The Cre/loxP system and gene targeting in the kidney

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Stricklett, Peter K., Raoul D. Nelson, and Donald E. Kohan. The Cre/loxP system and gene targeting in the kidney. Am. J. Physiol. Renal Physiol. 45: F651–F657, 1999.—The Cre/loxP and Flp/FRT systems mediate site-specific DNA recombination and are being increasingly utilized to study gene function in vivo. These systems allow targeted gene disruption in a single cell type in vivo, thereby permitting study of the physiological and pathophysiological impact of a given gene product derived from a particular cell type. In the kidney, the Cre/loxP system has been employed to achieve gene deletion selectively within principal cells of the collecting duct. Disruption of target genes in the collecting duct, such as endothelin-1 or polycystic kidney disease-1 (PKD1), could lead to important insights into the biological roles of these gene products. With selection of the appropriate renal cell-specific promoters, these recombination systems could be used to target gene disruption to virtually any renal cell type. Although transgenic studies utilizing these recombination systems are promising, they are in their relative infancy and can be time consuming and expensive and yield unanticipated results. It is anticipated that continued experience with these systems will produce an important tool for analyzing gene function in renal health and disease.

Cre recombinase; loxP; aquaporin-2

GENE TARGETING TECHNOLOGY can be a powerful tool for studying gene function in vivo. Although this technique has shed light on many developmental biological questions, it has not achieved the same success in explaining physiological and pathophysiological processes in mature animals. The reasons for such relatively poor results are largely twofold. First, it was initially not possible to control the timing of gene disruption. Originally, gene targeting typically involved insertion, using homologous recombination in mouse embryonic stem (ES) cells, of an exogenous DNA fragment into an exon critical for target gene function, resulting in gene “knockout” (37). Animals derived from these stem cells are affected by mutant gene dysfunction throughout ontogenesis, often yielding undesired effects. For example, endothelins-1 and -3 (ET-1 and ET-3) were initially implicated in blood pressure regulation (46, 47); however, homozygous ET-1 knockout mice die at birth from first pharyngeal arch malformation (20), and homozygous ET-3 knockout mice die shortly after birth due to failure to develop a myenteric plexus (3). In these cases, the biological roles of ET-1 and ET-3 could not be studied in mature mice. The second major reason why traditional gene targeting has had limited success is that the targeted gene is affected in all cell types. Thus, if one wanted to examine the biological significance of a targeted gene in a particular cell type, then this would be precluded by the confounding and potentially injurious effects of gene dysfunction throughout the body. Hence, the need arose to conditionally regulate gene targeting. This review will focus on recently developed techniques used to control the cell site, timing, and type of gene targeting. In addition, the utility of these new research strategies in studying renal function is discussed.

Cre/loxP and FLP/ FRT Reombinase Systems

Cre/loxP system. The Cre/loxP system mediates site-specific DNA recombination and was originally described in bacteriophage P1 (40). Two components are involved: 1) a 34-bp DNA sequence containing two 13-bp inverted repeats and an asymmetric 8-bp spacer region termed loxP ("locus of X-over in P1") that targets recombination; and 2) a 343 amino acid monomeric protein termed Cre recombinase that mediates the recombination event (40). As illustrated in Fig. 1, any DNA sequence flanked by two loxP sites will either be excised (loxP sites in same orientation) or inverted (loxP sites in opposite orientation) in the presence of Cre (14) (Fig. 1).

A major advantage of the Cre/loxP system lies in its relative simplicity. First, no cofactors are required for...
Cre activity. This is a result of the Cre/loxP complex providing the necessary energy through formation of phosphotyrosine intermediates at the point of strand exchange (1, 13). Second, loxP target sites are small and easily synthesized. Third, there are no apparent external energy requirements. Fourth, Cre is a very stable protein. Finally, and most important, it is easy to generate DNA constructs with any promoter of interest driving Cre expression (see below). This permits controlling the tissue site, and possibly the timing, of Cre expression and resultant gene disruption. It is not surprising, therefore, that this system has been increasingly employed in manipulating eukaryotic genes in vivo.

Utilization of the Cre/loxP system for gene targeting in vivo involves two lines of mice (Fig. 2). The first mouse line is generated using ES cell technology. Typically, the target gene is altered, by homologous recombination in ES cells, such that genomic regions critical for protein activity are flanked by loxP sites ("floxed" gene). Mice derived from these ES cells should ultimately contain floxed alleles in all cells. These mice should be phenotypically normal because the loxP sites were inserted into introns where they theoretically do not affect gene function. The second line of transgenic mice is generated by standard oocyte injection techniques. These mice express Cre under the control of a transgenic promoter. Mating of the two mouse lines should result in Cre-mediated gene disruption only in those cells in which the promoter is active.

Clearly, the power and versatility of the Cre/loxP system is largely a function of promoter activity. Such activity can be regulated by either 1) endogenous cell-specific elements or 2) exogenously administered regulatory (inducing) factors. Several promoters have been used to achieve tissue-specific Cre expression in mice, including the lck promoter (thymocytes) (12), the alpha A-crystallin promoter (eye lens) (21), the alpha calcium-calmodulin-dependent kinase II promoter (hippocampus and neocortex) (42), the whey acidic protein promoter (mammary gland) (44), the aP2 enhancer/promoter (adipose tissue) (2), the aquaporin-2 (AQP2) promoter (renal collecting duct) (28), and the mouse myogenin promoter (skeletal muscle) (11). Notably, most of the studies examining Cre activity only used a reporter system (a floxed STOP sequence is inserted 5' to a reporter gene; upon Cre-mediated recombination, the STOP is excised allowing for expression of the reporter). One group (12) did use the lck promoter coupled to Cre to disrupt the DNA polymerase b gene in T cells; however, only ~60% of T cells were affected. Thus, although there is precedent for tissue-specific Cre expression, these studies must be interpreted cautiously until it is clearly demonstrated that genes are disrupted only in the cell type(s) of interest and in the entire population of targeted cells (see discussion below).

Cre expression may also be temporally regulated using inducible promoters. This has the theoretical advantage of timing gene targeting events to a particular time in the animal's life, thereby avoiding potentially adverse consequences of defective gene function during earlier developmental stages. A few inducible systems have been coupled to Cre expression, including the interferon-responsive Mx1 promoter (19), a tamoxifen-dependent mutated estrogen receptor promoter (6), and...
and the tetracycline-regulated transactivator/tet operator system (39). Although this is an appealing prospect, these techniques have not yet demonstrated tight control of Cre expression in vivo and cannot, therefore, be recommended at this point.

Flp/FRT system. The Flp/FRT recombination system is essentially the eukaryotic homolog of the Cre/loxP system (34). Flp, a 423 amino acid monomeric peptide encoded within the 2-µm plasmid of Saccharomyces cerevisiae, is very similar to Cre in that it requires no cofactors, uses a phosphotyrosine intermediate for energy, and is relatively stable. FRT is also very similar to loxP in that it is composed of three 13-bp repeats surrounding an 8-bp asymmetric spacer region. The asymmetric region dictates whether excision (FRT sites in same orientation) or inversion (FRT sites in inverted orientation) of an intervening DNA sequence occurs after recombination.

Although not as widely used as Cre/loxP, the Flp/FRT system has been shown to cause site-specific DNA recombination in ES cells and transgenic mice (5). Interestingly, despite similar mechanisms of action and DNA recognition sites, the Cre/loxP and Flp/FRT systems do not exhibit significant cross-reactivity (26). The uniqueness of these two recombination systems may allow them to be used in concert to simplify the gene targeting process (26).

Conditional Gene Targeting in the Kidney

This section will discuss the AQP2-CreTag transgenic mice as an example of conditional gene targeting in the kidney (28). Our goal was to examine renal principal cell-specific gene function by targeting gene knockout to this particular cell type. Such mice would be useful for studying the principal cell-specific function of gene products that are widely expressed in the body and/or genes whose germline targeting leads to fetal or early neonatal death. For the reasons discussed earlier, the Cre/loxP recombination system was employed.

The first step toward achieving principal cell-specific gene targeting was creating a transgene in which the AQP2 5′-flanking region drove expression of Cre recombinase. The AQP2 water channel gene promoter was chosen, since it was thought to be selectively active in
renal principal cells (10, 29). In brief, 14 kb of the AQP2 gene 5'-flanking region, including the transcription initiation site, was linked to a Cre cassette with a eukaryotic translational initiation site, an amino-terminal SV40 nuclear localization signal (to ensure effective nuclear expression; Ref. 12), a carboxy-terminal HSV glycoprotein epitope tag (to facilitate immunodetection; Ref. 17), and an intronless SV40 late region polyadenylation signal (to enhance mRNA expression; Ref. 9). This transgene was termed AQP2-CreTag and is described in detail elsewhere (41).

AQP2-CreTag transgenic mice were generated by injection of the transgene into the male pronucleus of fertilized single cell embryos, and the embryos were implanted into pseudopregnant females (15). Three transgenic founder mice were identified by PCR amplification of the transgene from tail DNA, and, since transgene expression depends on integration site, each founder was individually bred as a line of mice for transgene expression analysis. Next, CreTag and AQP2 mRNA expression were determined by RT-PCR of whole organ RNA derived from each AQP2-Cre transgenic mouse line. Two lines expressed CreTag mRNA selectively in kidney, testes and vas deferens. Similarly, AQP2 mRNA was detected only in these organs. Thus AQP2 promoter activity, as determined by mRNA expression, appeared to be confined to renal principal cells and the male reproductive tract. CreTag and AQP2 protein expression were determined within the kidney and male reproductive system by immunocytochemistry and immunoblotting. As predicted by the mRNA studied, AQP2 and CreTag were detected in renal principal cells (Fig. 3), epithelial cells within the vas deferens, and seminiferous tubules. Double-label immunofluorescence of the kidney demonstrated that renal CreTag expression was limited to AQP2-expressing collecting duct principal cells. Notably, CreTag was not found in all renal principal cells (i.e., expression was variegated), a common finding when many transgene copies are chromosomally integrated and the transgene includes prokaryotic DNA (33).

It was next important to demonstrate that CreTag was active in vivo. This was accomplished by breeding the AQP2-CreTag mouse with a reporter mouse. The reporter mouse contained a loxP-STOP-loxP-lacZ transgene targeted to the RNA polymerase II gene promoter (RNA PolII promoter-loxP-STOP-loxP-lacZ; Ref. 30). Doubly heterozygous offspring of this mating should excise the STOP sequence in cells expressing CreTag, thereby permitting expression of lacZ (blue cells when stained by X-Gal), whereas cells not expressing CreTag should remain unstained by X-Gal. As predicted, mating female AQP2-CreTag mice with male lacZ reporter mice resulted in X-Gal-stained blue cells in testes, vas deferens, and kidney medulla (Fig. 4). Interestingly, when male AQP2-CreTag mice were bred with female

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Fig. 3. Immunofluorescent colocalization of aquaporin-2 (AQP2) and CreTag in renal inner medulla. AQP2 was immunostaining is cytoplasmic and appears red, whereas CreTag immunostaining is nuclear and appears green. Note that nuclear CreTag immunostaining is always associated with AQP2 immunostaining. Arrowheads, CreTag-positive cells; *, nonspecific immunostaining for the CreTag in non-nuclear regions around tubules and glomeruli; r, nonspecific staining of red blood cells; CD, collecting duct. Magnification, ×540.

Fig. 4. X-gal stained kidney sections from mouse carrying both AQP2-CreTag and lacZ transgenes (A) or lacZ transgene alone (B).
lact2 mice, the STOP was excised from all organs, suggesting that CreTag contained in sperm excised the STOP shortly after fertilization.

In summary, AQP2-CreTag mice express functionally active Cre in renal principal cells and the male reproductive tract. The finding of male reproductive tract AQP2-CreTag transgene expression was unexpected and underscores the difficulty in obtaining tissue-specific promoter activity. Nonetheless, we anticipate that these mice will still be highly useful in analyzing renal principal cell-specific gene function as long as coincident target gene deletion in the male reproductive tract does not have undesirable effects. As will be described below, it is anticipated that these mice will be highly useful in analysis of principal cell-specific gene function as well as in other applications.

Potential Applications of Conditional Gene Targeting for Renal Research

It is evident that genes might be targeted in almost any renal cell type. The ability to achieve cell-specific gene targeting depends upon the promoter driving Cre or Flp expression. Several renal cell-specific promoters have been identified, including those that may selectively target renal collecting duct intercalated cells (anion exchanger (AE1) or B-subunit of the proton ATPase; Refs. 27 and 35), renal collecting duct principal cells (vasopressin V2 receptor and vasopressin-regulated urea transporter; Refs. 22 and 38), thick ascending limb (Tamm-Horsfall protein and NKCC2 Na-K-Cl cotransporter; Refs. 16 and 31), thin limbs of Henle's loop (CIC-K1 kidney chloride channel; Ref. 43), proximal tubule γ-glutamyl transpeptidase, kidney androgen-regulated protein (KAP), the Na-P, cotransporter, or Heymann nephritis antigen gp330 (4, 25, 32, 36), and renal interstitial fibroblasts (erythropoietin; Ref. 23). Several important issues exist with regard to transgenic promoters driving Cre expression. First, the promoter may be active at unsuspected sites despite previous studies suggesting renal cell-specific expression. This may be due to an intrinsic property of the promoter (as in AQP2 promoter activity in the male reproductive tract) or to the chromosomal site of transgene integration. Second, promoters suspected to be active in multiple cell types may only drive expression in a single cell type, perhaps due to the promoter fragment utilized. For example, promoters driving expression of Na-glucose or Na-amino acid cotransport may be active in both colon and renal proximal tubule, but when incorporated into a transgene may only be active in the kidney. Third, transgene copy number may be important; higher copy numbers generally, but not always, correlate with higher transgene expression. Fourth, the possibility always remains that Cre recombinase activity may be affected by cell type; however, to our knowledge, this has not been described. Finally, it is worth recalling from the previous section (Cre/loxP and FLP/FRT Recombinase Systems) that transgenes can be constructed such that tissue-specific promoter activity would be regulated temporally by addition or removal of exogenous agents. This adds an additional layer of complexity to the system and has not yet been demonstrated to be a reliable technique in vivo.

Given that one could theoretically target virtually every cell type in the kidney, what genes would be likely candidates for knock out or knock-in? This is perhaps the most daunting challenge: selecting a gene of interest knowing that any gene that has been done can be targeted. Obvious candidate genes relevant to renal research include those encoding 1) transporter proteins; 2) intracellular regulatory or trafficking proteins; 3) secreted proteins suspected to function in an autocrine or paracrine manner in the kidney; 4) cell surface receptors for any number of autocrine, paracrine, or endocrine factors affecting renal cell function; or 5) proteins implicated in disease processes (e.g., polycystin, epidermal growth factor, transforming growth factor-β, etc.). Consider two examples. ET-1 has been implicated in the regulation of body volume homeostasis by virtue of its ability to modulate renal vascular tone and renal cell sodium and water transport (18). The peptide is produced by and binds to multiple renal cell types, each of which could contribute to the effects of ET-1 on renal salt and water excretion (18). The collecting duct is, however, the predominant nephron site of ET-1 production and binding; hence, we have been interested in the role of collecting duct-derived ET-1 in the physiological and pathophysiological regulation of renal fluid reabsorption. Unfortunately, no standard technique (ET-1 antibodies, antagonists, conventional gene targeting) selectively blocks collecting duct-derived ET-1. This problem could, however, potentially be circumvented by breeding the AQP2-Cre-expressing mouse with a mouse containing a floxed ET-1 gene, thereby deleting ET-1 production selectively in the collecting duct. As a second example, no animal model of autosomal dominant polycystic kidney disease due to PKD1 gene deficiency has been successfully developed. Conventional knockout of the PKD1 gene results in mice dying in the perinatal period associated with replacement of all normal renal parenchyma with cysts (24); hence, these mice cannot be used to study the progressive renal cyst formation that typically occurs during adulthood in this disease. One potential way to resolve this problem is to breed the AQP2-Cre-expressing mouse with a mouse containing a floxed PKD1 gene. Subsequent breedings should yield a mouse in which PKD1 gene function is disrupted only in the collecting duct. This may lead to localized renal cyst formation, thereby preventing perinatal mortality and permitting analysis of cyst progression during adulthood. Clearly, the possibilities are virtually limitless.

Potential Limitations of Conditional Gene Targeting

Although Cre- or Flp-mediated gene targeting holds much promise, significant potential limitations exist. There are several concerns with regard to the promoter-Cre gene mice. As alluded to above, because of chromosomal integration site and inherent promoter activity, many transgenic animal lines may need to be screened before one with the desired cell-specific expression is identified. In addition, not all targeted cells may pro-
duce Cre, i.e., Cre expression is variegated. The reasons for this are multiple (7, 8, 45), but again, many lines of transgenic animals may need to be evaluated if Cre activity in all targeted cells is desired (variegated expression may be useful in some circumstances to compare the biological effects of gene disruption in one cell versus a neighboring normal control cell). It is also critically important to document recombination at the floxed gene locus (e.g., mating loxP-expressing mice with IacZ reporter mice). This is particularly important since immunodetection may underestimate the number of cells with functional active Cre recombinase. With regard to mice with floxed genes, it is important to note that these are quite expensive and time consuming to generate. Several commercial organizations charge between $60,000–70,000 US to generate a mouse containing the floxed gene, and the process does not uncommonly take between 5–10 mo. Furthermore, if knockouts are being attempted, then the region of the gene to be flanked by loxP sites must be carefully selected, otherwise truncated or mutated proteins with biological activity may be synthesized. Finally, mice ultimately containing targeted gene disruptions may have phenotypes of uncertain significance. For example, if no unique phenotype is obtained, then does this reflect the biological insignificance of the targeted gene or potentially complex compensatory mechanisms? Also, if the usual system is devised such that the target gene is disrupted during embryogenesis, then unanticipated developmental changes may still occur.

Summary

The Cre/loxP and Flp/FRT recombination systems can be used to achieve cell-specific gene targeting in the kidney and other organs. These systems hold much promise for facilitating study of the contribution of genes products derived from specific cell types to physiological and pathophysiological processes. The power of these systems lies in 1) their ability to target any gene; and 2) their ability to target a given gene in any cell type for which a cell type-specific promoter exists. Conditional gene targeting should only be undertaken, however, after careful considerations of the time, expense, and potential complicating factors. This technique, while in its relative infancy, holds much promise as a means to explore heretofore unanswerable questions.

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