MINIREVIEW

Transgenic Animals as Tools in Hypertension Research (43685)

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Gene transfer technology was developed in murine embryos over 15 years ago, and since then, several thousand reports using transgenic animals have been published. A transgenic animal is defined as containing a segment of exogenous genetic material stably incorporated into the genome, which results in a new trait that can be transmitted to successive generations. These animals have been used as models to investigate basic mechanisms of gene regulation, to study mammalian development, and to construct models that emulate human diseases such as cancer, AIDS, Alzheimer's, and cardiovascular disease. The development of this technology affords an unprecedented opportunity for investigators to examine the pathogenesis, progression, and treatment of genetic diseases and disorders. With the use of recombinant DNA techniques, the expression of normal genes or their disease counterparts can be targeted to nearly any tissue, their regulation can be studied mechanistically or perturbed, and the pathophysiological consequences of these manipulations considered. In this review, the methodology required to generate transgenic animals and an assessment of how these tools have been used to forward research into our understanding of hypertension, a major risk factor associated with cardiovascular disease, will be discussed.

Methods for Generating Transgenic Animals

At the heart of generating transgenic animals is the methodology to manipulate the animals' genome during the earliest embryological stage. Three main methods to accomplish this are currently in use: (i) microinjection of 1-cell fertilized embryos, (ii) retroviral mediated transduction, and (iii) genetic manipulation of embryonic stem cells. Although the technology now exists to generate transgenic rats, sheep, pigs, and other agriculturally important species (1–8), nearly all of the reports published thus far have utilized mice. A number of factors have made mice the overwhelming choice for transgenic studies. First and foremost, mice have a long history as tools for classical genetics, a result of which is the availability of a wide range of mutant stocks, a large number of highly inbred strains, genetically distinct wild derived species, congenic strains, and a number of recombinant inbred sets. A large linkage map of all mouse chromosomes has been determined, and a host of tools are available to quickly map the location of new traits (reviewed in 9). Additionally, although the initial setup associated with a transgenic lab can be quite expensive ($50,000–$120,000), this does not begin to compare to the staggering, and ever increasing cost of purchasing mice and maintaining breeding colonies. Consequently, economic factors have played and continue to play an unfortunate role in making the mouse the most utilized animal thus far. This has far reaching ramifications since larger animals have classically been used for cardiovascular research, and much recent effort has been directed at generating larger transgenic animals. These issues notwithstanding, the mouse is clearly the easiest mammalian species to manipulate at the genetic level. Therefore, all of the technical aspects discussed below pertain to routine practices performed in mice.

Of the three methods mentioned above, microinjection is by far the most common approach used to deliver genetic material into mammalian embryos. The technique requires several important pieces of equip-

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ment, the most important of which is a good quality microscope outfitted with micromanipulators to hold and inject the embryos. An excellent discussion of the required equipment has been previously presented by Hogan *et al.* (10) and DePamphilis *et al.* (11) and therefore will not be discussed in detail here. Although the methods involved are technically demanding initially, a laboratory technician can generally become quite proficient within several months. Briefly, 1-cell fertilized mouse embryos are obtained from hormonally superovulated females the day after mating. Superovulation is first induced by the injection of pregnant mares' serum (follicle stimulating hormone) and then human chorionic gonadotropin (leutinizing hormone) 48 hr later. After a short incubation in tissue culture media, the embryos can be microinjected with a drawn capillary pipette (<1 μm diameter) filled with a DNA solution containing the transgene construct of interest. Following an overnight incubation, the surviving 2-cell stage embryos are surgically transferred into the oviducts of pseudopregnant foster mothers, made so by breeding with vasectomized or genetically sterile males. Although embryos from inbred mice, because of genetic uniformity, would be the preferred tool for many studies, mice from mixed genetic backgrounds have superior reproductive performance and embryo survival. Clearly, animal husbandry plays a critical role in the ultimate success of any transgenic program.

Once the technical details of microinjection are sufficiently mastered, transgenic frequencies ranging around 20% of live births can be common. Unfortunately, the major advantage of this technique, its relative ease, also presents significant drawbacks. Microinjected DNA segments generally integrate in a tandem head-to-tail array at a single random site in the genome. The consequences of this random insertion is 2-fold. First, the expression of the transgene can become altered by virtue of the juxtaposition of regulatory sequences at or near the insertion site, a so-called position effect. A simple example of this phenomenon is the expression of a gene, normally known to be specifically expressed only in a single tissue, in multiple tissues of the transgenic animal. Position effects are thought to be caused by the presence of tissue-specific regulatory elements controlling an endogenous gene near the site of transgene insertion. Indeed, al Shawi *et al.* (12) has shown that these effects are encoded *in cis*. In practice, these effects must be distinguished from true expression, by examining several different independent transgenic lines made by distinct insertion events. The other major impediment of the random integration of transgene sequences is a mutation caused by integration within an essential gene. Although the insertion into some genes may cause a lethal phenotype even in the heterozygous state, these mutations often remain undetected until the transgenic mice are bred to homozygosity. Occasionally, interesting mutations can result from these studies; a number of limb deformity and neurological mutants have been described (13–16).

In lieu of a detailed methodological description of retroviral mediated transduction and embryonic stem cells, important advantages and drawbacks of each approach will be briefly discussed. Retroviral vectors are viral genomes modified to carry exogenous DNA and can be used to deliver genetic material into the early stage embryo (17, 18). The advantages of this technique include: (i) the ability to infect preimplantation embryos at any stage between Day 1 and Day 8, (ii) the viral genomes integrate in a single copy, and (iii) the ease of cloning endogenous flanking DNA around the insertion site as compared with those from microinjected transgenes (important if insertional mutants are to be characterized). The major disadvantages of the retroviral system are: (i) a limitation placed on the size of the exogenous transgene DNA that can be packaged, and (ii) the presence of retroviral vector sequences at the site of insertion. Vector sequences are generally removed prior to microinjection because they can interfere with the normal expression of transgenes (19).

Embryonic stem (ES) cells are totipotent stem cells isolated from the inner cell mass of a blastula stage embryo. Under appropriate conditions, they can be propagated in culture while retaining their totipotency. These cells can be manipulated *in vitro* via transfection with transgene constructs, analyzed, reimplanted into foster blastocysts, and transferred into pseudopregnant foster mothers. Since ES cells can repopulate the embryo, the offspring are a mosaic formed partially of stem cell–derived cells and host blastocyst–derived cells (20–22). A fully transgenic (nonmosaic) animal results from successful germ line transmission of the transgene. Although transgenic mice generated by random integration can be created by this approach, the real impact and interest afforded by this technique is the ability to target specific chromosomal positions by homologous recombination, allowing investigators to generate site-specific gene knock-out mutations. Mutant mice generated with these genetically altered stem cells become extremely valuable tools for determining the role of the gene product *in vivo*. Mutations induced in the CFTR gene of embryonic stem cells lead to cystic fibrosis in the resultant mice (23, 24). Although ES cells have been used successfully to generate transgenic and knock-out mice by a number of laboratories, their manipulation is more difficult than microinjection. Despite these difficulties, their use as powerful genetic tools will eventually make this the preferred approach.

Design of a Transgenic Experiment

The outcome of a transgenic experiment is largely rooted in the design of the transgene construct. Experiments can be designed to overexpress a gene product, redirect endogenous protein expression to different tissue and cell types, or specifically induce the production of a novel protein in a specific tissue or cell type. For the purposes of this discussion we can consider a transgene to be composed of two important regions, the promoter/regulatory region and the protein coding region. The promoter/regulatory element is used to direct the tissue and cell specificity of transgene expression. The choice of promoter element is based upon the availability of cloned and characterized promoters and the desired site(s) of expression. For example, the renin promoter is active in kidney, reproductive tissues, and adrenal gland (25); the atrial natriuretic factor (ANF) and myosin heavy chain promoters are active in heart (26, 27); and the transthyretin promoter is active in liver (28). A summary of promoter elements used to target genes throughout the cardiovascular system has been previously reviewed (29, 30). In addition, a promoter's response to regulatory signals, such as feedback repression, or to physiologic inputs is also important. Consider the renin promoter, for example. In kidney, expression of the renin gene is sensitive to circulating angiotensin II (31) and responds to changes in arterial pressure and electrolyte balance (reviewed in 32). Therefore, if one wished to express a protein in the kidney independent of these physiologic changes, the use of the renin promoter would probably be inappropriate.

The other portion of the transgene consists of the protein coding region. This region can encode naturally occurring endogenous proteins, such as renin or ANF, or exogenous proteins, such as viral oncogenes or reporters. In many cases where gene expression or overexpression is desired, the promoter and coding regions can be contiguous sequences derived from a genomic clone. Indeed, the earliest experiments designed to overexpress the renin gene have been carried out using genomic clones isolated from mice and humans (reviewed in 25 and 33). In cases where genomic clones are extremely large, fusions between the genes promoter, isolated from the genomic clone, and a cDNA encoding the protein can be generated. Although these simple fusions are easy to make, they generally are not expressed as highly or as reliably as genomic constructs. A number of strategies can now be employed to stabilize or enhance the expression of cDNA or "mini-gene" transgenes (34–36); and these strategies are particularly useful when one wishes to use the promoter from one gene to drive the expression of a different gene product. Promoter-oncogene fusions have been extensively used as models to examine the mechanisms of tumorigenesis caused by targeting dysplastic or neoplastic growth to specific tissues (reviewed in 37 and 38). Reporter genes are defined as gene products, which by virtue of their uniqueness to the genome, act as sensitive and accurate markers of gene expression. Typical examples are *E. coli* LacZ, encoding β-galactosidase, and firefly luciferase. Simple biochemical and histochemical assays are available for each and their use in transgenic mice has been described (39–44).

Transgenic Animal Models for Studying Hypertension

Although many cardiovascular related genes have been examined in transgenic animals, no gene has received more attention than the renin gene. This is certainly due, at least in part, to its historical importance as a major player in arterial pressure regulation and in the pathogenesis of some forms of experimental and human hypertension (reviewed in 45–47). Therefore, the focus of the following discussion will be on the renin-angiotensin system and attempts to examine its expression and regulation, to develop novel tools to facilitate its examination *in vitro*, and to examine its role in the pathogenesis of hypertension, using transgenic animal models.

Transgenic Models for Studying the Expression of the Mouse Renin Gene. Transgenic mice have been used to examine the tissue- and cell-specific expression and regulation of the renin genes from mice and humans. Initial studies were performed with the aim of examining the complex differential expression pattern exhibited by different mouse renin genes and alleles. A summary of this differential expression is presented in Table I and has been previously reviewed (25, 33). Previous genetic and gene mapping studies have clearly described the presence of two distinct renin loci, closely linked on Chromosome 1, in some

Table I. Expression of the Renin Genes in Mice[a]

Tissue	*Ren-1*[c]	*Ren-1*[d]	*Ren-*[2]
Kidney	+ + +	+ + +	+ + +
Submandibular gland	+	−	+ + +
Adrenal gland (fetal)	+ +	+ +	+ +
Adrenal gland (adult)	−	+ +	+ +
Testes	+	+ +	+
Ovary	+	+	+
Anterior prostate	+ + +	−	−
Subcutaneous tissue (fetal)	+	+	+
Liver	−	−	−

[a] The relative level of renin mRNA in the tissues is indicated by + or −. + + +, high level expression; + +, easily detectable expression; +, low level expression; −, expression not detectable. Under normal conditions, renin mRNA cannot be detected in heart, spleen, lung, brain, thymus or uterus, using standard northern blotting or RNase protection assays.

strains of mice (48–50). Two separate alleles of one of these loci have been identified in common laboratory strains. Although the mouse renin genes are 97%–99% homologous in the coding region and exhibit extensive segmental homology upstream of the gene, their expression profiles vastly differ (reviewed in 25). Strains of laboratory mice can therefore be divided into two groups, based on whether they contain one or two renin loci; initial experiments were focused on generating a phenocopy of 2-renin gene mice, using a genetic background containing a single renin gene (*Ren-1*^{c}) and a transgene consisting of the duplicated renin locus (*Ren-2*). Tronik *et al.* (51) and Mullins *et al.* (52) reported two such models, differing in the background strain used, the genetic origin of the transgene, and the extent of transgene sequences employed (2.5 kb of 5′ flanking sequence in the former and 5.3 kb in the latter). Although both groups observed qualitative tissue-specific expression of the renin transgene, only Tronik *et al.* reported qualitatively correct expression. Transgene expression reported by Mullins *et al.* was low in some tissues, yet highly elevated in others. An important finding of both studies was that the transgenes were capable of being hormonally regulated in the appropriate target tissues. Neither study on its own, however, significantly localized the location of DNA sequence elements regulating tissue-specific renin expression. Nevertheless, the information obtained from these studies became important in localizing important regulatory elements when used in conjunction with data obtained from transgenic mice containing fusions between the mouse *Ren-2* promoter and a reporter gene (discussed below).

In order to assess more directly the location of important transcriptional regulatory elements controlling mouse renin expression, Sigmund *et al.* (53) and Sola *et al.* (54) generated transgenic mice with constructs consisting of the mouse renin (*Ren-2*) promoter fused to the SV40 T antigen gene. These experiments were performed with the goal of specifically targeting the expression of the T antigen oncoprotein to renin expressing cells. In these initial experiments, T antigen provided a valuable reporter function, since its mRNA could be detected using a unique probe and the accumulation of protein could be assayed in specific tissues by immunohistochemistry. In the first study, a transgene consisting of only the most proximal 2.5 kb of *Ren-2* 5′ flanking sequence was used (54). Recall that transgenic mice containing 2.5 kb of 5′ flanking *Ren-2* sequence in a genomic context (containing all exons, introns and 3′ flanking sequences) was sufficient for qualitative and quantitative tissue-specific and hormonally responsive expression (51, 55). On the contrary, in the context of a reporter construct inappropriate tissue-specific expression was observed, and although tumors developed in some tissues (due to the effects exerted by the oncoprotein), neither these tissues nor the tumors themselves expressed renin endogenously (54). These results strongly suggest that the proximal 2.5 kb of 5′ flanking information is insufficient to target expression of a reporter gene to renin expressing tissues *in vivo*. Furthermore, the data from both reports suggest that the important regulatory elements are located either further 5′ of the gene, within the gene itself (exons or introns), or in the 3′ flanking region of the gene.

Experiments to address this question were performed by Sigmund *et al.* (53). In this case, a construct containing 4.6 kb of *Ren-2* 5′ flanking sequence exhibited qualitative tissue- and cell-specific expression essentially indistinguishable from the endogenous renin gene. The transgene was also hormonally responsive in submandibular gland (SMG, 53), followed the normal developmental program as the renin gene in kidney (56), and was responsive to the effects of angiotensin converting enzyme inhibitors during early postnatal life (57). Although these results strongly suggest that important transcriptional regulatory elements lie between -2.5 and -4.6 kb, they do not rule out the possibility that redundant elements lie either 3′ of the gene or within the body of the gene itself.

Transgenic experiments were also used to examine the genetic determinants regulating the differential tissue-specific expression exhibited by the *Ren-1*^{c} and *Ren-1*^{d} genes. Miller *et al.* (58) generated transgenic mice containing a *Ren-1*^{d} genomic construct on an otherwise *Ren-1*^{c} genetic background. They demonstrated that the expression profile of the *Ren-1*^{d} gene was the same irrespective of the genetic background used and that the elements controlling the differential expression of the two genes were located *in cis*. Their results are in agreement with genetic studies utilizing F_1 and congenic mouse strains (59).

Transgenic Models for Studying the Expression of the Human Renin Gene. Before performing the mouse renin expression studies discussed above, we already had a solid understanding of the tissue and cell specificity exhibited by the mouse renin genes (reviewed in 25). This is not so for the human renin (hRen) gene, however. The fact that the three mouse renin genes exhibit substantial differences in their tissue-specific expression profiles, and with significant differences existing between mouse and rat renin expression, it was difficult to tacitly assume that we could totally predict the pattern of renin expression in humans based on studies in rodents. For obvious reasons the human system has been the poorest studied to date. The most significant problem is the lack of easily obtainable fresh human tissues for the required RNA analyses associated with gene regulation studies. Therefore, transgenic mice became an important tool for examining the expression and regulation of the

hRen gene. Importantly, previous transgenic mouse models using genes from heterologous species suggested that they are expressed in mice comparable to the way they are expressed in their endogenous systems (60, 61).

As with the mouse system, studies on hRen expression were initiated with large genomic clones. Fukamizu *et al.* (62) and Seo *et al.* (63) first reported the tissue-specific expression of one hRen transgenic line, which contained a construct consisting of all 10 exons and introns, and 3.0 kb and 1.2 kb of 5′ and 3′ flanking sequences, respectively. Using an RNase protection assay, they were able to differentiate hRen mRNA from the endogenous mRen mRNA (the genes share 78% identity in message sequence). Transgene expression was evident in kidney, brain, heart, lung, pancreas, spleen, stomach, testes, and thymus. Although RNase protection is a sensitive assay of expression a major drawback to this analysis is the inability to determine the relative expression levels of the two species renin genes. In order to overcome this deficiency, we developed assays capable of distinguishing the message from the two species and accurately quantitating the relative contribution of each gene to the total pool of renin mRNA. We then used the assays to examine hRen expression in transgenic mice containing a smaller genomic construct with flanking sequences extending 892 bp upstream and approximately 400 bp downstream of the gene (64). The first assay is a modification of standard northern blot analysis using species-specific antisense RNA probes. Because of the homology shared by the mRen and hRen mRNAs, blots probed under standard conditions, and using either gene specific probe, allowed us to detect total renin (both species) mRNA. Subsequent RNase-A treatment and stringent washing removed probe involved in a heterologous species hybrid, allowing us to specifically detect either hRen or mRen mRNA. The second and more quantitative assay is based on sequence polymorphisms present in the two genes. Chain-terminating primer extension of a mixed population of hRen and mRen mRNA with a common oligonucleotide primer located immediately adjacent to a polymorphic region results in two distinct bands on a sequencing gel, each representing a single-species of renin message. The intensity of the bands reflects the relative contribution of that gene to the total pool (64).

We used these assays to measure the expression of human renin in kidney, adrenal gland, testes, ovary, lung, and adipose tissue of four independent transgenic lines (summarized in Table II). Approximately equal levels of hRen and mRen mRNA were evident in the kidney of all four lines. No transgene expression was detected in liver, heart, SMG, or brain. The results largely agree with the above studies (62) and predictions made based on similarities in the expression of the mouse and rat renin genes (25, 33).

Table II. Human Renin Expression in Transgenic Mice

Tissue	Human renin mRNA	Mouse renin mRNA
Kidney	+[a]	+[a]
Kidney + captopril[b]	++	+++
Adrenal gland	+	−[c]
SMG	−[d]	+
Liver	−	−
Testes	+	±
Ovary	+	±
Heart	−[d]	−
Brain	−	−
Lung	+	−
Adipose tissue	+	−

[a] Approximately equal levels of hRen and mRen mRNA was evident.
[b] Captopril treatment was for five days at 100 mg/kg/day in the drinking water.
[c] Adult mice of this genetic background do not express adrenal renin (see Table I).
[d] Low level expression of human renin mRNA was detected in one of four transgenic lines.

The transgenic studies also were used to confirm the cellular localization of hRen mRNA (64) and protein (65) to juxtaglomerular (JG) cells of the kidney in adults. In adrenal gland, hRen mRNA was detected in scattered pockets of cells in the outer adrenal cortex (zona glomerulosa, 64). Interestingly, although this is the predicted site of renin expression in humans and rats (66, 67), it is not the cellular site of adrenal renin in mice, which is zona fasciculata and X-zone (52). This provides an additional example of the retention of species-specific expression patterns in transgenic mice. Of particular importance for future physiological studies on the human renin gene is the question of whether these transgenes are properly regulated. In order to address this issue, we examined renal expression of the human and mouse genes by differential primer expression in pairs of animals either left untreated or treated with captopril, an angiotensin-converting enzyme inhibitor. Treated animals exhibited significantly higher levels of both the mRen (5–9-fold) and hRen (2–2.5-fold) mRNAs, strongly suggesting that they were responding, as expected, to the decrease in angiotensin-II mediated feedback repression of renin expression caused by decreased circulating angiotensin-II levels (64).

Recently, we have examined the expression of the hRen gene throughout fetal development of transgenic mice (68). Human renin mRNA was evident in kidney at 15.5, 17.5, and 18.5 days of gestation and in newborns; the level of its message appeared to be slightly elevated as compared to an adult transgenic. It was not detected at any late developmental stage in heart,

liver, or brain, and was only moderately expressed in the gastrointestinal tract, a possible site of renin expression in adults (69). The most interesting pattern of expression was observed in lung, a site exhibiting high-level hRen expression in adult transgenics (64, 70) as well as the reported location of some renin secreting tumors in humans (71, 72). Although no hRen mRNA was detectable at 15.5 days of gestation, its expression was visible at 17.5 days and increased throughout late development peaking at or around birth. These results suggest that the expression of hRen in fetal lung is developmentally regulated yet distinct from the regulation of the gene in kidney. Current studies are underway to examine the cell specificity of hRen in the lung and to map regulatory elements responsible for the developmental expression.

Development of Renin-Expressing Cell Lines From Transgenics. In many systems, the most efficient means for mapping DNA sequence elements which control the transcription of a gene is through the use of tissue culture cells. We previously suggested that a suitable cell line to study renin regulation should: (i) be derived from the kidney, since this is the site responsible for blood borne active renin, and where the gene is regulated by physiologic inputs, and (ii) express the renin gene endogenously, so that we can be assured that the cells express the correct complement of ubiquitous and gene-specific transcription factors necessary for proper expression of the renin gene. Unfortunately, no suitable tissue culture cells were available for the examination of the renin gene at the inception of the projects described above. Although a number of attempts have been made to isolate JG cells from the human and mouse kidney, the resultant cultures invariably ceased to secrete renin or failed to transcribe the gene shortly after being placed into culture (73, 74). Certainly the small population of JG cells in the kidney (<0.01%) is partially responsible for these difficulties.

Numerous previous studies have demonstrated that the specific targeting of oncogenes in transgenic mice could lead to tumor formation in some targeted tissues and that permanent cell lines could be derived from these tumors (reviewed in 37 and 38). Although the spectrum of tissues targeted for transformation is contingent on the promoter used to drive oncogene expression, some tissues are more refractory than others to oncogene-induced tumorigenesis. Therefore, factors such as the frequency of tumor formation, and the latency period between oncogene expression and tumor induction, may depend on the specific oncogene used and the level of its expression. SV40 T antigen appears to be robust in its ability to transform a wide range of cell types, and it has been used to target oncogenesis to, and to derive cell lines from, pancreatic beta cells (75), thymic epithelial cells (76), and cardiomyocytes (77), among others.

Transgenic mice containing a fusion between the mouse *Ren-2* promoter and the SV40 T antigen gene were generated in order to derive renin expressing cell lines from the mouse (78). As discussed above, the tissue-specificity of the transgene was essentially the same as the endogenous renin gene (53). Transgenic mice containing this transgene exhibited overt tumor formation in kidney, subcutaneous tissue, adrenal gland, and reproductive tissues (78, 79). The frequency of tumorigenesis at these sites markedly differed among two independent transgenic lines, with subcutaneous tumors being most prevalent in a line exhibiting high-level T antigen expression, and kidney tumors more prevalent in a line exhibiting very low levels of T antigen expression. The kidney tumors expressed high levels of endogenous renin mRNA, which could be maintained even after multiple passages of the tumor material in immunocompromised nude (nu/nu) mice. This observation, along with data showing the specific targeting of JG cells during early neonatal life (57) strongly suggested that the tumors were derived from renin-expressing cells and likely were derived from JG cells. A clonal cell line, designated As4.1, has been derived from one of these tumors, and it maintains expression of renin mRNA past 40 consecutive passages in culture. Interestingly, renin mRNA in As4.1 cells is regulated by serum, with the highest expression observed in cells incubated under serum free conditions (80). This cell line is currently being used to examine the regulation of renin storage and release, the regulation of mouse and human renin gene expression, as well as to isolate novel mRNAs expressed in JG cells. Attempts to isolate cell lines from other renin expressing tissues from the mouse are being performed by a number of laboratories, the overall goal being to develop a library of renin-expressing cell types that would aid in the study of tissue-specific, cell-specific, and differentially regulated expression *in vitro*.

Transgenic Mouse Models of Hypertension. Recently, a great deal of effort has been directed at identifying the genetic determinants of hypertension. Initially, studies were limited to candidate genes. In these studies, polymorphisms in genes expected to be involved in hypertension were examined to determine if they cosegregated with increased blood pressure in F_2 cohorts derived from crosses of hypertensive and normotensive rat strains. These genetic studies suggested that the renin gene may be linked to hypertension in some (81, 82) but not all (83) hypertensive rat strains. More recent studies using anonymous markers, gene-specific microsatellite probes, and simple sequence repeat polymorphisms distributed throughout the rat genome, suggested that another gene of the

renin-angiotensin system, angiotensin-converting enzyme, may be linked to hypertension in the stroke-prone spontaneously hypertensive rat (84, 85). Similarly, other studies suggest that angiotensinogen, the substrate for renin, may be linked to hypertension in humans (86).

One method to test the hypothesis that a gene is involved in a particular phenotype is to overexpress that gene, and its encoded protein, in a whole animal system. This is the quintessential transgenic experiment, and this model has now been used to examine the involvement of the renin-angiotensin system in the pathogenesis of hypertension. Ohkubo *et al.* (87) designed the first studies to specifically examine the result on blood pressure caused by elevated synthesis and release of components of the renin-angiotensin system. They designed two constructs in which the synthesis of rat renin (rRen) and rat angiotensinogen (rAng) was driven by the mouse metallothionein (MT) I promoter. The MT promoter is active in liver and is responsive to heavy metals such as zinc. The goal of their experiment was to overexpress the proteins in the liver, where they would be constitutively released into the systemic circulation, and to examine the effect on blood pressure. Expression of the proteins also could be elevated in animals treated with zinc or other heavy metals. Their strategy was to generate transgenic mice that contained either the rat renin (MT-rRen) or rat angiotensinogen (MT-rAng), and once positive transgenic lines were identified and characterized, they were bred to generate transgenic strains carrying both the rRen and rAng genes.

Expression of both messages was confirmed in the liver of the transgenic animals, and the level of plasma rAng was highly elevated in the transgenics containing that gene. Systolic blood pressure was recorded in the singly and doubly transgenic mice at 4–5 months of age using the tail cuff method on unanesthetized, restrained mice. Systolic pressure was unchanged in transgenics containing either the MT-rRen or MT-rAng genes as compared with age matched, nontransgenic littermates. However, systolic pressure increased from approximately 95.3 mmHg in nontransgenic males and 106.6 mmHg in nontransgenic females to 125.8 mmHg and 131.8 mmHg in doubly transgenic males and females, respectively. This modest increase in blood pressure was sustained throughout the life of the animals and could be further increased to an average of 141 mmHg after one month of zinc treatment in the drinking water. As expected for a angiotensin-II dependent phenotype, blood pressure was reduced to normal levels after treatment with captopril.

These results point to what is now considered an important feature of the enzymatic interaction between renin and its substrate: its species specificity (summarized in Table III). The experiment also served to functionally confirm earlier reports suggesting that the kinetics of the renin-angiotensinogen reaction could be dramatically altered, when the enzyme and substrate were from heterologous species (88, 89).

Kimura *et al.* (90) examined transgenic mice containing the entire rAng, but not the rRen, gene. Some lines of these singly transgenic mice exhibited a sustained increase in mean arterial pressure (52 mmHg in transgenic males and 24 mmHg in transgenic females) despite the absence of the rRen gene. There are several important differences in the design of this experiment which may contribute to the disparity between the findings of this and the Ohkubo *et al.* (87) report: (i) these transgenic mice consisted of a genomic rAng gene containing all five exons and introns, and flanking sequences extending approximately 1.6 kb upstream and 0.9 kb downstream of the gene. This transgene

Table III. Species Specificity of the Renin-Angiotensinogen Reaction[a]

Renin source	Angiotensinogen source					
	Biochemical assay			Increased blood pressure		
	Mouse	Rat	Human	Mouse	Rat	Human
Mouse	+++	+[b]	−[c]	—	−[f]/+[g]/++[h]	−[i]
Rat	−[b]	+++	−[e]	−[f]	—	−[e]
Human	−[bcd]	+[b]	+++	−[i]	−[e]	—

[a] Biochemical assay based on *in vitro* kinetics or increased levels of plasma angiotensin in transgenics. Increased blood pressure based on whether transgenic animals containing the indicated genes exhibit spontaneously elevated arterial pressure.
[b] Oliver WJ, Gross F (89).
[c] Fukamizu *et al.* (91).
[d] Sigmund *et al.* (64).
[e] Transgenic rats containing the human renin and human angiotensinogen genes—Ganten *et al.* (70).
[f] Transgenic mice containing the rat renin and rat angiotensinogen genes. The double transgenics were mildly but chronically hypertensive—Ohkubo *et al.* (87).
[g] Transgenic mice containing the rat angiotensinogen gene—Kimura *et al.* (90).
[h] Transgenic rats containing the mouse Ren-2 gene—Mullins *et al.* (1).
[i] Transgenic mice containing the human renin and angiotensinogen genes. The double transgenics were chronically hypertensive—Fukamizu *et al.* (92).

was highly expressed in liver and brain, and to a lower level in heart, kidney, and testes. Therefore, unlike the MT-rANG, expression of the genomic construct occurred in extra-hepatic tissues which normally express the angiotensinogen gene; (ii) mean arterial pressure measurements in the present study were performed by chronic intra-arterial catheter in conscious, freely moving animals, a vastly superior method for measuring pressure in mice; and (iii) plasma rAng was elevated 2-fold in this model, as compared with MT-rANG transgenic mice. Interestingly, renin levels in mice are relatively high (approximately 15 ng angiotensin I/ml/hr (57) suggesting the possibility that enhanced generation of angiotensin I (and angiotensin II) can occur if sufficient substrate is available. It also remains possible that the strict species specificity of the renin-angiotensinogen reaction is only evident when comparing more distant species such as rodents and humans and not when comparing more closely related species such as mice and rats. In support of this, mouse renin can cleave rat angiotensinogen *in vitro* (89).

This species specificity was more recently examined by Fukamizu *et al.* (91). They examined the kinetics of the reaction using purified mouse SMG renin and recombinant human renin, employing mouse (nontransgenic) liver extracts as a source of substrate. Recombinant human renin exhibited 2.5-fold lower K_m and 23-fold lower K_{cat} than mouse renin, suggesting that mouse angiotensinogen is not a suitable substrate for human renin. Further, they followed this with a study to determine whether human and mouse renin from kidney extracts of hRen gene transgenic and nontransgenic mice could cleave substrate isolated from liver extracts of nontransgenic or human angiotensinogen gene transgenic mice. The results strongly confirm the notion that human and mouse renins utilize two different substrates.

To test this *in vivo*, Fukamizu *et al.* (92) recently examined transgenic mice containing the human renin and human angiotensinogen genes. As in the transgenic models containing the rat genes above (87), the singly transgenic mice exhibited normal arterial pressure levels. The maintenance of normal blood pressure was evident despite a concentration of human renin in the plasma of the singly hRen transgenic mice 34-fold higher than that normally found in human plasma, and a level of plasma human angiotensinogen in the singly hAng mice 18-fold higher than that normally found in humans. The levels of AII were slightly elevated in the singly hAng (1.3-fold) and hRen (1.8-fold) mice as compared to nontransgenic control animals, suggesting a slow rate of cleavage of angiotensinogen by the heterologous renin. Restoring the interaction between human renin and human angiotensinogen in the double transgenics resulted in a 35–50-fold increase in plasma renin activity, a nearly 5-fold increase in plasma AII, and a sustained increase of approximately 30 mmHg in systolic blood pressure. Although the systolic blood pressure of all the experimental groups (nontransgenics, hRen, hAng, and doubly hRen/hAng) decreased after angiotensin-converting enzyme inhibition or by inhibiting the type-1 angiotensin receptor, only the systolic pressure of the double transgenic was effected by treating the animals with a human renin specific inhibitor. A summary of biochemical and *in vivo* data examining the species specificity of the renin-angiotensinogen reaction is shown in Table III.

Transgenic Rat Models of Hypertension. Although mice have been used most often in transgenic experiments, they are small in comparison with rats, rabbits, and sheep, which historically have been used for cardiovascular research. Although techniques for effecting detailed physiology are improving in mice, there has been a great deal of interest in generating transgenic animals from larger species, and indeed this has been made possible by a number of laboratories (1–3, 7). Several important developments have been made along these lines that have benefited and will continue to benefit hypertension research. These developments include the technology to generate transgenic rats and the creation of novel models from which to study hypertension. The first such model was created by Mullins *et al.* (1) and will be discussed later in this section. The rat is a particularly important model because it allows investigators to compare the physiology of transgenic animals containing single gene manipulations with a host of extremely well-characterized genetic models of hypertension.

As an extension of the transgenic mouse models discussed above, Ganten and colleagues (70) have created transgenic rats containing the hRen and hAng genes. As in the previous examples, transgenic rat strains were generated containing either the hRen or hAng genes, and then these singly transgenic strains were bred together to generate doubly transgenic animals. Human renin transgenic rats contained higher levels of plasma active hRen than nontransgenic littermates, and was as high as 6800 pg human renin/ml in some transgenic lines. There was no significant effect on the level of plasma rat renin. As expected, expression of hRen mRNA was evident in kidney and adrenal gland, but it was also detected in spleen, thyroid, thymus, brain, lung, and intestine. Expression of hRen in the latter two tissues agrees with previously published reports, which also show a similar pattern of human renin expression in transgenic mice (64, 69). Human renin levels in plasma were highly elevated after furosemide-induced sodium depletion and sodium restricted diet, and human renin was specifically localized to juxtaglomerular cells in the transgenic kidney, both indicating proper cell-specific and regulated expression of the transgene.

Similarly, several transgenic rat lines carrying the hAng were identified with varying levels of plasma human angiotensinogen. Several of these lines had plasma human angiotensinogen in excess of that found even in humans. Transgene mRNA was evident in liver, kidney, lung, intestine, heart, and brain, and *in situ* hybridization confirmed that the site of hAng expression in the transgenic liver corresponded to the site of endogenous angiotensinogen production. Interestingly, their preliminary results suggested that the doubly transgenic animals do not develop spontaneous hypertension. This may be explained by the fact that the transgenes were isolated from genomic clones, and therefore contain regulatory sequences which are designed to respond to physiological inputs regulating arterial pressure. Consequently, it becomes important to determine if the expression of the hRen and hAng genes is depressed in the double transgenics, where the homologous enzyme-substrate interaction can occur, as opposed to the single transgenics, where the transgene proteins should be essentially inert.

Although chronic effects on blood pressure were not observed, an acute pressor response was noted in experiments in which human renin protein was infused into transgenic rats containing the hAng gene. This response was sensitive to the human renin-specific inhibitor Ro 42–5892. No changes in arterial pressure were noted when human renin was infused into nontransgenic littermates. The infusion of excess rat renin also elicited a pressor response, but it could not be attenuated by the administration of the human renin inhibitor. Administration of the angiotensin type-1 receptor antagonist, DUP 753, relieved the pressor response caused by both human and rat renin. Accordingly, at the very least, these results demonstrate the utility of the system for performing acute blood pressure studies, and further point to the species specificity of the renin-angiotensinogen reaction. It should be clear at this point that this species specificity also provides an excellent experimental system from which studies on gene regulation and transgene-induced pathophysiology can be separated.

Certainly the most provocative model developed thus far is a transgenic rat containing the mouse *Ren-2* gene (1). The *Ren-2* gene represents the duplicated renin locus in mice and exhibits an extremely high level of expression in mouse SMG (93). Interestingly, although the mouse Renin-1^c and Renin-1^d proteins contain three potential N-linked glycosylation sites, all three sites are absent from Renin-2, suggesting that the protein may be unglycosylated. Indeed, differences in the thermostability of Renin-2 and Renin-1 *in vitro* suggest there may be significant differences in the biochemistry of the proteins (94). These observations notwithstanding, multiple lines of transgenic rats containing *Ren-2* become severely hypertensive, with blood pressures exceeding 210 mmHg in heterozygotes and approaching 300 mmHg in homozygotes (1, 95). Initial studies revealed that plasma renin activity, angiotensin I, and angiotensin II all were depressed in the transgenic animals. Furthermore, the levels of renal renin were severely attenuated in the transgenics. *In situ* hybridization identified few juxtaglomerular cells expressing renin, and a nearly complete absence of renin secretory granules were found in the transgenic kidney after ultrastructural analysis (96). Elevated Renin-2 levels were observed in adrenal gland, and plasma prorenin, consistent with renin release from a nonrenal tissue, was highly elevated (97). The observation that angiotensin-converting enzyme inhibition and angiotensin type-1 receptor blockade decreased blood pressures to near normal levels strongly suggests that elevated blood pressure in this model is dependent upon angiotensin II (1, 95). Apparently, these animals represent a monogenic model of low plasma renin hypertension, despite the fact that they contain an additional renin gene.

This model has now become an important resource for investigating low renin hypertension and is the focus of intense effort by laboratories world wide. Based on the data presented above, it should not come as a surprise that much of the focus initially was directed at examining the transgene expression in the adrenal gland. Indeed, both blood pressure and plasma prorenin levels fell significantly after bilateral adrenalectomy, although the levels of each were still elevated as compared with nontransgenic animals (96). It remains unclear whether blood pressure and plasma prorenin levels remained diminished long term in the adrenalectomized animals.

In the mouse, *Ren-2* is expressed in two inner cortical zones, the zona fasciculata and X-zone (52). This differs significantly from the cell specificity of renin in the rat adrenal, which is primarily the zona glomerulosa (66, 98). Therefore, it was important to assess the localization of adrenal *Ren-2* expression in the transgenic rats. Yamaguchi *et al.* (99) reported high levels of prorenin and active renin both in extracts from adrenal capsule, containing essentially zona glomerulosa, and in decapsulated adrenal glands, containing the remainder of the cortex and adrenal medulla. In contrast, renin activity in Sprague-Dawley rats was limited to the capsular region. The results were confirmed by *in situ* hybridization studies showing highly elevated levels of renin mRNA localized in zona glomerulosa as well as in the outer zona fasciculata (96). That *Ren-2* expression was evident in both cortical zones suggests that the species-specific expression of *Ren-2* in adrenal gland is at least partially conserved in the transgenic rat, but that other factors contribute

to the overall expression of the transgene in the adrenal.

The synthesis of several hormones involved in electrolyte homeostasis occurs in the adrenal gland. Of particular importance is the conversion of corticosterone to aldosterone (a potent mineralocorticoid) in the zona glomerulosa (100). The renin-angiotensin system may play a role in regulating aldosterone biosynthesis or release (101), and therefore highly elevated adrenal renin levels may affect steroid metabolism by enhancing local production of angiotensin II and elevating aldosterone production. Indeed, the levels of urinary deoxycorticosterone, corticosterone, 18-hydroxycorticosterone, and aldosterone were elevated in transgenic rats during the initial phases of hypertension (6–18 weeks of age) (102). The difference in hormone excretion between transgenics and controls tended to diminish with age and was not significantly different in adults. The excretion of deoxycorticosterone and the increase in plasma prorenin in response to ACTH administration were significantly increased in the transgenic animals (102), as was the response to ACTH in primary cultures of glomerulosa cells (99). Although this data may suggest a mechanism by which hypertension develops in this model, spironolactone (a mineralocorticoid receptor antagonist) was not sufficient to lower blood pressure in 4-week-old transgenic rats or 18-week-old transgenic or stroke-prone, spontaneously hypertensive rats (95, 102). Thus, elevated mineralocorticoids, on their own, cannot explain the pathogenesis of hypertension in this model. Clearly, both the role of increased steroid biosynthesis and the adrenal renin-angiotensin system toward the development of hypertension in this model must be fully explored. An additional complication of this model is the expression of the *Ren-2* transgene in other extrarenal tissues, such as thymus, gastrointestinal tract, reproductive tissues, brain, and lung (95). It remains possible that *Ren-2* expression in some of these tissues plays a role in the development or maintenance of hypertension in this transgenic rat model.

Despite the uncertainty over the mechanism by which these animals become hypertensive, these rats may still provide an additional important tool for testing or developing therapeutic interventions for hypertension. As in other models of hypertension, and in hypertensive patients, pathological lesions including increased renal arterial and arteriolar wall thickening, renal glomerulosclerosis, and hypertrophy of thoracic aorta and coronary arteries and arterioles, develop as the disease progresses (96).

Prospectives for the Future

The development of gene transfer systems in mice and rats has allowed investigators to examine complicated multigenic systems in an experimentally amenable setting. What once was used almost exclusively to examine the regulation of cardiovascular-related gene expression has now moved forward into modeling human cardiovascular disease. Indeed, the examples presented above only scratch the surface of current models, which include hypotension (28, 103), renal vascular hypertrophy (57), polycystic kidney disease (104), glomerular sclerosis (105, 106), cardiac hypertrophy (107–109), cardiac arrhythmias (26, 110, 111), venous occlusions (112), and vascular remodeling (113). An increasing number of models also have been created to study atherosclerosis (114–119). Clearly, an enormous amount of information will be gained from an analysis of these preexisting models and the creation of new ones. New models will undoubtedly be generated with genes identified by molecular geneticists to be linked to high blood pressure. Also, the increasing use of methods to either ablate gene expression or create null mutations in specific genes *in vivo* is of particular importance. For example, the use of antisense RNAs in transgenic animals affords the potential to ablate the expression of a specific gene in the entire animal or in specific tissues. Transgenic animals containing antisense RNA constructs have been used to examine glucocorticoid-receptor function (120), create neurological mutations (121), and to study leukemia (122). Moreover, the development of catalytic antisense RNAs, termed ribozymes, may further facilitate their use in transgenic animals. As discussed earlier, the generation of null mutations in embryonic stem cells provides investigators with the ability to "knock-out" specific genes by insertional mutagenesis and will be used eventually to induce specific point mutations. The power of this technology to emulate human genetic diseases characterized by a loss of function is exemplified by the recent development of an animal model for cystic fibrosis (23). Without question, the generation of null mutations to probe the cardiovascular system represents a particularly exciting frontier that will allow investigators to gain insight into the function played by specific genes or disease alleles. Moreover, important advances are being made in the field of somatic gene transfer, or "gene therapy" that will strongly affect how we think about treating cardiovascular diseases. Finally, important new tools are now available that combine molecular genetics, molecular biology, and medicine, that will greatly impact our understanding of human cardiovascular diseases, eventually leading to advances in therapy or possibly cures.

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