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Basic science review

Transgenic animal models as tools for studying renal developmental physiology

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Abstract. Transgenic animal technology, which allows the germline insertion of exogenous genes or the alteration or disruption of endogenous genes, has emerged as a powerful tool for the in vivo analysis of gene function. Since the primary strategy of transgenic techniques is to examine the biological results of lifetime overproduction or underproduction of particular gene products, perhaps no field is better suited for such technology than developmental biology. Indeed, many new phenotypes observed in novel transgenic models involve the alteration of some aspect of development or growth. Considerable information regarding genes involved in the regulation of renal developmental physiology and pathophysiology has emerged from the use of transgenic technology over recent years. We will review the use of traditional transgenic approaches and the resulting animal models, as well as describe more recent advances that allow tissue-specific, cell-specific, and temporal control of genes involved in kidney development.

Key words: Development - Knockout - Transgenic Mice - Renal Physiology - Gene Targeting

Transgenic methodology

Traditional approaches

Transgene Delivery. Most commonly, linear exogenous DNA or the "transgene" (free of prokaryotic vector sequences) is microinjected into one of the two pronuclei of a one-cell fertilized mouse embryo, resulting in integration of that DNA into the host genome of every cell, including the germ cells, of the developing organism [1, 2]. Eggs injected with the transgene are cultured to the two-cell stage and then implanted into the oviduct of a pseudo-pregnant female (a female mated to a vasectomized male), typically resulting in 30% – 50% of injected embryos developing to term, 10% – 25% of which have integrated the DNA into their germline, thus qualifying as transgenic "founders". Typically 1 – 200 copies of the exogenous transgene integrates into the host genome at an apparently random site, probably at points of chromosomal breakage, in head-to-tail configurations. Because the integration is random, transgene expression can be influenced by *cis*-acting regulatory elements present at the integration site. Factors such as DNA purity, buffer conditions, and concentrations of linearized DNA influence the efficiency of transgene integration. Breeding of transgenic founders with normal mice generates F1 heterozygotes, and brother/sister matings of these animals can be performed to produce homozygotes if desired.

Retroviral vectors have also been used as delivery systems for transgenes into the fertilized egg (3). Although integration of the DNA into the genome is still random, this technically simpler approach does allow delivery of a single copy of the transgene. Since infection can occur at the one-cell or later stage embryo, some temporal control over introduction of the gene at a particular embryological developmental phase can be obtained. However, there is a size limitation on the transgene that can be infected (<10 Kilobases), and mosaicism is prevalent since infection can occur after the first or later cell divisions.

Transgene construction and promoter selection. Construction of the DNA to be transferred is critical in obtaining transgenic lines which exhibit appropriate expression of the transgene. In addition to the coding region of the gene of interest, the transgene must contain the appropriate transcription-driving promoter in order to obtain the desired targeting of the gene both spatially and temporally. By utilizing the promoter of a ubiquitous gene, widespread transgene expression occurs. Viral promoters such as simian virus 40 T antigen (SV40 [4]), human cytomegalo-

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virus (CMV [5]) and mouse mammary tumor virus (MMTV [6]) are examples of strong promoters which drive expression of transgenes in many tissues including the kidney. Medullary and cortical tubules (CMV), glomerular epithelium (MMTV and SV40), glomerular endothelium (SV40), and mesangium (SV40) are examples of cell types within the kidney targeted by these ubiquitous promoters. Promoters of genes encoding proteins that exist in many tissues are also good choices for driving widespread transgene expression. The metallothionein-1 (MT-1) promoter is highly active in various tissues, including the kidney, and it has the added feature of being inducible by heavy metals such as zinc [7]. Expression of MT-1-driven transgenes in mice is markedly increased upon administration of zinc in the drinking water [7]. Control of transgene expression has also been accomplished with MMTV-driven transgenes, since this promoter has been shown to be glucocorticoid inducible [8].

While widespread expression of certain transgenes may be appropriate to address some questions concerning renal development, a more tissue- and cell-specific targeting may also be desirable. Promoters that are preferentially active in specific renal cell compartments have recently been identified. The promoter for the human erythrocyte-regulating glycoprotein hormone erythropoietin has been shown to drive high level expression of genes in transgenic mice in peritubular interstitial cells of the renal cortex, the physiological source of erythropoietin [9]. Similarly, the rat phosphoenolpyruvate carboxykinase (PEPCK) promoter directs expression of transgenes to renal proximal tubular epithelium [10]. Although the PEPCK promoter does not exclusively target the kidney, the extrarenal cell types where expression is detected (e.g., hepatocytes and villar epithelium of the small intestine) are as expected. This is important since transgenes driven by some of the more classically used promoters such as SV40 and MT-1 have been detected in cell types that do not normally express the gene product (e.g., MT-1 in neurons) or at non-physiological levels. Renal and extrarenal vascular endothelial cells and mesangial cells have recently been targeted by the murine preproendothelin-1 promoter [11], and proximal tubule epithelium (and physiological extrarenal tissues) expresses transgenes driven by the rat α-glutamyl transpeptidase promoter [12].

We have shown that the human renin transgene, driven by its endogenous promoter, is expressed at a high level and with exquisite specificity in renal juxtaglomerular cells of transgenic mice [13]. Extrarenal human renin was also detected in adrenal gland, ovary, testis, submandibular gland, and heart, indicating that the human renin promoter directed appropriate tissue-specific expression of the transgene in mice. Similarly, high level human angiotensinogen (hAng) mRNA was detected in renal proximal convoluted tubules (PCTs), the physiological source of renal Ang in both humans and mice, utilizing a genomic hAng construct driven by its endogenous promoter [14]. These transgenic mice also exhibit appropriate extrarenal localization of hAng, including high level expression in the liver, the primary site of Ang synthesis and release into the circulation. We are currently examining the ability of the kidney androgen-induced protein (KAP) promoter to target expression of the hAng transgene exclusively to renal PCTs of transgenic mice [15]. KAP is an abundant protein that is expressed in PCTs of mice [16] and mRNA levels are highly responsive to androgen stimulation [17]. While the function of KAP is currently unknown, we hypothesized that we could utilize its promoter to drive PCT-specific expression of the hAng gene in order to examine the role of kidney-derived Ang independent of the circulating (liver-derived) source.

The recent refinement of transgenic technology, particularly the increased use of more specific promoters, has afforded the investigator utilizing these traditional transgenic approaches greater control over the analysis of gene function than ever before. However, it should be noted that whether a tissue-specific, cell-specific, or ubiquitous promoter is used, the level and often the location of expression of transgenes can differ from one transgenic founder to another. This underscores the random nature of this technique, in that the regulatory elements present at the location on the chromosome where integration occurs can influence the transgene expression pattern [18]. As such, characterization of expression of multiple founders and transgenic lines is critical.

Gene targeting: homologous recombination

Specific non-random targeting of DNA to particular chromosomal locations in order to precisely alter, mutate, or disrupt (“knockout”) a single gene of interest distinguishes homologous recombination techniques from traditional transgenic technology [19]. This approach is the coalescence of several fundamental advances, including development of methods to culture totipotent cells from the inner cell mass of the blastocyst, so-called embryonic stem (ES) cells [20], and increased understanding of the mechanisms of recombination of exogenous DNA at chromosomal locations in mammalian cells [21]. ES cells can be isolated from the mouse blastocyst, genetically altered, cultured, and microinjected back into an intact mouse blastocyst, giving rise to a “chimeric” embryo that contains cells derived both from the manipulated ES cells and host blastocyst. Since some of the manipulated ES cells will contribute to the germ cells of the embryo, the altered genome can be propagated and homozygotes generated by interbreeding.

Since the aim of gene targeting is to insert into a specific gene of interest an exogenous fragment of DNA which either disrupts or causes a mutation or alteration of that gene, the DNA fragment must contain flanking regions of homology with sequences surrounding the gene in order to ensure that the fragment will recombine into the appropriate region on the chromosome. While the frequency of homologous recombination is very low, cells in which gene replacement has occurred can be identified by including sequences that encode for selectable marker proteins. For example, disruption of the mouse Ang gene was accomplished by homologous recombination-mediated replacement of exon 2 with a gene conferring neomycin resistance [22]. In this case, the neomycin resistance gene served a dual purpose as both mutagen and selectable marker.

Clearly, the ability to examine deficits present in mice in which there is a lifetime knockout or mutation of a particular gene increases our understanding of the role of that gene and its products in normal and pathological states. Perhaps no field has benefited more from these novel techniques than developmental biology. The analysis of phenotypes produced by mutation and inactivation of genes involved in fetal development and organogenesis has been particularly powerful.

Applications of transgenic technology in the study of renal development

Growth factors and cytokines

Transgenic animal models have been utilized extensively to examine the role of proteins involved in the growth hormone (GH) cascade in renal growth and pathophysiology. Chronic overexpression and elevated serum levels of GH results in mesangial hypercellularity and increased glomerular size with eventual glomerular sclerosis, tubular lesions, and synechiae in transgenic mice [23]. Since transgenic mice that chronically overexpress insulin-like growth factor-I (IGF-I) have increased glomerular size but no sclerosis or tubular pathology, it is suggested that GH-related disorders in the kidney are not mediated solely by the IGF-I component of the GH axis [23]. Studies employing transgenic models also have shown that the various polypeptide forms of GH derived from the single GH molecule have differing effects on growth. Whereas transgenic mice that overexpress full length [22-kilodalton (kDa)] or slightly shortened 20-kDa variants of GH exhibit increased overall body, liver, and kidney size, mice transgenic for a 5-kDa amino terminus variant are not phenotypically different from non-transgenic littermates. The role of GH in diabetic glomerular pathology has also been investigated with GH and GH antagonist transgenic mice [24]. Streptozotocin-induced diabetic mice that overexpress GH exhibit severe hypertrophy and sclerosis of the glomeruli, whereas mice transgenic for a GH antagonist peptide are protected from diabetes-induced nephropathy.

Our understanding of the role of various growth factors and cytokines in renal development and organogenesis has also been increased with the use of transgenic models. Previous in vitro evidence has shown that expression of the cytokine and major mitogen platelet-derived growth factor (PDGF) and its receptors are important in the development of major renal structures, including glomeruli, nephrons, and renal vasculature [25, 26]. The critical role of PDGF in growth, migration, and function of mesenchymal cells in vivo has recently been demonstrated with the generation of PDGF-B knockout mice [27]. These mice die perinatally, at least in part due to abnormal glomerular tuft formation, presumably due to the absence of mesangial cells. Similarly, mice lacking the PDGF-β receptor exhibit severe kidney abnormalities at the level of the glomeruli during various stages of gestation [28]. The capillary tuft normally consisting of mesangial cells and podocytes was completely absent in the glomerulus, and was replaced with blood cells. Interestingly, these abnormal glomeruli were present in normal numbers, suggesting that the loss of the receptors leads to a defect in maturation rather than in the initial commitment to form the glomerulus. Recent knockout studies of another growth factor receptor, epidermal growth factor (EGF), suggest that this factor plays an important role in normal tubule development [29]. Finally, mice transgenic for the murine tumor necrosis factor (TNF)-β or TNF-α genes show high level expression of these cytokines in the kidney, but only TNF-β mice exhibit renal pathology including mononuclear infiltration around proximal tubules [30,31].

Receptors and transcript factors

The development of a multicellular organ such as the kidney requires precise modulation of factors that regulate tissue- and cell-specific gene expression. Several genes encoding transcription factors and signaling molecules involved in coordinating the early events of renal epithelium induction and differentiation have been identified, and recent transgenic models exhibiting either overexpression or disruption of these genes have been generated.

The Wilms' tumor suppresser gene, WT-1, encodes a transcription factor that is mutated in a subset of embryonal tumors, and is now thought to be critical for modulating normal kidney mesenchymal-epithelial cell transition [32]. For example, WT-1 expression is first detected in condensing renal vesicles as proliferation declines and epithelium differentiation commences, and again in epithelial cells as they differentiate into nephric tubules [33]. WT-1 has also been implicated in the process of outgrowth and branching of the ureteric bud [34]. Indeed, a gene-targeted mutation of the murine WT-1 gene is lethal in homozygotes, perhaps due to failure of kidney development [35]. Examination of murine fetuses at 11 days' gestation revealed a complete absence of the ureteric bud with no induction of the renal epithelium. Although the precise mechanisms by which WT-1 regulates renal developmental processes are not known, these results establish a crucial role for WT-1 in early murine urogenital development. Similarly, Wnt-4, the gene encoding a secreted glycoprotein that has been localized in the developing kidney, also appears crucial for renal development. Wnt-4 knockout mice fail to form pretubular cell aggregates and exhibit arrested tubulogenesis [36].

The conversion of mesenchyme to epithelium in the developing kidney may also be critically dependent on expression of one of the paired domain (Pax) gene family members, Pax-2. Pax gene products are transcription factors now known to be critically involved in mammalian development [37]. The Pax-2 gene is expressed in the ureteric epithelium, is activated in the mesenchyme in response to induction, and is subsequently downregulated in mesenchyme-derived differentiated cells [38]. The activation and subsequent suppression of Pax-2 appears critical since chronic overexpression of the human Pax-2b gene in transgenic mice generates severe proximal tubule abnormalities [39]. While Pax-2 knockout mice have yet to be generated, Rothenpieler and Dressler [38] reported that inhibition of Pax-2 protein accumulation in the mesen-

chyme with specific Pax-2 antisense oligonucleotides in mouse kidney organ cultures inhibits aggregation of mesenchymal cells [38].

The critical role of signaling molecules in renal development is demonstrated by a mouse model in which there has been a targeted mutation of c-*ret* [40]. The proto-oncogene c-*ret* encodes one of a family of cell surface molecules that transduce cell growth and differentiation signals, known as receptor tyrosine kinases (RTKs). Although the normal function of the RTK for which c-*ret* encodes is not currently understood, a role in renal development is suggested by its substantial expression in the nephric duct, ureteric bud epithelium, and the developing tips of the renal collecting ducts at various gestational stages [41]. Indeed, c-*ret* knockout mice die before weaning, showing a failure of the ureteric bud to grow and branch appropriately [40]. Taken with the findings from the WT-1 knockout mice that a signal from the mesenchyme may be required for the initial formation of the ureteric bud [35], Schuchardt et al. [40] suggested that c-*ret* encodes the receptor that transduces this signal.

The renin-angiotensin system

It is well established that the renin-angiotensin system (RAS) is a major regulator of arterial blood pressure and volume homeostasis. Angiotensin II (AII) is the primary effector of this system and results from the processing of the substrate Ang first by the protease renin to form the physiologically inactive decapeptide angiotensin I (AI), and further by angiotensin converting enzyme (ACE) to form the octapeptide AII. The vasoactive and volume homeostatic properties of AII are mediated through its interaction with specific AII receptors (AT-1 and AT-2) that are present in vasculature, kidney, heart, adrenal gland, brain, and reproductive tissues [42]. In its classical definition the RAS acts predominantly by endocrine mechanisms; i.e., the substrate and enzymes are synthesized and released from specific tissues, giving rise to blood-borne AII. However, recent data suggest that individual tissue RAS may play an equal or perhaps more important role than the circulating system in numerous physiological processes. The renal RAS has received particular attention in this regard, since the kidney contains all RAS components necessary for the generation and action of AII [43 – 45]. In addition to its proposed role in blood pressure regulation and volume homeostasis, pharmacological evidence suggests that the renal RAS may be important in mediating normal embryonic and postnatal kidney development. For example, AT-1 receptor blockade [46, 47] or ACE inhibition [48] in neonatal rats and in tadpoles undergoing prometamorphosis [47] results in abnormal kidney morphology that includes reduction in number and size of afferent arterioles and glomeruli. These in vivo findings are supported by in vitro data showing that AII applied to cultured renal arteriolar smooth muscle cells increases DNA synthesis and cell numbers [49]. Further evidence supporting a role for the RAS in achieving and maintaining normal morphology and function of the kidney comes from recent studies in which gene-targeted deletions of the murine Ang gene were made [22, 50, 51]. Kim et al. [22] reported a highly significant deficiency in the number of homozygous knockout mice that survived to weaning, and occasional survivors that were homozygous for a deletion of the Ang gene developed abnormal renal morphology, including glomerular atrophy, cortical thinning and tubule loss, interstitial fibrosis and inflammation, and interestingly, hyperplasia-mediated arterial wall thickening. Papillae of kidneys from homozygous knockouts were also reduced in size, and this was accompanied by a downregulation of PDGF-A expression in this structure [50]. That the defects were due primarily to a loss of AII and not secondary genetic events is supported by our recent observations that the lethality and renal abnormalities in Ang knockout mice are rescued (genetic complementation) by transfer of both the hRen and hAng genes (R.L. Davisson and C.D. Sigmund, unpublished observations; [52]).

The abnormal renal structures in Ang knockout mice are consistent with the RAS being an important growth regulator during the development of the kidney. However, the finding of hyperplasia in arterial walls of kidneys of knockout mice is paradoxical if Ang and/or AII are growth-promoting factors. One possible explanation is that a certain level of Ang is required for normal kidney development, and that suppression of Ang may lead to the activation of some growth factors in certain renal cell types. Alternatively, the arterial wall thickening may be in response to the hypotension that is observed in these mutant mice. A third possibility is that AII is required to cease smooth muscle cell replication, a finding consistent with the proposed role of AII and the AT-2 receptor in apoptosis [53]. While the precise mechanisms by which the RAS regulates kidney development are not well understood, it appears that a loss of AII-mediated growth may explain the renal abnormalities exhibited by the Ang knockout mice. Indeed, mice homozygous for a gene-targeted deletion of the ACE gene have changes in kidney morphology similar to the Ang knockout mice [54]. It is important to point out that the phenotypic similarity between the Ang and ACE knockout mice implies that the absence of A II, and not accumulation of bradykinin which may occur in the ACE knockouts, is responsible for the renal abnormalities observed. However, the finding that kidney morphology is normal in mice that are homozygous for a deletion of the AT-1 (subtype a) receptor gene [55] suggests that the effects of AII on renal growth and development are not mediated by its activation of this receptor subtype. Similarly, Hein et al. [56] reported that histological sections of kidneys from AT-2 receptor gene knockout mice were normal compared with heterozygous knockouts or non-mutants. While these studies demonstrate the importance of components of the RAS in regulating normal renal development, further study is required to delineate the exact mechanisms involved.

Future prospects

As evident from the small sampling of transgenic animal models described in this review, the enormity of the potential for transgenic technology to increase our under-

standing of the role of gene products in normal and pathophysiological development of the kidney has just begun to be realized. As increasingly precise targeting of particular renal cell types becomes possible with the use of more tissue- and cell-specific promoters, exact gene products, structures, and mechanisms involved in kidney development will be elucidated. In addition, recent advances which allow gene knockouts to be conditional and restricted to particular cell or tissue types throughout ontogeny ("cre-lox" system) have been described [57]. This technology will be particularly advantageous in cases in which the complete loss of a gene product in all cell types (as with genetargeting techniques utilized thus far) results in a lethal phenotype. The "cre-lox" technology will allow analysis of gene function at later stages of development, as well as in specific cells and tissues without the confounding influence of global loss of the gene product. In addition to increased spatial control of gene expression, greater temporal control over the activation or inactivation of specific genes in vivo will also be possible with recent advances in the use of inducible promoters, such as tetracycline-responsive promoters in transgenic mice [58]. This technique, which utilizes promoters containing binding sites for transcription factors that can be activated or suppressed by systemic administration of tetracycline or its derivatives, will allow genes to be "switched" on or off in a controlled, reversible manner. Clearly, this technology has great potential to increase our understanding of the molecular genetics of mammalian development and organogenesis.

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Literature abstract

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Shunt nephritis associated with *Moraxella bovis*

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Moraxella bovis was repeatedly isolated from cerebrospinal fluid in a girl with two episodes of shunt nephritis. Clinical remission of nephritis was achieved only after shunt replacement. A list of about 20 infectious agents reported to date in patients with shunt nephritis is given. *M. bovis* is yet another agent previously not reported in patients with shunt nephritis.