Inducible Gene Silencing in Podocytes: A New Tool for Studying Glomerular Function

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Abstract. Glomerular filtration is one of the primary functions of the kidney. Podocytes, a highly specialized cell type found in glomeruli, are believed to play a critical role in that function. Null mutations of genes expressed in podocytes like WT1, nephrin, and NEPH1 result in an embryo and perinatal lethal phenotype and therefore do not allow the functional analysis of these genes in the adult kidney. Here is describes the generation of a model that will allow such studies. We have engineered transgenic mice in which the disruption of targeted genes can be induced in a temporally controlled fashion in podocytes. For this, a transgene encoding the mutated estrogen receptor-Cre recombinase fusion protein was introduced into the mouse genome. Animals were crossed with Z/AP reporter mice to test for efficient and inducible recombination. We found that, after injection of inducer drug tamoxifen, Cre fusion protein translocates to the nuclei of podocytes, where it becomes active and mediates recombination of DNA carrying loxP target sequences. These animals provide for the first time a tool for silencing genes selectively in podocytes of adult animals.

Glomerular visceral epithelial cells otherwise known as podocytes play a critical role in glomerular filtration. Several lines of evidence show that a failure in podocyte function is involved in glomerular diseases leading commonly to end-stage renal failure. Until recently, this highly specialized cell population has been difficult to study. The emergence of new tools such as optimized conditions for cell culture (1) and transgenic mouse models (2) are beginning to address this problem. Indeed, Eremina et al. (2) have recently described the generation of transgenic animals in which the Cre recombinase is expressed under the control of Nphs1 promoter, these animals express Cre exclusively in podocytes during development and throughout adulthood, which will allow selective silencing of genes within this cell type. In this report, we describe the generation of animals also expressing Cre selectively in podocytes in the kidney but in an inducible fashion, thereby providing an extra level of control for gene silencing. We have used the mutated oestrogen receptor-Cre fusion protein, MerCreMer (3), to generate transgenic animals. In this system, Cre is expressed and remains in the cytoplasm of expressing cells, where it is in an inactive form bound to HSP90. After injection of tamoxifen to the animal, Cre translocates to the nucleus, where it becomes active and mediates recombination of DNA carrying loxP target sequences. MerCreMer contains two mutated estrogen receptor–binding domains, which confers tight dependency on tamoxifen binding for translocation to the nucleus and recombinase activity (3). This level of control becomes critical when inactivating genes whose absence during development results in an embryonic lethal phenotype as exemplified in the WT1−/− mouse (4).

Materials and Methods

Constructs and Generation of Transgenic Animals

MerCreMer was placed under the control of CMV enhanced-chicken βactin promoter pCAGGS (5). The HindIII insert from pAMA (a kind gift from M. Reth [3]) was cloned into the EcoRI site of pCAGGS.

Transgenic mice were generated by pronuclear injection of (C57BL/6 × DBA) F1 oocytes. Tail tips of progeny were genotyped by PCR, and founders were crossed with C57BL/6 mice.

For complete characterization, animals were crossed with Z/AP reporter mice kindly provided by C. Lobe. Z/AP animals were genotyped as described (6).

Animals and Tamoxifen Treatment

Mice were maintained in CBS in accordance with Home Office guidelines (Animals [Scientific Procedures] Act 1986). Animals were injected intraperitoneally with 33 mg/kg tamoxifen (ICN, UK) solubilized in sunflower oil daily for 5 d or as indicated for kinetic and dose response studies. Various tissues were cryo-embedded in OCT (Raymond Lamb, UK) for immunohistology.

Tissue Staining

Seven-micrometer cryosections of various tissues were dried and fixed in 4% paraformaldehyde in PBS for 10 min. Sections were incubated with a blocking solution (1% BSA, 0.2% Triton X100, 10%
Cre expression in various tissues of \( \beta MCM_{86} \) line. Tamoxifen (33 mg/kg) was given daily for a period of 5 d to adult animals. Tissues were collected 24 h after the last injection. Seven-micrometer cryosections from various tissues of induced animals were immunostained using biotinylated-Cre antibody. These pictures are representative of tissues observed in these animals and has been repeated in tissues from several mice. Tissues from wild-type animal were used as negative control. Scale bars, 50 µm.

*Figure 1.* Inducible Gene Silencing in Podocytes
mouse serum, and 10% donkey serum [Sigma, UK] in PBS) for 30 min. For immunofluorescence analysis, slides were incubated overnight with goat, WT1-reactive antibody (N20; Santa Cruz Biotechnology, Santa Cruz, CA). Sections were then incubated with a FITC-conjugated donkey anti-goat Ig (Jackson ImmunoResearch, Pine Grove, PA) for 1 h. Second antibody-staining procedure was performed with either a rabbit polyclonal Cre antibody (Covance, CA) at 1:3000 or with a human Alkaline phosphatase (hAP) antibody (AHP537; Serotec, UK) at 1:50 overnight. Finally, sections were incubated with TRITC-labeled donkey anti-rabbit Ig (Santa Cruz Biotechnology) at 1 μg/ml in block solution for 1 h. Sections were mounted in Vectashield media (Vector, UK) for analysis.

For light microscopy analysis, Cre staining was followed by a biotinylated goat anti-rabbit Ig (Vector, UK) at 1:100 and developed with ABC and AEC kit (Vector, UK). In some experiments, hAP was also detected by adding a substrate kit solution to the sections (Vector, UK) and as described (6).

Results

Cre Expression in βMCM<sub>86</sub> Animals

Founders carrying the transgene as detected by PCR were analyzed for MerCreMer transcript expression profiles in a

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Figure 2. Dose dependence recombination in heart of βMCM<sub>86</sub> line. Animals were injected with 3.3, 33, or 333 mg/kg tamoxifen for 1, 3, or 5 d as indicated. Tissues were collected 24 h after the last injection, and 7-μm cryosections were stained for hAP with enzyme substrate. N.D., not determined due to sickness of animals induced by this high dose of drug over time. Scale bar, 50 μm.
wide variety of organs. Of nine different lines generated, 7 showed mRNA expression at varying levels (data not shown). We then examined Cre translocation to the nucleus after induction with tamoxifen. Animals derived from two different lines showed good translocation of Cre to the nucleus of cells in some tissues. One line, βMCM<sub>70</sub>, expressed Cre in an ubiquitous fashion (data not shown), whereas the other line, βMCM<sub>86</sub>, shows a patchy pattern of expression. The results are similar to those obtained by others for transgenes driven with the pCAGGS promoter (6,7). Hemizygous βMCM<sub>86</sub> animals were healthy, developed and bred normally, and had a normal lifespan. Moreover, histology of all tissues showed normal morphology (Figure 1). Expression of Cre was analyzed in a variety of tissues of the βMCM<sub>86</sub> line after induction with tamoxifen (Figure 1). We observed a relatively low frequency of cells expressing Cre overall with the highest expression in skeletal and heart muscle tissue. Interestingly, in the kidney, we noticed a pattern of expression that corresponded to that of podocytes observed in these animals and has been repeated in kidneys from four mice. Scale bars, 10 μm.

**Figure 3.** Cre co-localized with WT1 in kidney from induced βMCM<sub>86</sub> animals. Tamoxifen (33 mg/kg) was given daily for a period of 5 d to adult animals. Tissues were collected on the last day of treatment. 7-μm cryosections from kidneys of induced animals were immunostained using TRITC for Cre and FITC for WT1; images were merged for colocalization analysis. (A) Staining obtained from a kidney of an induced wild-type animal; WT1 staining shows a typical podocyte pattern while no Cre staining was detectable. (B) Result obtained with a kidney from a βMCM<sub>86</sub>-induced animal. Cre staining was detected in nuclei, and Cre-positive cells were also WT1-positive. This picture is representative of glomeruli observed in these animals and has been repeated in kidneys from four mice. Scale bars, 10 μm.

Inducible Cre Recombination in βMCM<sub>86</sub> Line

To assess the ability of Cre in induced animals to trigger appropriate recombination of target DNA, βMCM<sub>86</sub> animals were crossed with the reporter line Z/AP (6). Z/AP animals carry a floxed LacZ gene under the control of a pCAGGS promoter followed by a hAP cassette. hAP activity is dependent on recombination between the two <i>loxP</i> sites. Double transgenic animals were induced with 3.3, 33, or 333 mg/kg tamoxifen for 1, 3, or 5 d, and tissues were analyzed for expression of the hAP reporter gene. Figure 2 shows the result obtained for the heart; there was very little hAP detectable at low doses of tamoxifen (3.3 mg/kg) or when tamoxifen was only injected for 1 d. After 3 d of tamoxifen, hAP was readily detectable with greatest recombination being obtained when drug was injected for 5 d. Recombination plateaus with a tamoxifen dose of 33 mg/kg. Animals injected with 333 mg/kg for 5 d showed signs of sickness, with two animals dying on days 4 and 5. This result indicates that Cre expressed in βMCM<sub>86</sub> animals is able to recombine target DNA in an inducible fashion.

To assess if recombination was completely tamoxifen-dependent, we analyzed untreated double transgenic embryos and adult animals for hAP expression. In no tissue analyzed, including whole embryo (day 14), muscle, heart, or pancreas, was hAP observed (data not shown).

Cre Translocates to the Nucleus of Podocytes in Kidneys of βMCM<sub>86</sub> Mice

To clarify which cells in the kidney expressed Cre, we stained kidney sections with Cre and WT1 antibodies sequentially. As shown in Figure 3, after induction in vivo with tamoxifen, no Cre staining could be seen in the nuclei of kidneys harvested from wild-type animals. In the βMCM<sub>86</sub> line, strong staining was seen in the nuclei of cells. Furthermore, no Cre staining was detected in nuclei of cells from uninduced βMCM<sub>86</sub> animals (data not shown). When Cre and WT1 staining were performed on the same section, we found that the expression of these two proteins was coincident in induced βMCM<sub>86</sub> mice. Between 60 and 90% of WT1-positive cells were also positive for Cre. All Cre<sup>+</sup> cells observed in the glomerulus were also WT1<sup>+</sup>. WT1 is believed to be an exclusive marker for podocytes (8); therefore, this result shows that...
in the kidney of our animals Cre is expressed in and translocates to the nucleus of podocytes after induction with tamoxifen.

**Inducible DNA Recombination in Podocytes**

Double (Cre, Z/AP) transgenic animals were induced with tamoxifen for 5 d, and kidneys were analyzed for expression of the hAP reporter gene. As shown in Figure 4, hAP staining was restricted to the glomeruli of kidneys collected from tamoxifen-induced animals. Recombination was completely tamoxifen-dependent; no hAP staining was detected in untreated double transgenic animals (Figure 4, A and C). When we compared hAP with WT1 staining, we found that hAP staining was seen in a distinctive pattern in the extended cytoplasmic

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**Figure 4.** *In vivo* injection of tamoxifen induced hAP expression in podocytes after DNA recombination of target DNA mediated by Cre. Animals double transgenic for βMCM86 and Z/AP were left untreated (A and C) or injected *in vivo* with tamoxifen (B, D through F) before tissue analysis. Kidney sections were stained for hAP substrate (dark brown) (A and B) or using FITC for WT1 (green) and TRITC for hAP (red) (C through F). A and B show general views of kidney cortex where hAP staining is observed in glomeruli only (black bars, 100 μm). Panels C and D show representative glomeruli (white bars, 10 μm). E and F show details of glomeruli where hAP is detected in the cytoplasm of podocytes (white bars, 10 μm).
processes of cells whose nuclei were positive for WT1. The percentage of cells positive for hAP corresponded to that of Cre-positive podocytes, which, as mentioned above, was less than 100%.

Discussion
In this report we, describe the βMCM86 line of transgenic mice, which provides a new tool to study the role of podocytes in kidney function via inducible gene inactivation. Our results show, first, that Cre was expressed in podocytes of the βMCM86 line; second, that after treatment with tamoxifen, Cre translocates to the nucleus of podocytes; and third, that DNA containing loxP sequences is recombined successfully by Cre in these cells. Importantly, no background recombination was observed in animals that were not treated with inducer, demonstrating the tight regulation of Cre activity in these mice.

A recent report has described the generation of transgenic animals expressing Cre exclusively in podocytes by driving expression of Cre with nephrin (Nphsl) promoter (2). This model will clearly be very useful in situations in which recombination of the target gene is desired at early stages of development. However this could also be problematic because constitutive silencing of genes in podocytes and/or neighboring cells may alter the developmental program in such a way as to obscure the real function(s) of the targeted gene. Our model circumvents this problem by triggering the silencing of the gene of interest at the desired time.

βMCM86 animals express Cre in podocytes but also in a variety of tissues, including striated and smooth muscles, some pancreatic cells, and cultured primary fibroblasts derived from the ear. Although this has to be taken into account, it should nevertheless not present a problem for most genes involved in kidney and/or podocyte function, especially because Cre acts solely in an inducible manner in our animals. An ideal candidate target gene for our model is WT1. During embryogenesis, WT1 is expressed in tissues in a complex pattern. Its expression is completely downregulated in the adult in all tissues except for podocytes. Mutations in the human WT1 gene are responsible for the development of embryonic kidney cancer (Wilms tumor); in mice, a null mutation in this gene results in an embryo lethal phenotype (4,8). The function of this gene in the adult kidney has therefore been impossible to study. Our model provides a direct way to address this issue. Other genes like nephrin and its homolog NEPH1 are also important targets because null mutation in both results in perinatal lethality with serious defects in foot process formation of the podocyte (9,10). Again, inactivating these genes in adult kidney should reveal key functions for these genes, which so far have been impossible to study.

We observed a small percentage of podocytes that did not show Cre or hAP staining after induction with tamoxifen, suggesting that 100% recombination might not be achievable. This is in line with other workers findings when using inducible recombination systems in which, nevertheless, phenotypes are readily observed.

In conclusion, we have developed a transgenic mouse with an inducible form of the Cre recombinase that operates within the kidney exclusively in podocytes. This mouse model provides for the first time an opportunity to silence genes specifically in podocytes of the adult kidney thereby allowing analysis of several important genes in glomerular filtration in the adult mouse.

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References