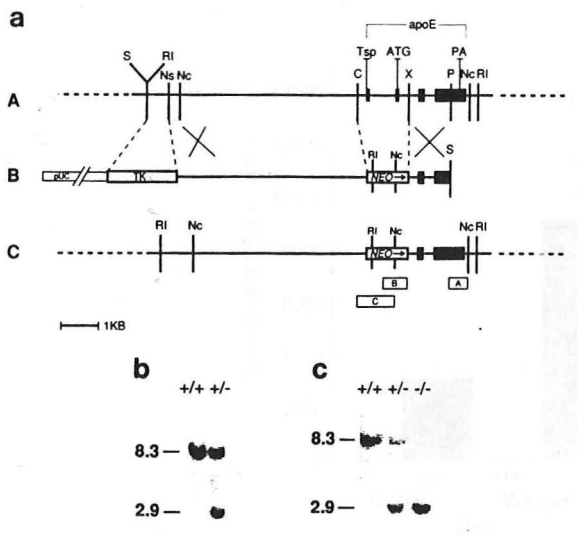


Figure 5. Gene-targeting at the murine apoE locus

(a) Line A represents the map of the endogenous murine *apoE* gene (Horiuchi et al., 1989) and its flanking sequence. The closed boxes denote exon sequence, and the solid line represents flanking and intron sequence. Restriction endonuclease sites used for cloning and screening are shown (S, SalI; RI, EcoRI; Ns, NsiI; Nc, NcoI; C, SacII; X, XbaI; P, PvuI). Important landmarks are also shown (Tsp, transcription start point; ATG, translation start site; PA, polyadenylation signal). Line B represents the vector used to target the *apoE* locus (pUC, pUC18; TK, herpes simplex virus thymidine kinase gene; *NEO*, neomycin resistance gene). The herpes simplex virus thymidine kinase and the *neo* genes were each driven off of the herpes simplex virus thymidine kinase promoter and a mutant polyoma enhancer (Mansour et al., 1988; Thomas and Capecchi, 1987). The 3' end of the construction is 5' to the *apoE* polyadenylation signal. Line C denotes the predicted organization of the locus after homologous recombination. The open box A under line C is the probe used to screen for homologous integrants, and boxes B and C are the two probes used to confirm the integrity of site-specific integration.
(b and c) Southern blot analysis of ES cell and mouse tail tip genomic DNA, respectively, digested with EcoRI and hybridized with probe A. DNA size indicated in kilobases. +/+, normal ES cell DNA and control mice; +/−, targeted ES cell DNA and heterozygous deficient mice; −/−, homozygous deficient mice.

The most prominent phenotype in these mice was the widespread appearance of atherosclerosis (even within the *pulmonary* arteries !) - which is normally very difficult to produce in mice even on a high-fat/cholesterol diet. Figure 7 illustrates the mean lesion area of the proximal aorta in animals fed either diet, and as is apparent there is a profound difference in the wild-type versus mutant animals. Histology confirmed the presence of lipid-laden macrophages within the walls of blood vessels throughout the animal, particularly in regions of high flow and presumed wall stress.

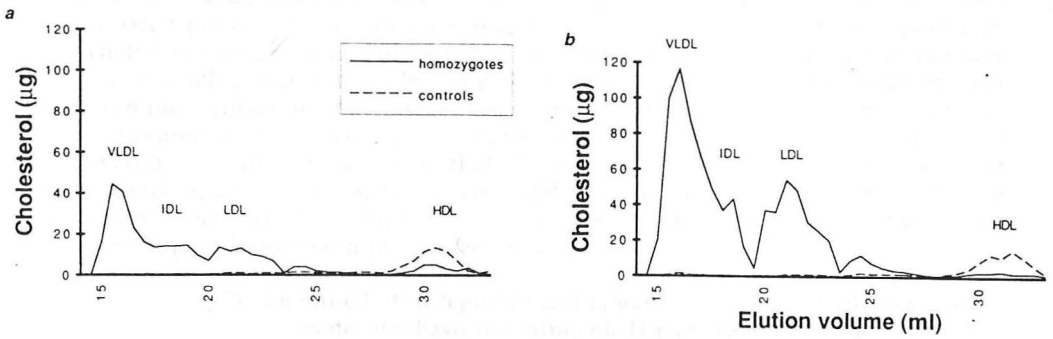


Figure 6. FPLC fractionation of plasma from apoE-deficient mice and control mice on two different diets

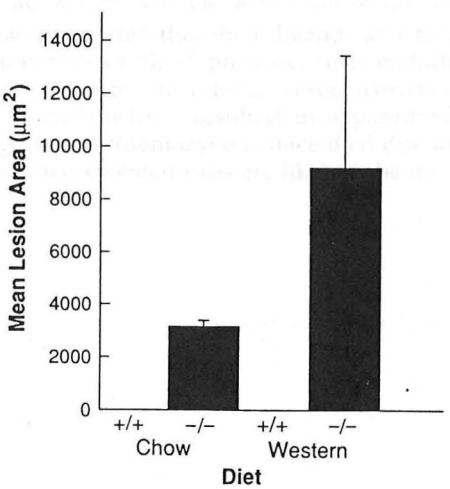
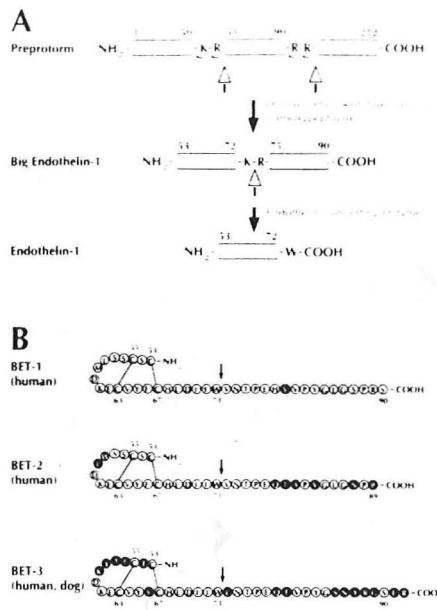


Figure 7. Quantitative analysis of atherosclerosis in apoE-deficient mice.

These mice have provided investigators with a relevant model of severe hypercholesterolemia which results in pathological lesions virtually identical to the human condition. In addition, they have proven to be a very useful model for strategies aimed at correcting hypercholesterolemia. Surprisingly, bone marrow transplantation in these mutant mice has led to complete correction of serum cholesterol and diet-induced development of atherosclerosis (26), indicating that apoE synthesized by bone-marrow derived cells can accelerate the clearance of lipoproteins (despite being present at only 12% of its normal level). This suggests that additional strategies aimed at promoting overexpression of apoE (and other secreted proteins) by tissues other than the liver may ameliorate the effects of high cholesterol on arteries. Furthermore, these animals will be important in defining the role other genes may play in promoting susceptibility to atherosclerosis. For example, crosses of these mice with mutant mice lacking P-selectin, a protein implicated in migration of monocytes across the endothelial cell layer, might establish a line of animals that is resistant to the untoward effects of hypercholesterolemia. Such animals might provide a model system for selection of drugs which inhibit the migration of monocytes into the intima - one of the earliest pathological lesions seen in atherosclerosis.

3. Endothelin-B receptor/ligand mutations - multicolored mice and molecular basis of multigenic Hirschprung's disease

The endothelins are a family of closely related peptides comprised of 21 amino acids (endothelin-1, -2, -3) that bind to one (or both) of two known receptors, endothelin-A and -B (27). Both of these receptors belong to the G protein-coupled superfamily; preferential interaction of the endothelins with the receptors appears to affect a number of signalling pathways, including the activation of phospholipase C-beta, inhibition of adenyl cyclase and activation of nonreceptor tyrosine kinase $p125^{FAK}$ (28). It was postulated that these ligands and their receptors played an essential role in maintenance of blood pressure, since endothelin-1 was discovered by virtue of its ability to promote intense vasoconstriction (29). Furthermore, targeted disruption of endothelin-1 resulted in a paradoxical increase in blood pressure in heterozygous mice (homozygous mice died due to a craniofacial defect). As you will see below, their essential roles are likely to be much more complex !

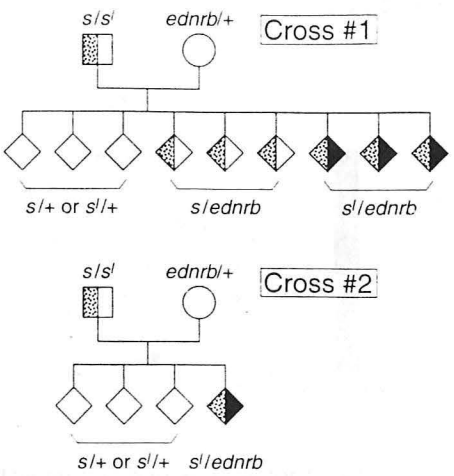


Posttranslational processing of prepro-ETs. A, 203-amino acid human prepro-ET-1 peptide is cleaved by dibasic amino acid endopeptidase action at two sites (arrows, top), followed by sequential carboxypeptidase activity (small curved arrows) to yield prepro-ET-1 (53 to 91), referred to as pro-ET-1 or big ET-1 (middle). (The COOH-terminal portion of preprop-ET-1 contains the ET-like peptide; see fig. 4.) Big ET-1 is then cleaved by ECE (arrow) at the Trp^{73} to Val^{74} bond to yield the final 21-amino acid product, ET-1 (53 to 73). B, amino acid sequences of the three human big ET-1 isoforms are shown. Arrows indicate the sites of ECE action. ●, amino acid sequence changes compared to the originally identified porcine big ET-1 sequence (Yanagisawa et al., 1988a,b). Illustrations taken from Phillips et al., 1992, and used with permission of Oxford University Press.

Figure 8. Endothelins

Late last year Yanigasawa (who originally identified endothelin-1) and his colleagues here at UT-SWMC began a dissection of the functions of these genes and their receptors using the KO mouse as one approach. They described a mouse mutant in the endothelin-B receptor (generated by homologous recombination) which developed two interesting phenotypes (30). One was immediately obvious at birth - white spotting despite the use of an ES cell line that should yield only Agouti (dark brown) colored mice.

White-spotting is a naturally occurring mutation described almost 30 years ago (and in which aganglionic megacolon was present). They went on to show that two alleles of white-spotting (*s*, white-spotting only; s^{l}, Piebald-lethal, white-spotting and aganglionic megacolon) were allelic with their induced mutation (ie, same gene); in the case of Piebald-lethal mutant mice the entire gene was missing. See Figure 9 for summary of crosses.



Two crosses between compound heterozygote *s^{l}/s* male and heterozygous *ednrb/+* female are shown diagrammatically. Closed symbols, megacolon phenotype; stippled symbols, spotted coat color. Inferred genotype of the pups is indicated below the diagrams

Figure 9. Allelism between *EDNRB* and Piebald.

The second phenotype was even more interesting : Hirschsprung's disease - aganglionic megacolon. Histological examination of the colons of the mutant mice revealed the absence of enteric neurons. Furthermore, they (along with collaborators from Case Western Reserve) demonstrated the presence of a missense mutation in exon 4 of the endothelin-B receptor gene in a large, inbred Mennonite kindred (31) in which recessive Hirshsprung's disease was prevalent. This mutation, which segregated with the disease, results in substitution of a Cys residue for a Trp at position 276 (W276C) and alters the ligand-induced calcium response in transiently transfected cells (Figure 10; careful controls ruled out the possibility of altered binding of ligand or instability of the protein). Thus, the mutant receptor lacks the ability to correctly convey signal transduction upon binding its ligand.

Since nearly 80% of Hirschsprung's disease is recessive (or of multigenic inheritance) they speculated that naturally occurring mutations in the endothelin-B gene (or ligand) result in susceptibility to aganglionic megacolon in humans

(mutations in endothelin-B now designated *HSCR2* ; *HSCR1*, a dominant form of the disease, appears to be due to mutations in the RET proto-oncogene).

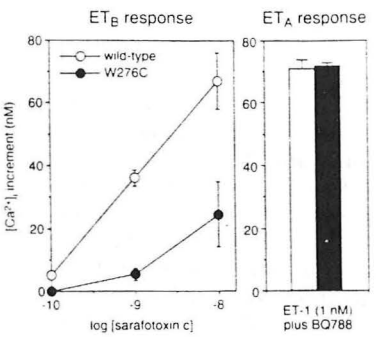


Figure 10. The mutant human receptor fails to mediate appropriate Ca transients.

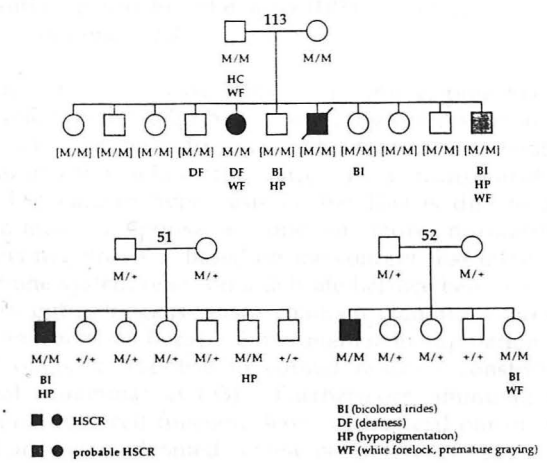


Figure 11. Pleiotropy of the W276C Mutation

Three Mennonite nuclear families with segregation of megacolon and other nonenteric phenotypes are shown. Although confirmed or probable megacolon is observed in 5 of 17 W276C homozygotes, 6 additional homozygotes have pigmentary anomalies or deafness. The genotypes of individuals within brackets are predicted.

In a subsequent report Yanigasawa and colleagues (32) also showed that endothelin-3 was the biologically relevant ligand for the endothelin-B receptor, since targeted disruption of endothelin-3 resulted in the same phenotype - white-spotting and aganglionic megacolon. Indeed, the KO mice proved to be allelic with lethal-spotting (*ls*), a previously characterized mutation that results in the identical phenotype (*ls* mutant mice carry a point mutation in the endothelin-3 gene that abolishes processing of the prepropeptide; ref 32).

This demonstrated conclusively that endothelin-3 is required for normal development of enteric ganglia and epidermal melanocytes (despite equivalent binding of endothelin-1, -2 and -3 with the endothelin-B receptor, neither endothelin-1 nor -2 could compensate for the mutation). These studies have thus defined an essential role for endothelin receptors and one of their ligands in the normal development of two neural crest derived lineages, the enteric ganglia and epidermal melanocytes, and have established a novel molecular basis for Hirschsprung's disease in humans. This isn't the final chapter, however, since the presence of the disease in humans is only 74% penetrant in homozygotes with the W276C mutations (24% in heterozygotes) with a male preponderance. There are obviously other genes that may lead to susceptibility to Hirschsprung's disease in humans (see Figure 11).

4. Inflammatory bowel disease (IBD) - cytokine imbalance in the immune system

Inflammatory bowel disease (IBD) is a chronic, presumably noninfectious, inflammation limited to the large bowel (ulcerative colitis) or anywhere along the gastrointestianl tract (Crohn's disease). The former is a relatively superficial, ulcerative inflammation while the latter is a transmural, granulomatous inflammation. The current hypothesis is that IBD is due to an abnormal and uncontrolled immune response to one or more normally occurring gut constituents. This hypothesis is based on the concept that immune homeostasis in the mucosal immune system relies on a delicate balance between the ability to react to the presence of gut pathogens versus common ubiquitous gut constituents (e.g., food proteins and bacterial flora). Experimental manipulation that results in a heightened and ongoing response to normal mucosal constituents can lead to chronic intestinal inflammation (33). Furthermore, immunosuppressive drugs (which affect primarily T cell function) have a beneficial outcome in patients with the diseases. (For a more detailed review see reference (33) and accompanying notations).

Again, the results of gene-targeting in murine ES cells has led to elucidation of some of the underlying mechanisms that may be operative in human disease. One of these, a null mutation in IL-10, will be the subject of this discussion.

IL-10 was initially identified as an activity produced by T-helper subset 2 (Th2) which inhibited the synthesis of cytokines by Th1 cells (34). It was subsequently

found to be produced by B cells (specifically B-1 lymphocytes), macrophages, thymocytes and keratinocytes (35), and is a potent supressor of macrophage activation *in vitro* , inhibiting the production of IL-1, IL-6 and TNF by LPS-stimulated macrophages (36). In short, its effects in the immune system are myriad, but its precise role(s) in disease remained elusive.

The IL-10 gene was disrupted in mice by the same techniques described previously (37). Surprisingly, the development and function of T and B lymphocytes was completely normal (and in particular the B-1 subset of B cells, see Table next page). The most apparent phenotype became obvious at about 7-11 weeks of age when it was found that homozygous mice were growth-retarded and anemic (Figure 12). Microscopic examination of the mice revealed normal histology for all major organs except the intestinal tract where regionally variable mucosal inflammation was observed associated with hyper regenerative or degenerative lesions of the epithelium. Immunostaining for expression of MHC class II molecules, normally absent in regular house mice, was altered in the IL-10 KO mice. Most of the affected mice died by three months of age due to a chronic debilitative state.

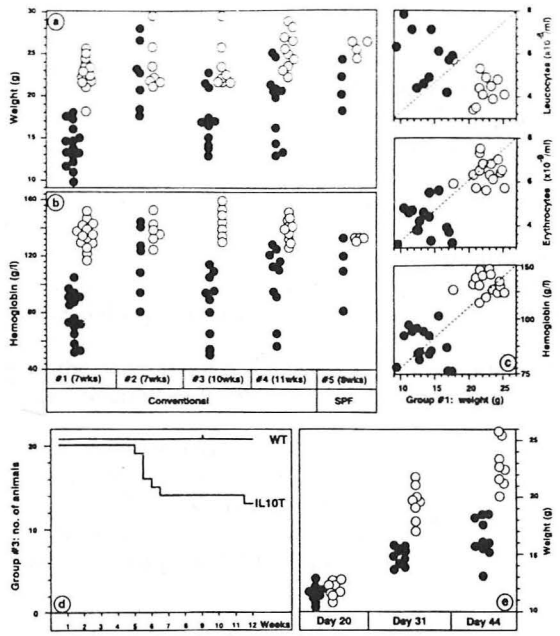
Table 2. Analysis of T and B Cell Subsets in IL10T and Control Mice

Thymus							Spleen				
Mouse Number	Mouse Strain	Body Weight (g)	Cell Number ($\times 10^{-6}$)	$CD4^+$ (%)	$CD8^+$ (%)	$CD4/CD8^+$ (%)	Cell Number ($\times 10^{-6}$)	Lymphocytes (%)	$CD4^+$ (%)	$CD8^+$ (%)	B Cells (%)
1	IL10T	ND	85	8	2	82	35	95	21	13	54
2	Wild type	ND	24	13	3	74	36	93	14	7	49
3	IL10T	13	40	15	4	77	21	72	22	8	51
4	IL10T	12	39	6	2	87	37	82	19	5	39
5	Wild type	25	70	9	2	87	50	87	13	6	49
6	Wild type	25	70	7	2	89	32	91	13	4	29

Bone Marrow							Peritoneum			
Mouse Number	Mouse Strain	Cell Number ($\times 10^{-6}$)	Lymphocytes (%)	Pre–B Cells (%)	Newly Generated B Cells (%)	B Cells (%)	Cell Number ($\times 10^{-6}$)	Lymphocytes (%)	CD5 B Cells	Conventional B Cells
1	IL10T	14	57	33	15	6	1	45	54	14
2	Wild type	14	32	31	13	5	2	45	31	35
3	IL10T	14	15	38	12	8	1	28	55	22
4	IL10T	20	31	23	10	6	2	20	32	26
5	Wild type	19	46	35	16	8	1	34	29	32
6	Wild type	15	41	40	14	4	1	37	40	30

Mice 1 and 2 were 4 weeks old, and mice 3–6 were 6 weeks old. Single cell suspensions were prepared from thymus, spleen, and bone marrow, and cell numbers were determined in a hemocytometer. Cells were stained with labeled antibodies and analyzed by flow cytometry as described in Experimental Procedures. The percentages of cells in the lymphocyte gate are shown. T cell subsets were analyzed by staining with anti-CD4 and anti-CD8 antibodies. B cell subsets were analyzed by staining with anti-CD45R/B220, anti-IgM, and anti-IgD antibodies. B cells in spleen were identified as CD45R/B220 and surface IgM-positive cells. Pre–B cells in bone marrow were $CD45R/B220^{low}$ and surface immunoglobulin negative; newly generated B cells were $CD45R/B220^{high}$ and surface IgM positive but negative for surface IgD. B cells were $CD45R/B220^{high}$ and IgM and IgD positive. CD5 B cells and conventional B cells in the peritoneum were identified as $CD5^+$,$CD45R/B220^{low}$ or $CD5^-$,$CD45R/B220^{high}$ cells, respectively. ND, not determined.

Table 3. Analysis of T and B cell subsets in control and IL-10 KO mice.



Body Weight, Hemoglobin Concentrations, and Mortality of IL10T Mice

(a and b) The body weights (a) and hemoglobin concentrations in the blood (b) of several groups of IL10T (closed circles) and littermate control (open circles) mice at the indicated age are shown. Each symbol represents the value obtained from one animal. Males and females are not distinguished.

(c) Correlation of the body weight with the concentration of hemoglobin, the number of erythrocytes, and the number of leukocytes in the blood of the individual animals of group 1.

(d) Mortality diagram of IL10T and control animals of group 3 from birth to the age of 3 months.

(e) Postnatal growth of the individuals of a group of IL10T mice and littermate controls between day 20 and day 44.

Figure 12. Growth retardation and anemia in IL10-KO mice.

However, when the mice were kept under specific pathogen free conditions (SPF, a state whereby certain specific pathogens such as mouse hepatitis virus, Sendai virus, and others are notably absent - but the mice are not in a completely sterile environment) they failed to develop the rampant disease seen in the conventional colony. A mild local colitis was observed but none of the animals died. In addition, the alterations in the hemopoietic tissues were similar between the two groups (thus IL-10 deficiency causes a mild anemia compounded by the iron deficiency seen in the conventionally housed animals).

Thus, these investigators suggested the absence of IL-10 - normally a potent suppresser of cytokine production by macrophages, natural killer cells, and T cells - led to a continuous overproduction of TNF-alpha, IL-1 and others leading to a

chronic inflammatory state. Secondly, enhanced epithelial MHC class II expression and antigen presentation led to a massive exposure of lymphoid cells in the mucosa to luminal antigens and bacterial cell wall components, essentially reinforcing the inflammatory response. Finally, the finding that SPF animals failed to develop a similar disease may allow these investigators to identify the pathogens implicated in development of the intestinal disease.

Breeding of these mutant mice with animals specifically deficient in T or B cell populations (again generated primarily by gene-targeting) should allow for identification of the cell types and cytokines that are crucial for development of the enterocolitis.

(Of note, in the same issue of Cell two additional mutant mice also developed IBD-like syndromes, references 38, 39).

In summary, it seems likely that many additional mutations in murine ES cells and creation of mutant mice (and cell lines) will be described during the coming years. Indeed, there is likely to come a time when the description of any new gene will be accompanied by the question - " What did the KO show ? " It is truly a revolution in developmental biology.

IV. Development of the technology in other species

Pluripotential ES cells have been derived from hamster (40), porcine (41), bovine (42), mink (43), rabbit (44) and rat (45) preimplantation embryos. However, the mouse is the only species to date in which germline pluripotency has been demonstrated, and the rat and rabbit are the only species in which chimeras have been produced from continuously passaged cell lines. The reasons for failure to demonstrate germline transmission of parental cells in these additional species is not yet clear, and may relate to the culture conditions used for their maintenance.

Despite these limitations, some additional techniques are being employed for production of mutant animals. One of the most promising is nuclear transplantation (46). In this method, early cleavage stage embryonic cells (typically blastomeres, but also inner cell mass cells) are individually isolated and electrically fused with enucleated mature oocytes. Nuclear transfer with blastomeres has resulted in viable progeny in several species, including mouse (ref 47, but only up to the two cell stage), sheep (48, 49), cattle (50), pigs (51) and rabbits (52, 53).

Nuclear transplantation of mouse ES cells does not give rise to viable offspring, probably because these cells are true inner cell mass cells incapable of contributing to extraembryonic development. Recently, Sims and First succeeded in generating viable cattle from briefly cultured ICM cells (ref 54; they were unable to generate true cell lines since they overtly differentiated after passage onto feeders). In my own laboratory, we have successfully generated normal blastocysts from *continuously passaged* rabbit ES cells (55). Future studies will determine if these blastocysts can give rise to normal viable progeny. If this proves to be the case, then it will bypass the cumbersome technique of blastocyst injection, and the time-consuming matings of chimeric animals to determine if the lines are germline

capable. Additionally, if mutated cell lines display totipotential properties, then heterozygous animals can be generated in *comparable* time that it currently takes in the mouse.

V. Embryonic stem cells from preimplantation human embryos

In 1994 the NIH established the Human Embryo Research Panel, composed of 19 individuals with broad expertise in the fields of basic and clinical research, ethics, law, social science, public health and public policy. Their purpose was to establish guidelines for research involving the "preimplantation human embryo" and to provide advice on areas viewed to be acceptable for Federal funding, unacceptable for Federal funding, or warranting additional review. The Panel's charge encompassed only research involving the human embryo produced by *in vitro* fertilization (test tube embryos) or parthenogenesis (initiation of ovum development in the absence of sperm). The preimplantation human embryo was defined as a fertilized ovum *in vitro* that had never been transferred to or implanted into a uterus.

The Panel concluded that certain areas of research involving the preimplantation human embryo were acceptable for Federal funding within a framework of stringent guidelines. Three considerations led the panel to this conclusion : (1) the promise of the human benefits from the research is significant, (2) the preimplantation human embryo warrants serious moral consideration as a developing form of human life, but it does not have the same moral status as infants and children because of the absence of developmental individuation, the lack of even the possibility of sentience and most other qualities considered relevant to the moral status of persons, and the very high rate of natural mortality at this stage, and (3) Federal research funding of such research would provide consistent ethical and scientific review at the national level.

The Panel considered numerous areas that would be acceptable for research funding, and in particular the potential benefits from **research with embryos remaining from infertility programs**. These are embryos that have been stored at liquid nitrogen temperatures for up to a decade and are not likely to be used in humans anyway. These potential benefits included (1) improvements in the treatment of infertility, (2) preimplantation diagnosis of genetic diseases, (3) understanding and prevention of birth defects, and (4) *advancing the development of embryonic stem cells for therapeutic purposes.*

The latter area, as you can deduce, is founded primarily on much of the work in the mouse that I have already presented. The Panel reasoned that " culture conditions might be defined that allow pluripotent ES cells to differentiate into specialized cell types that give rise to blood cells, primitive nerve cells, or muscle cells. In the future, such differentiated cells might be used for bone marrow transplantation in the treatment of cancer or for the repair of damaged or degenerated tissue including, for example, in the nervous system. In the nearer

term, the study of embryonic stem cells might also lead to important insights into the regulation of embryo growth and development. "

Thus, areas of research that were deemed acceptable for Federal funding with respect to human ES cells were as follows :

(1) research involving the development of ES cells but only with embryos resulting from IVF for infertility treatment or clinical research that have been donated with the consent of the progenitors.

(2) nuclear transplantation into an enucleated, fertilized or unfertilized (but activated) egg, without transfer, for research that aims to circumvent or correct an inherited cytoplasmic defect.

The latter of these two (and there were several others not dealing with ES cells) was approved by a narrow majority of the Panel.

1. Experimental basis

Given these guidelines, it is prudent to examine the experimental basis for such a proposal. As you can already surmise, injection of undifferentiated ES cells into an extrauterine site would likely give rise to a tumor. However, the isolation of a pure population of *committed* stem cells, for example hematopoietic stem cells injected into the bone marrow, probably would not. I could find only a few papers on this subject, all dealing with the development of hematopoietic stem cells from murine ES cells (56-62). In one of these papers a mixed population of differentiated "ES fetuses" (an unfortunate name, but basically embryoid bodies) were immortalized with oncogene-containing retroviruses, and subsequently injected intraperitoneally into lethally irradiated mice (57). Most of the animals died as a result of tumors, but three groups of mice survived (Table 4). These mice all had macrophages, T and B cells derived from the EBs (albeit at low actual numbers of cells). The authors state they cannot be certain they were dealing with a committed stem cell versus a pluripotent stem cell (or both), and many of the mice appeared to be sickly despite the reconstitution.

Percentage of lymphoid and myeloid cells of donor or host origin in the chimeric F_1 (group 2) mice

Cell surface marker	Donor (d^-)	Host (d^+)
Ig^+	20.4	79.6
$CD3^+$	48.7	51.3
$CD4^+$	38.5	61.5
$CD8^+$	42.9	57.1
$Mac\text{-}1^+$	19.6	80.4

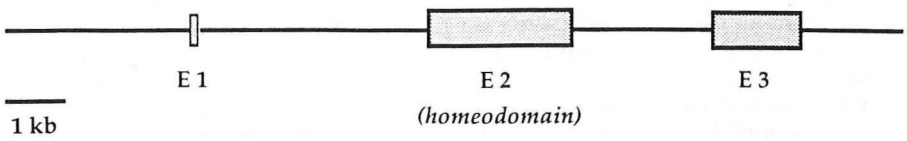
d, $H\text{-}2D^d$ class I antigen recognized by mAb 15.5.5 (52).

Table 4. Adoptive transfer of embryoid body cell cultures reconstitute specific lymphoid and myeloid cells in irradiated mice.

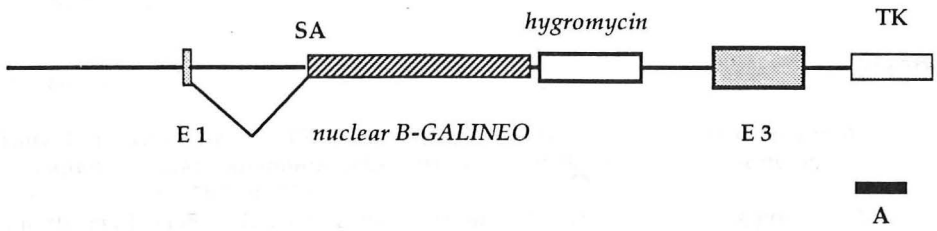
Nonetheless, it seems plausible to propose that one might be able to select from the population of differentiated ES cells a clone of committed stem cells that might safely reconstitute the bone marrow (or other tissues). One strategy for doing so would be to engineer a targeting vector designed to yield expression of a selectable gene only upon the earliest differentiation into a committed stem cell, thus obviating the need for immortalization with oncogenes (assuming the committed stem cell would continue to divide).

An example of this is shown below for gene-targeting at the murine homeobox gene NKx2.5. This gene is the earliest known marker of the procardiac mesoderm, where its expression is detected at day 7.25 postcoitum. Interestingly, its expression stays on throughout the remainder of cardiac development even into the adult animals. We hope to take advantage of these patterns of expression in attempts at immortalizing committed cardiac progenitor cells that are capable of continuous growth.

Genomic structure of NKx2.5 - a cardiac specific homeobox gene
Earliest known marker of procardiac mesoderm (on at day 7.25 and stays on into adult stage hearts)



" Dual selection " targeting vector for NKx2.5 locus



STRATEGY

(1) Target the locus and select for hygromycin resistant colonies

(2) Work-up correctly targeted clones (probe A)

(3) Differentiate the correctly targeted clones in culture to form embryoid bodies; at appropriate time interval begin selection in both hygromycin and neomycin

(4) Stain for the presence of blue nuclei that also stain for cardiac troponin C in dividing cells

Figure 13. Strategy for immortalization of cardiac progenitors (similar strategy could be applied to any gene with a known pattern of expression).

VI. Summary

Thus, the development of ES technology in the mouse and other mammals has allowed investigators to begin the molecular dissection of complex developmental and biochemical pathways in the context of the intact animal. No doubt the advent of this technology will continue to provide insight into a host of cellular processes; some predictable, and some that would never have been discerned by any other method. Indeed, if strategies are developed that allow for isolation and culture of committed stem cell populations from developing organs, one could imagine a time when the utility of these cells might be extended to use in humans for therapeutic purposes. These considerations can only be made by an informed society, and much of the purpose of todays' Medical Grand Rounds is to provide that information.

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