Review

Newer Approaches to Genetic Modeling in Mice

Tissue-Specific Protein Expression as Studied Using Angiotensin-Converting Enzyme (ACE)

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Virtually all scientists are aware of the tremendous progress in genetic modeling brought about by targeted homologous recombination in embryonic stem cells. Often called a “knockout mouse”, the ability to modify the mouse genome has created animal models for a large number of disease processes. Typically, targeted genetic modifications are used to inactivate a gene resulting in a mouse null for the corresponding protein. While this approach has been tremendously useful, the most recent work in this area makes use of more subtle genetic modifications to probe the functional role of a protein in an organ-specific or developmental fashion. This review will touch on work from my group and several other laboratories that are using newer approaches to gene targeting with the goal of creating mouse models that ask questions not addressable through the simple inactivation of a gene.

Gene Targeting: Advantages and Disadvantages

The first technology widely used to modify the mouse genome was the transgenic mouse. Here, exogenous DNA is injected into the pro-nucleus of a fertilized mouse ovum.¹ Typically, one or several copies of the injected construct will integrate into a single random location within the mouse genome. This approach produced mice with added genetic information. In fact, since the integration of the exogenous DNA occurs at apparently random locations within the mouse genome, every founder mouse created using a transgenic approach is unique. There are advantages to this approach, including the relative rapidity of the experiment and the occasional incorporation of many copies of the exogenous construct. Also, occasionally, the integration of the transgene fortuitously interrupts

an intrinsic mouse gene responsible for some interesting aspect of mouse development. For example, the *inv* gene (responsible for left-right body asymmetry) was discovered through its chance interruption during transgenesis.^{2,3} However, the transgenic approach also has drawbacks. It is a method that can only add DNA; it is not capable of eliminating a gene or modifying the underlying mouse genome in a planned fashion. Despite this, the classical approach of creating transgenic mice by pronuclear injection remains important, both for studies using tetracycline-responsive genes (discussed below) and for possible use in expressing small interfering RNA.^{4,5}

As compared to transgenesis, targeted homologous recombination in embryonic stem cells can modify the mouse genome in a planned fashion; the function of over 7000 genes have been investigated using this approach.⁶ Historically, the development of targeted homologous recombination was the result of two areas of investigation. One was the study of teratomas and the realization that the many different tissues comprising these tumors originated from pluripotential stem cells.⁷ While this was conceptually important, it was the isolation of embryonic stem (ES) cells from the inner cell-mass of strain 129 mouse embryos that was critical for the practical development of this technique.^{8,9} These ES cells are cultured *in vitro* and yet remain undifferentiated such that when reimplanted into a mouse blastocyst, they contribute to all of the tissues of the resulting mouse pup. Virtually all commonly used ES cells are derived from male mouse blastocysts so that when they are reimplanted into a recipient blastocysts, the resulting pups are male. If the ES cells participate in the formation of testis and, specifically, to the germ cells of the pups, these animals can be

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used to breed mice homozygous for a change induced in the genome of the ES cell.

The DNA of ES cells is modified *in vitro* using targeted homologous recombination. Indeed, it was the detailed mechanistic study of homologous recombination in single cells that was the second intellectual progenitor of the gene-targeting approach. Several previous reviews have discussed the principals involved in creating targeting vectors capable of making specific changes in a particular gene.^{10,11} This methodology is extremely powerful in that it allows the creation of virtually any genetic modification that can be envisioned by an investigator. While the classic approach is to eliminate a particular gene, the technology is capable of many other modifications, including the duplication of genes and the selective introduction of point mutations.^{12,13} Thus, the knockout approach is far more plastic than the transgenic approach in that it modifies the underlying mouse genome through genetic addition, genetic elimination, or precise and subtle genetic modification.

Before discussing modern approaches to gene targeting, it is worthwhile noting some of the disadvantages of using knockout technology to eliminate a protein. Specifically, what are the strengths and weaknesses of a genetic knockout approach as compared to inhibiting a particular protein using a pharmaceutical? The genetic creation of a knockout mouse eliminates the protein of interest from the moment of conception until the death of the animal. Theoretically at least, during the embryonic period, overlapping physiological systems may compensate for the lack of the protein of interest. Also, during the entire life of the mouse, the knockout strategy produces an animal completely null for the targeted protein. This is quite different from pharmacological inhibitors which are typically used after birth and thus after a normal embryogenesis. Pharmaceuticals can be varied in concentration and in duration of administration to experimentally regulate the onset and duration of effect. Thus, the classical genetic (knockout) approach is digital in the sense that the gene of interest is either functional or eliminated by targeting.¹⁴ In contrast, pharmaceutical inhibitors are analog in that they can be used to achieve a partial or complete inhibition of activity for a period of time and then withdrawn to restore normal function. As will be discussed below, newer approaches to gene targeting have attempted to create a more analog environment for the genetic regulation of gene expression.

Perhaps one of the most significant results of the typical knockout mouse experiment is that the resulting animal is homozygous for the desired genetic mutation in every tissue of that animal. When gene targeting is used to eliminate protein expression, the resulting mouse completely lacks expression of that protein. While a powerful methodology, null mice are massively different from a wild-type animal. Thus, in creating a knockout mouse, a scientist is studying the extreme phenotype induced by the absolute lack of functional protein production. While these extreme phenotypes are often quite interesting,

newer approaches to gene targeting aim to make more selected and subtle genetic changes.

ACE Knockout Mice

Our lab studies the renin-angiotensin system, a series of enzymes and substrates designed to produce the eight-amino-acid vasoconstrictor angiotensin II. This peptide is the final product of the sequential degradation of angiotensinogen by renin and by angiotensin-converting enzyme (ACE). While angiotensin II is a well recognized vasoconstrictor, studies preceding the creation of knockout mice suggested many different physiological roles for this peptide. These included effects on the central nervous system, metabolic effects on the liver, the induction of aldosterone production, direct effects on renal handling of salt and water, growth effects on cells and even poorly understood effects on reproduction.^{15,16}

Animals null for ACE production are markedly changed from normal mice. This extreme phenotype is characterized by body-wide elimination of ACE, the extensive reduction of angiotensin II production and unpredictable changes of other peptides (such as bradykinin and acetyl-SDKP) that are substrates for ACE.^{17,18} While ACE knockout mice survive, they have systolic blood pressures that average 35 mm Hg below that of wild-type mice. Specifically, as measured using a tail manometer, systolic blood pressure in a wild-type mouse was approximately 110 mm Hg. In contrast, ACE null mice had systolic blood pressures that averaged 73 mm Hg. These animals also produced large amounts of a dilute urine. Their inability to concentrate urine accompanied a widened urinary space with a reduced renal medulla and papilla. Surprisingly, renal arterioles and small arteries were thickened, a paradoxical finding given the very low systolic blood pressure. The renal changes in ACE knockout mice were completely unanticipated, but have now also been noted in angiotensinogen and angiotensin II-receptor knockout mice.¹⁹

ACE null mice are also anemic.²⁰ This was confirmed through analysis of hematocrit as well as determination of red-cell volume using ⁵¹chromium-labeled red blood cells. While the precise mechanism of the anemia is not clear, restoration of angiotensin II levels using an implanted osmotic minipump corrected the hematocrit to near normal. These and other data suggest a role of angiotensin II in facilitating erythropoiesis.

In mammals, males produce two isozymes of ACE. One isozyme, somatic ACE, is made by endothelium and other somatic tissues. It is this form of the protein that is typically associated with blood pressure control. A second isozyme, called testis ACE, is made by developing male germ cells and corresponds to the carboxyl half of the somatic ACE protein. Both ACE isozymes originate from the same genetic locus which contains two separate promoter regions. While the somatic ACE promoter is upstream of the first exon of somatic ACE, the testis ACE promoter is located within the 12th intron of the ACE gene.^{21,22} This promoter is highly tissue-specific and is used only by developing male germ cells. However, it is

Figure 1. In a wild-type mouse, ACE is expressed by many different tissue types including the endothelium, vascular adventitia, proximal tubular epithelium of the kidney, macrophages and areas of the gut, brain, and testis. Contrast this with a knockout mouse that is null for all ACE expression (Knockout - No ACE). To achieve a more selective expression of ACE, one can eliminate the protein from one or a few tissues (No ACE in Kidney). A second approach is a mouse lacking ACE except for selected ACE expression in one or a few tissues (ACE only in Kidney). Thus, instead of removing protein expression selectively, the second approach envisions selectively restoring protein expression in an animal generally lacking ACE.

quite active in these cells, resulting in large amounts of testis ACE mRNA and protein. The functional role of testis ACE was unclear until the creation of mice lacking this protein. We now know that testis ACE plays a critical role in male reproductive capacity. Male mice lacking this protein reproduce less frequently and with much smaller litters than wild-type animals. The exact mechanism by which this protein contributes to male fertility is not yet understood.

Tissue-Selective ACE Expression

ACE null mice present with a complex phenotype characterized by reduced blood pressure, abnormalities of blood electrolytes, structural lesions of the kidney, the inability to concentrate urine, anemia, and reproductive defects. This list of structural and physiological abnormalities underlines the many roles played by the renin-angiotensin system in normal physiology. To understand the role of the renin-angiotensin system in a more tissue-selective fashion, one can envision two different strategies (Figure 1). One is an experimental approach in which ACE is eliminated from one or, at most, a few organs of the mouse. Such an approach is technically feasible but probably not optimal for ACE, a protein expressed by many different tissues. The renin-angiotensin system is highly controlled through the regulated production of renin by the kidney. Given both the compensatory potential of the renin-angiotensin system and the wide distribution of the ACE protein, it seemed unlikely that the selected removal of ACE expression in one organ would result in an abnormal phenotype. A second approach to analyzing tissue-specific function of the renin-angiotensin system is to produce a mouse lacking ACE except for selected ACE expression in one or a few tissues. Thus, instead of removing protein expression selectively, the second approach envisions selectively restoring protein expression in an animal generally lacking ACE.

Wild-Type Allele

Construct

ACE.3 Modified Allele

Figure 2. The wild-type ACE allele contains two promoter regions (thick arrows). These are the somatic promoter, which directs expression to somatic tissues, and the testis promoter, which controls the expression of testis ACE in male germ cells. ACE exons are indicated with solid boxes. Between the 12th and 13th exons of the somatic ACE gene is a gray box that indicates the start site of the testis ACE isozyme. A unique BssHII restriction site is located immediately preceding the translation start site of somatic ACE. The ACE targeting construct (Construct) contains two homologous arms corresponding to the ACE gene. A neomycin resistance cassette (Neo^R) and albumin promoter were inserted into the BssHII site. TK is a thymidine kinase resistance cassette used for negative selection of ES cells. A properly targeted ACE allele (ACE.3 Modified Allele) positions the neomycin cassette to block the somatic ACE promoter and the albumin promoter to control somatic ACE expression.

One method to selectively express proteins on a null background is to use transgenic technology to reinsert the ACE protein under the transcriptional control of a tissue-specific promoter. This construct must then be bred onto the ACE knockout background to create the final animal model.²³ Such an approach can be successfully used but is somewhat cumbersome, particularly given the reproductive difficulties of male ACE knockout mice. A different experimental approach results from modeling the ACE gene in quite simple terms. While the exons of the ACE gene encode the ACE protein, it is the somatic ACE promoter that regulates the tissue distribution and the temporal expression of the ACE protein. Change the promoter and, in theory, the patterns of expression change. Thus, we envisioned an approach in which the endogenous somatic ACE promoter would be disabled and control of the ACE gene would be regulated instead by novel, exogenous promoters that are highly tissue-specific in their expression patterns. To test this approach, we made a mouse (termed ACE.3, this gene mutation was the third ACE mutation studied in our laboratory) in which control of the ACE locus was disassociated from the somatic ACE promoter and placed under the control of an albumin promoter.²⁴ The targeting vector used to create such a mouse is shown in Figure 2. This

Figure 3. ACE.3 wild-type (+/+) and ACE 3/3 (indicated as -/-) mice were sacrificed, and organ extracts were analyzed by Western blot using a rabbit anti-mouse ACE antibody. Somatic ACE is detected as a protein of approximately 170 kd while testis ACE is a more diffuse band centered around 95 kd. The organs studied were liver, lung, heart, spleen (spl), kidney (kid), aorta, small intestine (S Int), large intestine (L Int), striated muscle (Mus), brain, seminal vesicles (SV), and testis. ACE 3/3 mice express ACE in the liver but not in the lung or aorta. However, the mice do express about 15% of the ACE found in a wild-type kidney.

vector contained two homologous arms, each composed of genomic DNA identical to that found in the ACE gene. The left arm (2.4 kb) encoded the ACE promoter region. The right arm (8.3 kb) contained the first twelve exons of the ACE gene. In a wild-type mouse, these two DNA sequences are separated by a unique BssHI restriction site. In the targeting construct, this restriction site was used to insert a neomycin resistance cassette followed by an albumin promoter construct containing both promoter and enhancer regions of the mouse albumin gene.²⁵ These added DNA cassettes have two functions. The neomycin resistance cassette physically separates the somatic ACE promoter from the ACE gene. In addition, this cassette contains a powerful transcriptional terminator.²⁶ The net effect is that the neomycin cassette functionally blocks influence of the somatic ACE promoter on the expression of the ACE gene. In this construct, we also placed an albumin promoter downstream of the neomycin cassette and immediately 5' of the ACE coding regions. The hypothesis was that expression of ACE protein would be restricted to the liver due to the control of the gene by the albumin promoter. Mice bearing two copies of this modified ACE allele are referred to as ACE 3/3, and evaluation showed a tissue pattern of ACE expression highly consonant with the design of the experiment. This was best observed with a tissue Western blot developed using an anti-ACE antibody (Figure 3). Wild-type mice produce little ACE in the liver; in contrast, ACE 3/3 mice have easily identifiable ACE protein. Wild-type mice express abundant ACE in lung due to the large amount of endothelium present in this tissue. ACE 3/3 mice have essentially no ACE protein within lung tissue as measured by both Western blot and ACE enzymatic activity. In fact, the ACE 3/3 mice lack ACE in the

aorta, gut, muscle, seminal vesicle, and virtually every other organ with the exception of the kidney. In the kidney, ACE 3/3 mice have minor ACE expression by proximal tubular epithelium that measures approximately 14% of levels found in the kidneys of wild-type mice. This breakthrough in protein expression is probably due to the use of an artificial (man-made) albumin promoter that does not contain the complete sequences controlling albumin expression *in vivo*. However, the ACE 3/3 mouse represents a unique model in which ACE protein expression was shifted from vascular endothelium to liver hepatocytes. This was dramatically demonstrated by immunohistochemistry (Figure 4). While wild-type mice have marked ACE expression in endothelium and in the lung, the ACE 3/3 mice completely lacked vascular endothelial ACE, but expressed this protein on the surface of hepatocytes.

Apart from the technical achievement of targeting a protein to a novel pattern of tissue expression, what are the scientific reasons for creating the ACE 3/3 mouse? The importance of this model centers on the question of systemic *versus* the local vascular production of angiotensin II. While it is easy to envision the systemic renin-angiotensin system (circulating renin and ACE produce angiotensin II within the blood), many investigators have described separate, organ-based (local) production of angiotensin II.²⁷ This concept is based on tissue expression of all of the components necessary for the local production of angiotensin II. In the ACE 3/3 mouse, the novel pattern of tissue ACE expression precluded many normal local renin-angiotensin systems. In particular, it was thought that the localized expression of ACE on the surface of vascular endothelium was responsible for the local delivery of angiotensin II to vascular smooth muscle,

Figure 4. Tissues from wild-type (+/+) and ACE.3 (indicated as -/-) mice were prepared and stained with rabbit anti-ACE antibody. **A** and **B**: Sections of liver. The high-power inset in **B** shows abundant ACE within the cell membranes of hepatocytes. **C** and **D**: Sections of lung. In contrast to wild-type mice, the lung from ACE 3/3 mice has no detectable ACE. **E** and **F**: Sections of kidney. Wild-type kidney has abundant ACE in proximal tubular epithelium. ACE 3/3 mice show much-reduced levels of ACE staining within this same tissue. Renal arterioles from wild-type mice have easily observed endothelial and adventitial immunoreactivity for ACE (arrow in **G** indicates endothelium). In contrast, renal arterioles from ACE 3/3 mice (**H**) show no such staining. In fact, no endothelial immunoreactivity for ACE was present anywhere in ACE 3/3 mice. **A** and **B**: Photographed with a $\times 10$ objective. **C** to **F**: Photographed with a $\times 20$ objective; **G** and **H**, as well as the insert in **B**, were photographed with a $\times 100$ oil objective.

Figure 5. The systolic blood pressure of mice is measured using a tail cuff manometer. The blood pressures of wild-type (+/+), ACE 3 homozygous (3/3) and heterozygous (3/+ and 1/3) mice are indistinguishable. In contrast, mice homozygous for an ACE null allele (1/1) or a tissue ACE null allele (2/2) have systolic blood pressures less than 75 mm Hg. The tissue ACE null mice (ACE 2/2) are a strain modified to contain a stop codon in the coding region between the amino terminal and carboxyl-terminal catalytic domains of ACE.⁵² These mice lack the carboxyl-terminal catalytic domain and the carboxyl-terminal hydrophobic domain that retains ACE in the plasma membrane of cells. Thus, the ACE 2/2 mice export all ACE from cells into blood and extracellular fluids. While they have about 33% normal plasma levels of ACE activity (as measured by the ability to convert angiotensin I to angiotensin II), organs such as the lung and kidney have virtually no ACE activity. Interestingly, ACE 1/3 and ACE 2/2 have very similar plasma levels of circulating ACE, but very different blood pressures.

and ACE expression by endothelium was often postulated as being central to the control of blood pressure.²⁸ To investigate this, one needed a way to study the *in vivo* role of endothelial ACE expression. However, until recently, there was no way to truly test the functional role of protein expression by a particular tissue type. Now, targeted homologous recombination facilitates creation of a mouse (such as the ACE 3/3 mouse) containing substantial quantities of ACE, despite the complete absence of ACE expression by blood vessels. Surprisingly, the phenotype of this mouse was indistinguishable from that of a wild-type mouse. ACE 3/3 mice have a normal blood pressure and a normal renal histology. They responded to fluid restriction by concentrating urine to a level indistinguishable from wild-type, and the hematocrit of the ACE 3/3 mouse was normal. Thus, endothelial expression of ACE is not obligatory for normal renal function or blood pressure control.

Compound Heterozygous Mice

In the ACE 3/3 model, one may argue that normal function is the result of two separate factors. First, ACE 3/3 mice have approximately 80% normal plasma ACE activity. This protein, probably resulted from hepatocyte shedding in the ACE 3/3 animal akin to the endothelial shed-

ding observed in wild-type mice. Second, though these animals have a marked reduction of renal ACE expression, there was 14% residual activity. Fortunately, there is a simple way of addressing these concerns by creating a compound heterozygote mouse in which one ACE allele was derived from the ACE knockout model (a null allele that we term *ACE.1*) while the second ACE allele was derived from the *ACE.3* model (liver ACE expression). These compound heterozygous mice were easily created through the mating of *ACE.1* heterozygous mice with ACE 3/3 mice.²⁹ Since the compound heterozygote (termed ACE 1/3) contained one null ACE allele and one ACE allele targeting expression to the liver, the tissue patterns of ACE expression were quite predictable. The compound heterozygote has roughly half the liver ACE present in the ACE 3/3 mouse, but still lacks all ACE expression by vascular endothelium or vascular adventitia. Thus, like the ACE 3/3 animal, the ACE 1/3 mouse has no detectable ACE within the lung. Renal ACE was roughly half that measured in the ACE 3/3 model and thus was only 6 to 7% wild-type values. Finally, circulating ACE activity in the ACE 1/3 mouse was about 40% that of wild-type mice.

To our surprise, the phenotype of the ACE 1/3 mouse was indistinguishable from a wild-type mouse. Blood pressure, hematocrit and renal structure/function were completely normal (Figure 5). To investigate how the ACE 1/3 mouse maintained a normal physiology, we measured blood levels of angiotensin peptides. The conclusion of these studies was that the ACE 1/3 mouse has elevated levels of plasma angiotensin I, angiotensin II, and renin (discussed further below). The ACE 1/3 mouse nicely illustrates the inherent plasticity of normal blood pressure regulation. In addition, the physiological comparison of the ACE 1/3 mice to wild-type mice suggests that in the wild-type animals, both a local and a systemic production of angiotensin II contribute to blood pressure control. Thus, in wild-type mice, there is a contribution of both systemic and local systems to the formation of angiotensin II such that blood pressure, as determined by the kidney, remains normal. In the ACE 1/3 mouse, the endothelial production of angiotensin II was virtually nonexistent. In this instance, the kidney detected and compensated for the changed tissue distribution of ACE in such a fashion that plasma elevations of angiotensin I, angiotensin II, and renin resulted in a final tissue concentration of angiotensin II sufficient for maintaining normal physiological indices. Thus, while the plasma levels of angiotensin II are elevated in this model, the operational levels in tissues resulted in normal physiological function.

In evaluating the ACE 1/3 mice, we wondered whether the normal physiology was the result of these animals existing under laboratory conditions where food and water are plentiful. In reality, the renin-angiotensin system is designed for effectiveness in stress situations of dehydration or blood loss. To test the response to stress, wild-type and ACE 1/3 mice were put on a salt-free diet for 2 weeks. This resulted in a marked stimulation of the renin-angiotensin system, and wild-type mice responded with a marked reduction of urinary sodium excretion. For instance, wild-type mice excreted 64.2 ± 12.3 mil-

Figure 6. Plasma renin. Mice were exsanguinated and the plasma renin concentrations (PRC) of wild-type (+/+), ACE 3 homozygous (3/3), and heterozygous (3/+ and 1/3) mice were measured (**solid bars**). ACE 3/+ and 1/3 mice were also studied after >2 weeks on a zero-sodium diet (**striped bars**). On the normal diet, the plasma renin concentration of ACE 1/3 mice averaged $681 \pm 83\%$, $323 \pm 39\%$, and $204 \pm 25\%$ that found in wild-type, ACE 3/+, or ACE 3/3 mice, respectively. These differences were statistically significant ($P < 0.01$, indicated by *). In addition, the ACE 3/3 mice were significantly different from the wild-type controls ($P < 0.01$, indicated by *). On the zero-sodium diet, the plasma renin activity of ACE 1/3 mice was elevated more than 15-fold as compared to wild-type mice on a normal diet. This measurement was highly significant as compared to littermate ACE 3/+ mice on the zero-sodium diet or to ACE 1/3 mice at baseline (** $P < 0.001$). The number of mice in each group was as follows: +/+ normal salt, 7; 3/+ normal salt, 8; 3/3 normal salt, 8; 1/3 normal salt, 8; 3/+ zero salt, 9; 1/3 zero salt, 6. All data presented are the group mean $\pm$ SEM.

liequivalents of sodium per day on a normal diet, but only 5.0 ± 1.2 milliequivalents per day after 2 weeks on a salt-free diet. The ACE 1/3 (compound heterozygote) mice also reduced urinary sodium excretion, but to a level that was slightly higher than that of the control animals (10.0 ± 1.9 milliequivalents per day). This small additional excretion of sodium was reflected in the blood pressure change observed at the end of the experiment. In this experiment, the wild-type mice reduced average blood pressure by only 0.6 mm Hg on the sodium free diet. In contrast, ACE 1/3 mice had a reduction of systolic blood pressure of 9.7 mm Hg (from a baseline of 102.0 ± 2.2 to 92.3 ± 2.0 after the salt-free diet). While significantly different from that of control animals, it was remarkable that the ACE 1/3 mice were able to maintain systolic blood pressures above 85 mm Hg. Determination of plasma renin levels gave great insight into how this was achieved (Figure 6). All animals showed elevation of plasma renin activity in response to the absence of dietary salt. What was remarkable was the marked up-regulation present in the ACE 1/3 model. The novel tissue-pattern of ACE expression forced this animal to maximally activate renin-angiotensin system activity to achieve physiological conditions just capable of maintaining near normal homeostasis. The conclusion of these studies is that an animal has no absolute need for vascular production of ACE in the presence of normal kidney function. As noted by Harry Goldblatt in the 1930s and John Hall and Arthur Guyton in the 1970s,³⁰ the kidneys are the central regulators of systemic blood pressure; normal kidneys vigorously guard physiological homeostasis, even under substantial physiological stress.

Figure 7. In a TET-off scheme, a tissue-specific promoter (PROMOTER) drives the production of a TetR/VP16 fusion protein. In the absence of tetracycline (Tet), the fusion protein binds to the *TetO* sequence and stimulates transcription of the gene of interest (GOI). The addition of tetracycline causes binding of the drug to the TetR/VP16 fusion protein, a conformational change, and the loss of binding to the *TetO* sequence. In a TET-on scheme the binding of tetracycline to a modified TetR/VP16 protein (rTetR/VP16) causes binding to *TetO* and the induction of transcription. In these schemes, the regulated, tissue-specific expression of a protein (the GOI) is achieved.

The renin-angiotensin system has been implicated in several different physiological systems apart from blood pressure control. For example, angiotensin II has been reported to stimulate vascular smooth-muscle proliferation, enhance neointimal formation, and play a role in the pathophysiology of atherosclerosis.^{31–33} While much of this work is based on solid physiological investigation, *in vivo* investigations have the intrinsic difficulty of discriminating between direct effects of angiotensin II and effects secondary to blood pressure changes induced by angiotensin II. For example, there is substantial evidence in ApoE knockout mice that angiotensin II is deleterious to blood vessels.³⁴ Whether this is due to the effects of changing blood pressure or to local angiotensin II effects mediated within the blood-vessel wall is a point of scientific discussion. The ACE 3 model offers a means to discriminate between different pathophysiologic mechanisms of vascular injury. These mice have no vascular ACE and no ACE expression by inflammatory cells, but do maintain normal blood pressure. Presumably, the breeding of the ACE.3 allele onto an ApoE knockout background will give insight into the pathophysiology of vascular injury. In this and other pathological investigations, the utility of mice containing abnormal tissue patterns of protein distribution becomes increasingly apparent.

Digital versus Analog Gene Control

Other investigative groups have developed and used genetic techniques for the temporal or positional regulation of protein production. Some of these studies are driven by the desire to achieve organ-specific effects similar to that described in our work.^{35,36} A major area of interest concerns the problem that most gene-based modification approaches are functionally equivalent to digital switches: the genes exist either in a wild-type or in a modified state. There is no possibility of a partial modification or of reversibility in such approaches. To address this, many studies have investigated inducible-promoter systems. These systems use a regulating agent (often a small molecule such as tetracycline) to induce or to silence a gene. Such systems are analog in the sense that the response is graduated, being dependent on the concentration of the regulating agent. Also, such systems are reversible with the withdrawal of the regulating agent. As reviewed by Weber and Fussenegger,¹⁴ optimal gene-regulatory systems seek 1) a low leaky expression in the absence of the regulating agent but high levels of induction in the presence of the agent, 2) a lack of toxicity of the regulating agent, 3) a reasonably linear dose-dependence for the regulating agent, 4) high reversibility, and 5) good bioavailability yet low immunogenicity of the regulating agent. While a number of approaches have been investigated, including cold-inducible gene regulation systems, and single regulated promoters (such as those regulated by heavy metals, heat shock, or steroids), finer regulatory control has been achieved by binary systems composed of an effector molecule (the regulator) and a target transgene.³⁷ The prototypic system is a tetracycline-responsive system originally developed by Gossen and Bujard (Figure 7).³⁸ This uses an effector protein composed of a fusion between the *Escherichia coli* tetracycline-repressor protein (TetR) and the *Herpes simplex* VP16 transactivation domain. In the absence of tetracycline, the fusion protein binds the 19-bp operator sequence, *TetO*. The scheme then is to incorporate two separate genetic modifications into an animal. One of these is the gene to be regulated under the control of a promoter containing seven repeats of the *TetO* sequence and a minimal version of the human cytomegalovirus immediate early promoter. The second genetic component encodes the VP16-TetR fusion protein. In the absence of tetracycline, the fusion protein binds to the tetracycline operator sequences and activates the promoter. This leads to gene expression; however, with the addition of tetracycline, the VP16-TetR fusion protein binds the antibiotic and disassociates from the promoter region. This so-called "Tet-off" system terminates transcriptional activity in the presence of the antibiotic. Often, doxycycline (Dox) is used because of its low cost, availability, and affinity for the tetracycline-repressor protein at concentrations well below toxic levels.

In the Tet-off system, protein expression is repressed by the antibiotic. Protein induction is dependent on clearance of the antibiotic from tissues, a process that can take 24 hours or longer. To address this potential problem, "Tet-on" systems were developed in which the VP16-

TetR protein binds to the promoter region only when tetracycline is present; in the absence of the drug, there is no transcription.³⁹ In such a system, protein induction is far more rapid. Another variation of this approach is the fusion of the *TetR* gene to a repressor domain from the Kox1 zinc finger protein. In the absence of tetracycline, the fusion protein binds a tetracycline-operator sequence inhibiting promoter transcription (Kox1 is a transcriptional repressor).^{40,41} The addition of a tetracycline-like antibiotic disassociates the fusion protein, resulting in depression of the gene.

In addition to tetracycline-based systems, there are other similar approaches based on the streptogramin resistance operon, rapamycin-inducible regulation systems, and steroid-regulable systems.¹² One of the most interesting uses of regulable systems has been in the field of carcinogenesis where several studies have provided evidence that oncogenes are necessary for both the induction of experimental tumors and for their maintenance. For example, Chin et al⁴² showed that, in a carcinogenesis model, melanoma genesis and maintenance were strictly dependent on expression of H-RasV12G. When the oncogene was down-regulated by doxycycline withdrawal, both the tumor and the host-derived endothelial cells underwent marked apoptosis, even in the presence of enforced expression of vascular endothelial growth factor. Similar studies by Yamamoto et al⁴³ have demonstrated the reversibility of neuropathology and motor dysfunction in a conditional model of Huntington's disease.

Site-Specific Recombinases

Another powerful approach used for the temporal or positional regulation of protein production exploits site-specific recombinases. Two such enzymes are widely used: Cre from the bacteriophage P1 and FLP from *Saccharomyces cerevisiae*. The site-specific recombinases catalyze recombination between two 34-bp recognition sites (*loxP* for Cre and *FRT* for FLP). As reviewed by Lewandoski,⁴⁴ these recombinases do not require specialized accessory proteins for activity and thus are very suitable for use in mammalian tissues. Indeed, these recombinases are becoming increasingly important in mediating chromosomal engineering.

Recombinases are also suitable for strategies resulting in tissue-specific gene knockout. The very same recombinases can also be used to initiate gene expression in selected tissues. As an example of tissue-specific gene knockout, consider that targeted homologous recombination in ES cells requires the presence of an antibiotic resistance cassette (such as neomycin) to facilitate selection of targeted ES cell clones. However, once the selection of targeted ES cell clones is completed, the continued presence of the resistance cassette may interfere with the proper genetic regulation of the targeted gene. To address this problem, the traditional approach has been to flank the antibiotic resistance cassette with *loxP* sites and then to remove the cassette through Cre mediated recombination. Often this involved an extra experimental manipulation (such as the transient expres-

Figure 8. A: Schematic of a self-excising cassette. The testis ACE promoter (tACE) controls CRE expression while a polymerase II promoter controls neomycin resistance (Neo). The entire construct is flanked with two *loxP* sites. **B:** Use of the cassette. This cassette is stable in ES cells since the tACE promoter is silent. Thus a founder chimeric mouse will also contain the entire cassette in the DNA of tissues derived from the ES cells. In male germ cells derived from ES cells, spermatogenesis activates the testis ACE promoter leading to CRE expression (scissors), recombination of the two *loxP* sites and excision of the cassette. Only a single 34-bp *loxP* site (▶) remains in the DNA of mature sperm derived from ES cells. Thus, F1 heterozygous mice (+/–) lack the neomycin cassette.

sion of Cre in ES cells) or additional animal breeding (such as the mating of a targeted animal with a different mouse line expressing Cre).^{45,46}

To approach the problem, we created a modified neomycin cassette consisting of two functional parts.⁴⁷ One part contained the neomycin resistance gene with appropriate promoters for expression in mammalian tissues. The second portion of the cassette consisted of the gene encoding Cre recombinase under the control of a highly tissue-specific promoter active only in developing male germ cells. This promoter is the testis ACE promoter, normally responsible for the production of the testis ACE isozyme. The entirety of this modified neomycin cassette is flanked by two *loxP* sites (Figure 8). We envisioned the cassette as useful with many different targeting vectors. While this cassette is larger than a typical neomycin-resistance construct, it has the useful property of being self-excising. In ES cells, the testis ACE promoter is silent, no Cre is produced, and the modified cassette is functionally equivalent to a standard neomycin-resistant cassette. When ES cells are re-injected into a blastocyst, which in turn gives rise to a chimeric mouse, the modified neomycin cassette is stable and again functionally equivalent to a standard neomycin-resistance construct. However, during spermatogenesis, the testis ACE promoter is activated, and there is excision of the cassette. Thus, tissue-specific Cre expression leads to self-excision of the cassette in developing sperm such that all further offspring of the chimeric mice lack the neomycin cassette. The use of a tissue-specific promoter induces specific genetic recombination in a tissue-specific fashion.

The expression of Cre in a tissue-specific pattern has obvious uses in the selective modification of a gene. Indeed, a number of laboratories have created strains of mice

stably expressing Cre recombinase under the control of tissue-specific promoters (a list of such lines is available at http://www.mshri.on.ca/nagy/Cre_pub.html). If a gene of interest is modified by the introduction of two *loxP* sites within or surrounding the gene, the mating of this animal with mice expressing Cre in selected tissues results in a tissue-specific elimination of the targeted gene.

A similar approach can also be used to create mice expressing a particular protein in a restricted, small subset of tissues.⁴⁸ Such an experimental approach requires ES cell targeting to inactivate the gene of interest, perhaps through the introduction of an additional piece of blocking DNA (for example, blocking promoter activation of a gene). If gene inactivation is arranged so that the blocking sequences are flanked by *loxP* sites, then the mating of this animal with strains of mice expressing Cre in a tissue-specific fashion results in tissue-specific reestablishment of a functional gene and thus selected tissue expression of a protein. This approach is similar to what we achieved through the modification of the somatic ACE promoter. The Cre-based approach has the advantage that a single mouse containing the interrupted gene of interest can be mated to many different mouse strains expressing Cre in unique tissue patterns. The disadvantage of this approach is the extra mouse-matings required to achieve the final genetic result. This is because the desired mouse needs not only two copies of the modified gene of interest, but also a copy of the transgene directing tissue-specific Cre expression. This approach is also limited in that the gene of interest is expressed under the control of its endogenous promoter. Thus, if one wishes to express a protein, for example ACE, in a location not specified by the endogenous somatic ACE promoter, then additional manipulation of the gene is necessary. To address this problem, investigators have developed strategies to position the gene of interest within genetic loci (such as *Rosa26*) that are ubiquitously expressed.^{49–51}

Conclusion

The past 20 years have brought a revolution in our ability to manipulate the genomes of model systems. In particular, homologous recombination in ES cells of the mouse has permitted investigations possible through no other technical means and knockout-mouse technology has become ubiquitous for scientific research. The goal of this review was to draw attention to what may represent the next step in the use of this technology, namely the creation of more subtle models to investigate organ and tissue-specific functions. While most pathologists understand the approach of eliminating a particular protein using knockout-mouse technology, the newer uses of this technology offers the possibility of creating models with subtler changes in protein expression patterns. If we envision the knockout mouse as representing an extreme phenotype, then these subtler mutations represent the type of genetic change that may more closely mimic the onset of human disease. Undoubtedly, a greater understanding of what is possible using genetic manipulation in model systems will result in increasingly elegant

studies examining the physiology and pathology of individual organ systems.

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